

(19) **DANMARK**



Patent- og
Varemærkestyrelsen

(12)

Oversættelse af europæisk patentskrift

(10) **DK/EP 3204399 T3**

-
- (51) Int.Cl.: **C 07 H 21/00 (2006.01)** **C 12 N 15/00 (2006.01)** **C 12 N 15/64 (2006.01)**
C 12 P 19/34 (2006.01)
- (45) Oversættelsen bekendtgjort den: **2025-02-24**
- (80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2025-01-22**
- (86) Europæisk ansøgning nr.: **15848462.6**
- (86) Europæisk indleveringsdag: **2015-10-08**
- (87) Den europæiske ansøgnings publiceringsdag: **2017-08-16**
- (86) International ansøgning nr.: **US2015054780**
- (87) Internationalt publikationsnr.: **WO2016057850**
- (30) Prioritet: **2014-10-09 US 201462062098 P** **2015-05-13 US 201562161107 P**
- (84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**
- (73) Patenthaver: **Seattle Children's Hospital, dba Seattle Children's Research Institute, 1900 9th Avenue, Seattle, WA 98101, USA**
- (72) Opfinder: **SCHARENBERG, Andrew M., 1222 NW Norcross Way, Seattle, WA 98177, USA**
JACOBY, Kyle, 1717A 27th Avenue, Seattle, WA 98122, USA
GRIER, Alexandra E., 2209 Yale Avenue E 103, Seattle, WA 98102, USA
- (74) Fuldmægtig i Danmark: **Novagraaf Brevets, Bâtiment O2, 2 rue Sarah Bernhardt CS90017, F-92665 Asnières-sur-Seine cedex, Frankrig**
- (54) Benævnelse: **LANGE POLY (A)-PLASMIDER OG FREMGANGSMÅDER TIL INDFØRING AF LANGE POLY (A)-SEKVENSER I PLASMIDET**
- (56) Fremdragne publikationer:
WO-A1-2014/089290
WO-A1-2016/005004
WO-A2-99/61605
US-A1- 2008 260 706
US-A1- 2009 093 433
US-A1- 2009 263 873
US-A1- 2010 129 877
US-A1- 2014 179 770
S. HOLTKAMP ET AL: "Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells", BLOOD, vol. 108, no. 13, 15 December 2006 (2006-12-15), pages 4009 - 4017, XP055044965, ISSN: 0006-4971, DOI: 10.1182/blood-2006-04-015024
FORD L P ET AL: "THE POLY(A) TAIL INHIBITS THE ASSEMBLY OF A 3'-TO-5' EXONUCLEASE IN AN VITRO RNA STABILITY SYSTEM", MOLECULAR AND CELLULAR BIOLOGY, AMERICAN SOCIETY FOR

Fortsættes ...

MICROBIOLOGY, WASHINGTON, US, vol. 17, no. 1, 1 January 1997 (1997-01-01), pages 398 - 406, XP000857790, ISSN: 0270-7306

D R GALLIE: "The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency.", GENES AND DEVELOPMENT., vol. 5, no. 11, 1 November 1991 (1991-11-01), US, pages 2108 - 2116, XP055452703, ISSN: 0890-9369, DOI: 10.1101/gad.5.11.2108

PROMEGA.: "pSP64 Poly(A) Vector", December 2005 (2005-12-01), XP055426567, Retrieved from the Internet <URL:<https://www.promega.com/~media/files/resources/protocols/technical%20bulletins/0/psp64%20poly%20a%20vector%20protocol.pdf>>

DESCRIPTION

Description

CROSS-REFERENCED TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application Serial No. 62/062098, entitled "LONG POLY(A) PLASMIDS AND METHOD FOR INTRODUCTION OF LONG POLY(A) SEQUENCES INTO THE PLASMID" filed October 9, 2014, and U.S. Provisional Application Serial No. 62/161,107 entitled "LONG POLY(A) PLASMIDS AND METHOD FOR INTRODUCTION OF LONG POLY(A) SEQUENCES INTO THE PLASMID" filed May 13, 2015.

REFERENCE TO SEQUENCE LISTING, TABLE, OR COMPUTER PROGRAM LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled SCRI.098WO.TXT, created October 7, 2015, which is 180 kb in size. The information is the electronic format of the Sequence Listing.

STATEMENT REGARDING FEDERALLY SPONSORED R&D

[0003] This invention was made with partial support under NICHD U19 AI096111, awarded by the US Department of Health and Human Services and the National Institutes of Health.

FIELD OF THE INVENTION

[0004] Aspects of the invention as described herein, include compositions and methods relating to genes that allow for the generation of a nucleic acid having a poly(T) tract or poly(A) tract of a desired length. Included herein are methods of increasing the stability of a nucleic acid transcript, enhancing gene transcription, and enhancing protein translation by providing one or more of the aforementioned nucleic acids. Also described herein, are methods of enhancing gene transcription, and enhancing protein translation by providing one or more of the aforementioned nucleic acids comprising a gene encoding a nuclease and methods of treating a disease with compositions or cells comprising the nucleic acids.

BACKGROUND OF THE INVENTION

[0005] Increased demand for large scale synthesis of *in vitro* transcribed (IVT) mRNA is being driven by an increasing use of mRNA for transient gene expression in *ex vivo* cell engineering or direct *in vivo* therapeutic applications. One of the most important determinants of potency of an IVT mRNA is the 3' poly-adenosine (poly(A)) tail, the length of which directly correlates with efficiency of translation. However, present methods for large scale generation of IVT mRNA rely on templates derived from circular plasmids, in which homopolymetric tracts are highly unstable, thus limiting encoded poly(A) tail lengths to less than approximately 120 base pairs. These methods are needed to generate mRNA that are highly stable for translation.

SUMMARY OF THE INVENTION

[0006] As provided herein are novel methods for the generation of extended poly(A) tracts using a previously described linear plasmid system. It is found that the linear plasmid is able to stably propagate poly(A) tracts up to at least 500 base pairs in length. To facilitate generation of IVT mRNA with precisely defined encoded poly(A) tracts and 3'termini, alternatives are provided with modified linear plasmid, by removing extraneous restriction sites, adding a T7 promoter sequence upstream from an extended MCS, and adding a unique type IIS restriction site downstream from the position of the encoded poly(A) tract. The resulting plasmid, designated p(Extended Variable Length) (pEVL), can be used to generate IVT mRNA with consistent, defined lengths and terminal residue(s). The invention is set out in the appended claims.

[0007] Some alternatives relate to a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0008] In some alternatives, not forming part of the invention, a method of making a polynucleotide having a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, and wherein said template strand comprises a plurality of thymine nucleotides, is provided. Some methods can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0009] In one aspect of the invention, a method of making a nucleic acid is provided. The method can comprise providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The method comprises providing a first nucleic acid sequence comprising at least one endonuclease recognition site, and providing a second nucleic acid sequence comprising a plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises

the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. The polynucleotide further comprises telomeres. The polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The polynucleotide can comprise a first nucleic acid sequence comprising at least one endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the endonuclease cleavage site, and wherein the endonuclease cleavage site is within the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 1-55 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives,

the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0010] In some alternatives, a nucleic acid is provided. The nucleic acid can be manufactured by a method of any of the alternatives described herein. The method of making a nucleic acid can comprise providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. Some methods can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site,

wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. Some polynucleotides can comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a

nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is a RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminates with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic

sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0011] In some alternatives, not being part of the invention, a method of enhancing transcription of a gene is provided. The method can comprise providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of any one of the alternatives provided herein and contacting said polynucleotide with a RNA polymerase in the presence of nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. Some methods can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is

within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the method is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0012] In a further aspect of the invention, a method of stabilizing an RNA during in vitro translation at 30-37 degrees Celsius for at least 100 hours is provided. The method can comprise providing the nucleic acid of any one of the alternatives herein or a nucleic acid manufactured by a method of any one of the alternatives herein and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours (e.g., at least or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. The method can of making the nucleic acid can comprise providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in

the presence of nucleotides. Some methods can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. Some polynucleotides a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the

plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250,

275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is a RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a RNA. In some alternatives, the contacting is performed at a temperature equal to 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes an endonuclease. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0013] In a further aspect of the invention, a method of stabilizing RNA during in vivo translation at 30- 37 degrees Celsius for at least 100 hours in a cell, is provided. The method can comprise transfecting into a cell the nucleic acid of any one of the alternatives herein or a nucleic acid manufactured by a method of any one of the alternatives herein, placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation and incubating the cell for at at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow translation. In some alternatives, the method of making the nucleic acid or RNA comprise providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. Some methods can comprise providing a first nucleic acid sequence

comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm wherein the left arm

comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0014] In a further aspect of the invention, a method of increasing and/or stabilizing expression of a protein is

provided. The method can comprise generating RNA from the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and translating said RNA into a peptide. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the method of making the polynucleotide can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32,

33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the restriction endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the translating step further comprises providing ribosomes, tRNA and amino acids. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene encoding a protein. In some alternatives, the protein is a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0015] A method of enhancing stability of a replicating gene not being part of the invention is provided, wherein the method comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured

by any method any one of the alternatives herein, wherein said polynucleotide further comprises a gene transforming a cell with said polynucleotide; and propagating the cell, wherein the polynucleotide is replicated. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the r endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the method of making a polynucleotide having a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, and wherein said template strand comprises a plurality of thymine nucleotides, is provided. The method for making the polynucleotide can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for

the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C or any other temperature between any these values. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0016] In some alternatives, not forming part of the invention, a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm.

In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0017] In some alternatives, not forming part of the invention, a method of making polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The method can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino

acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0018] In some alternatives, not forming part of the invention, a method of making a nucleic acid encoding a nuclease, is provided, wherein the method comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic

acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0019] In some alternatives, which is not being part of the invention, a method of enhancing transcription of a gene encoding a nuclease, is provided. The method can comprise providing polynucleotide of any one of alternatives herein, or a polynucleotide manufactured by a method of any one of alternatives herein and contacting said polynucleotide with a RNA polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine

nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is BsaI or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is BsaI or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally

joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the method is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0020] In some alternatives, not forming part of the invention, a nucleic acid encoding a nuclease manufactured by any one of the methods of any of the alternatives herein, is provided. The method for manufacturing the nucleic acid comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising

a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0021] In some alternatives, not forming part of the invention, a method of stabilizing translation of an RNA encoding a nuclease during in vitro translation at 30- 37 degrees Celsius for at least 100 hours is provided. The method can comprise providing the nucleic acid of any of the alternatives herein or a nucleic acid manufactured by a method of any one of the alternatives herein and contacting said nucleic acid with a ribosome and tRNAs in the presence of amino acids, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours. The method of making the nucleic acid can comprise providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours (e.g., at least or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the

at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some methods of making a polynucleotide can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5,

10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the nucleic acid further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature equal to 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid comprises a plurality of adenines, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0022] In some alternatives, not forming part of the invention, a method of stabilizing translation of an RNA encoding a nuclease during in vivo translation at 30- 37 degrees Celsius for at least 100 hours is provided. The method can comprise transfecting into a cell the nucleic acid of any of the alternatives herein or a nucleic acid manufactured by a method of any of the alternatives herein, placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation and incubating the cell for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow translation. The method for manufacturing the nucleic acid comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the

template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. Some methods of making a polynucleotide can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site,

wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is BsaI or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0023] In some alternatives, not being part of the invention, a method of expression of a nuclease or increasing expression of a nuclease is provided. The method can comprise generating an mRNA from the polynucleotide of any

one of the alternatives herein or a polynucleotide manufactured by a method of any one of any one of the alternatives herein and translating said mRNA into a peptide. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence

comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the mRNA comprises a plurality of adenines, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400,

425, 450, 475, or 500 covalently linked adenines. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0024] In some alternatives, not being part of the invention, a method of replicating a gene comprising a nuclease or enhancing stability of a replicating gene comprising a nuclease, is provided. The method can comprise providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein, wherein said polynucleotide comprises a gene encoding a nuclease, transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the

endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction

endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, or 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, or 37°C or any other temperature between any these values. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0025] In some alternatives, not forming part of the invention, an RNA having a plurality of adenine nucleotides, is provided. The RNA can comprise a first sequence comprising at least one complement sequence to an endonuclease recognition site covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides, a second sequence that comprises a plurality of adenine nucleotides, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines and wherein said complement sequence to an endonuclease recognition site of said first nucleic acid is covalently linked to said plurality of adenine nucleotides of said second nucleic acid at the 3' end of said plurality of adenine nucleotides of said second nucleic acid. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA complement is a complement to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site or a StuI recognition site. In some alternatives, the gene is codon optimized for expression in a human cell. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0026] In some alternative, a cell manufactured by the methods of any one of the alternatives herein is provided, but not being a part of the present invention. The method can comprise transfecting into a cell the nucleic acid of any of the alternatives herein or a nucleic acid manufactured by a method of any of the alternatives herein, placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation and incubating the cell for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow translation. The method for manufacturing the nucleic acid comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease

cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the restriction endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the

endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by any method any one of the alternatives herein, wherein said polynucleotide further comprises a gene transforming a cell with said polynucleotide; and propagating the cell, wherein the polynucleotide is replicated. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29,

30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some methods of making a polynucleotide can comprise providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49,

50, 51, 52, 53, 54 or 55 base pairs, of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine or 5' terminal thymine in the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotide comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C or any other temperature between any these values. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. The method can comprise providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein, wherein said polynucleotide comprises a gene encoding a nuclease, transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to

said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a Bsal restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is Bsal or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Bsal restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is Bsal or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine,

cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, or 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, or 37°C or any other temperature between any these values. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0027] In some alternatives, not being part of the invention, a pharmaceutical composition is provided. The pharmaceutical composition can comprise a pharmaceutical vehicle and a nucleic acid of any one of the alternatives herein or mRNA of any one of the alternatives herein. The nucleic acid can be manufactured by a method of any one of the alternatives described herein. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the

template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right

arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotide comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently

linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method for manufacturing the nucleic acid comprising a nuclease can also be provided. The method comprises providing the polynucleotide of any one of the alternatives herein or a polynucleotide manufactured by a method of any one of the alternatives herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some

alternatives, the sequence encoding the nuclease is optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the plurality of covalently linked thymines comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the first nucleic acid sequence encoding the nuclease is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. The RNA can comprise a first sequence comprising at least one complement sequence to an endonuclease recognition site covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides, a second sequence that comprises a plurality of adenine nucleotides, wherein the plurality of

adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines and wherein said compliment sequence to an endonuclease recognition site of said first nucleic acid is covalently linked to said plurality of adenine nucleotides of said second nucleic acid at the 3' end of said plurality of adenine nucleotides of said second nucleic acid. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site or a Stul recognition site. In some alternatives, the gene is codon optimized for expression in a human cell. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0028] In some alternatives, not being part of the invention, the nucleic acid described herein is for use in a method of treating, ameliorating, or inhibiting disease in a subject is provided. The method can comprise introducing into a cell the nucleic acid of any one of the alternatives herein or the mRNA of any one of the alternatives herein and delivering the cell to the subject. The nucleic acid can be manufactured by a method of any one of the alternatives described herein.

[0029] In some alternatives, a pharmaceutical composition is for use in treating, ameliorating, or inhibiting a disease in a subject is provided, wherein the method comprises delivering the pharmaceutical composition of any of the alternatives herein, to the subject. The pharmaceutical composition can comprise a pharmaceutical vehicle and a nucleic acid of any one of the alternatives herein or mRNA of any one of the alternatives herein. The nucleic acid can be manufactured by a method of any one of the alternatives described herein.

[0030] In some alternatives, not being part of the invention, a method of treating, ameliorating, or inhibiting disease in a subject is provided, the method can comprise introducing into a cell the mRNA or RNA of any one of the alternatives herein and delivering the cell to the subject. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic

sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the mRNA encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a human cell. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0031] In some alternatives, not being part of the invention, a pharmaceutical composition is provided, wherein the composition comprises a pharmaceutical vehicle and the mRNA of any one of the alternatives herein. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminates with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the mRNA encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or

3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0032] In some alternatives, not being part of the invention, the pharmaceutical composition is for use of treating, ameliorating, or inhibiting a disease in a subject is provided wherein the method comprises delivering the pharmaceutical composition of any of the alternatives herein to the subject. In some alternatives, the composition comprises a pharmaceutical vehicle and the mRNA of any one of the alternatives herein. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the mRNA encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0033] In some alternatives, not being a part of the invention, the cell for use in treating, ameliorating, or inhibiting a disease in a subject is provided, wherein the method comprises delivering the cell of any one of the alternatives, to the subject. In some alternatives, the cell is manufactured by transfecting into a cell the nucleic acid of any one of the alternatives herein or a nucleic acid manufactured by a method of any one of the alternatives herein, placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation and incubating the cell for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow translation. In some alternatives, the method of making the nucleic acid or RNA comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is BsaI or Stul. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one

covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from a group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0034] In some alternatives, a polynucleotide that encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-

14, wherein said polynucleotide comprises an endonuclease site at the 3' end of said polynucleotide, preferably outside of the coding region for said protein, and said endonuclease site is joined to at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500 or 550 covalently linked thymine residues or an amount of covalently linked thymine residues that is within a range defined by any two of the aforementioned values, is provided. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0035] In some alternatives, an mRNA that encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the composition comprises a pharmaceutical vehicle and the mRNA or mRNA of any one of the alternatives herein. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the mRNA encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in

the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0036] In some alternatives, not being part of the invention, a method of treating, ameliorating, or inhibiting disease in a subject is provided wherein the method comprises introducing into a cell any one or more of the polynucleotides or nucleic acids set forth in any of the aforementioned claims; and delivering the cell to the subject. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic

sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives the nucleic acid provided, is manufactured by a method of any one of the alternatives described herein is provided. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic

sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotide comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene

encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some

alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0037] In some alternatives, not being part of the invention, the polynucleotides or nucleic acids is for use in treating, ameliorating, or inhibiting disease in a subject is provided, wherein the method comprises administering any one or more of the polynucleotides or nucleic acids set forth in any of the aforementioned claims to a subject. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises

telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives the nucleic acid provided, is manufactured by a method of any one of the alternatives described herein is provided. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some

alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the

polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third

nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0038] In some alternatives, a method of increasing and/or stabilizing expression of a protein is provided wherein the method comprises generating an mRNA from any one or more of the polynucleotides of any one or more of the aforementioned claims and translating said mRNA into a peptide. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease

recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives the nucleic acid provided, is manufactured by a method of any one of the alternatives described herein is provided. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third

nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides,

wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some

alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminates with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The

polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0039] In some alternatives, not being part of the invention, a method of enhancing transcription or expression of a gene is provided, where the method comprises providing any one or more of the polynucleotides or nucleic acids of any one or more of the aforementioned claims, wherein said polynucleotide further comprises a gene, such as a nuclease gene, for example SEQ ID NOs. 6-14 and contacting said polynucleotide or nucleic acid with a RNA polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning

region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a Stul restriction endonuclease recognition site, and wherein the endonuclease is BsaI or Stul. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives the nucleic acid provided, is manufactured by a method of any one of the alternatives described herein is provided. In some alternatives, the method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located

between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a third sequence, wherein the third sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently

linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a fourth sequence, wherein the fourth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the polynucleotide further comprises a promoter for a polymerase. In some alternatives, polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the gene is codon optimized for expression in human cells. In some alternatives, the nucleic acid is a DNA. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminate with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the gene is codon optimized for expression in human cells. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage

site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the sequence encoding the nuclease is optimized for expression in human cells. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). More alternatives concern a linear polynucleotide comprising a first nucleic acid sequence that comprises at least one endonuclease recognition site; and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides; and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease

cleavage site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. Alternatives also include, wherein the polynucleotide above comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. Alternatives also include, wherein the polynucleotide above further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. Alternatives also include, wherein the gene above encodes a protein, a nuclease, or an endonuclease. Alternatives also include, wherein the nuclease above comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. Alternatives also include, wherein the at least one endonuclease recognition site above is a restriction endonuclease recognition site. Alternatives also include, wherein the restriction endonuclease recognition site above is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. Alternatives also include, wherein the at least one endonuclease recognition site above is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. Alternatives also include, but are not a part of the invention, wherein the endonuclease recognition site above is inverted. And, wherein the plurality of thymine nucleotides terminates in a cysteine, an adenine, or a guanine. Alternatives also include, wherein the polynucleotide is a linear polynucleotide. More alternatives concern methods of making a nucleic acid, comprising providing a polynucleotide above; and contacting said polynucleotide with a polymerase in the presence of nucleotides. The nucleic acid is RNA. Alternatives also include, wherein the RNA above comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. More alternatives concern methods of stabilizing an RNA during *in vitro* translation at 30- 37 degrees Celsius for at least 100 hours comprising providing the nucleic acid manufactured by the method above; and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30-37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). The contacting can be performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). The nucleic acid is an RNA. The RNA comprises a plurality of adenine nucleotides and, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the RNA further comprises a gene and, wherein the gene encodes, a protein for therapy, an endonuclease or a nuclease. More alternatives involve methods of stabilizing an RNA during *in vivo* translation at 30- 37 degrees Celsius for at least 100 hours in a cell, comprising transfecting into a cell the nucleic acid providing the nucleic acid manufactured by a method above; placing the cell into a culture vessel with media; supplying the cell with nutrients and amino acids for translation; and incubating the cell for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow for translation. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from the group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells. More alternatives concern methods of increasing and/or stabilizing expression of a protein comprising generating an RNA from the polynucleotide of claim 278; and translating said RNA into a peptide. In some alternatives, the translating further comprises providing an RNA polymerase. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease or a Type IIS restriction endonuclease and, preferably, the endonuclease is BsaI or StuI. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. More alternatives concern the RNA for use in treating, ameliorating, or inhibiting a disease in a subject comprising introducing into a cell the RNA manufactured by a method described above; and delivering the cell to the subject. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the cell is a human cell and preferably, the cell is CD4+ and/or CD8+.

[0040] Some alternatives relate to a polynucleotide. Some polynucleotides comprise a first nucleic acid sequence that comprises at least one endonuclease recognition site and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or

20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease cleavage site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein, a nuclease, or an endonuclease. In some alternatives, nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the at least one endonuclease recognition site is a restriction endonuclease recognition site. In some alternatives, the restriction endonuclease recognition site is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the plurality of thymine nucleotides terminates in a cysteine, an adenine, or a guanine. The polynucleotide is a linear polynucleotide.

[0041] Some alternatives relate to a method of making a nucleic acid. Some alternatives of the method comprise providing the polynucleotide of any of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. Some polynucleotides comprise a first nucleic acid sequence that comprises at least one endonuclease recognition site and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease cleavage site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein, a nuclease, or an endonuclease. In some alternatives, nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the at least one endonuclease recognition site is a restriction endonuclease recognition site. In some alternatives, the restriction endonuclease recognition site is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the plurality of thymine nucleotides terminates in a cysteine, adenine, or a guanine. The polynucleotide is a linear polynucleotide. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines.

[0042] Some alternatives provided herein are methods for stabilizing an RNA during *in vitro* translation at 30-37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times) comprising providing the nucleic acid manufactured by a method of any of the alternatives herein, and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30-37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times).

Contacting can be performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times. Some methods of making the nucleic acid comprise providing the polynucleotide of any of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. Some polynucleotides comprise a first nucleic acid sequence that comprises at least one endonuclease recognition site and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease cleavage site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein, a nuclease, or an endonuclease. In some alternatives, nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the at least one endonuclease recognition site is a restriction endonuclease recognition site. In some alternatives, the restriction endonuclease recognition site is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the plurality of thymine nucleotides terminates in a cysteine, adenine, or a guanine. The polynucleotide is a linear polynucleotide. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the nucleic acid is an RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides and, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the RNA further comprises a gene and, wherein the gene encodes, a protein for therapy, an endonuclease or a nuclease.

[0043] In some alternatives, a method of stabilizing an RNA during in vivo translation at 30- 37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times) in a cell is provided. In some alternatives, the method comprises transfecting into a cell the nucleic acid, providing the nucleic acid manufactured by a method of any of the alternatives provided herein, placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation; and incubating the cell for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times so as to allow for translation. Some methods of making the nucleic acid comprise providing the polynucleotide of any of the alternatives provided herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. Some polynucleotides comprise a first nucleic acid sequence that comprises at least one endonuclease recognition site and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease cleavage site is within 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein, a nuclease, or an endonuclease. In some alternatives, nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the at least one endonuclease recognition site is a restriction endonuclease recognition site. In some alternatives, the restriction endonuclease recognition site is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the plurality of thymine nucleotides terminates in an cysteine, adenine, or a guanine. The polynucleotide is a linear polynucleotide. In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the cell is a human cell. In some alternatives, the cell is selected from the group consisting of primary cells, human primary T cells, CD4+ cells and CD8+ cells.

[0044] In some alternatives, a method of increasing and/or stabilizing expression of a protein is provided, wherein the method comprises generating an RNA from the polynucleotide of any of the alternatives herein and translating said RNA into a peptide. Some polynucleotides comprise a first nucleic acid sequence that comprises at least one endonuclease recognition site and a second nucleic acid sequence that comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises an endonuclease cleavage site that is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides from the 5' terminal thymine in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid and, wherein said endonuclease recognition site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides of the endonuclease cleavage site and said endonuclease cleavage site is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides of the 5' terminal thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises a left arm that comprises a left telomere; a right arm comprising a right telomere; and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a third nucleic acid and, wherein the third nucleic acid comprises a gene. In some alternatives, the gene encodes a protein, a nuclease, or an endonuclease. In some alternatives, nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the at least one endonuclease recognition site is a restriction endonuclease recognition site. In some alternatives, the restriction endonuclease recognition site is a Type II restriction endonuclease recognition site or a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the plurality of thymine nucleotides terminates in a cysteine, adenine, or a guanine. The polynucleotide is a linear polynucleotide. In some alternatives, the translating further comprises providing an RNA polymerase, ribosomes, tRNAs and amino acids. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045]

Figure 1A and 1B are illustrations of the linear DNA vector comprising a site encoding a poly(A) tail and a BsaI

endonuclease cleavage site within the poly(A) tail encoding region (**Figures 1A and 1B**). As shown, the BsaI cut site is within the polyA encoding region. The BsaI recognition site is inverted and lies downstream from the polyA encoding region so that the endonuclease can cut within the polyA encoding region. A zoomed-in region of **Figure 1A** is shown in **Figure 1B**, which illustrates the region of interest, the multicloning site (MCS) with a poly (A) tail encoding region.

Figure 2 is a series of 3% agarose gel separations demonstrating the fidelity of DNA replication of the poly(A) encoding region of the plasmids that encode for poly(A) tracts of 100, 200, 300, or 500 covalently linked adenines. The plasmids encoding for the poly(A) tracts were generated from the linear vector shown in **Figures 1A and 1B**. After replication, the plasmids from the bacteria were cut on either side of the poly(A) encoding tract and the product was run on the 3% gel to examine the sizes of the poly(A) tract after replication. **Figure 2** demonstrates the shortening poly(A) encoding region of the DNA vector itself for the plasmids pEVL100, pEVL200, pEVL300, and pEVL500, when the plasmids are continuously replicated in bacteria grown at temperatures of 30°C and 37°C, and for times of 1 day, 6 days and 14 days.

Figure 3A and 3B are a series of Lonza FlashGels (Lonza) of 1.2% agarose gel, where separations of mRNA were run to illustrate the use of a pEVL linear vector as a template to produce highly homogenous mRNA. The pEVL linear template comprised a site encoding a poly(A) tail in which a BsaI endonuclease cleavage site is within the poly(A) tail and is compared to a standard method of adding a poly(A) tract by a transcription vector and a polyadenylation enzyme. The linear vectors used for transcribing the mRNA are derived from a linear vector, as shown in **Figures 1A and 1B**.

Figure 4 is a bar graph illustrating the expression of blue fluorescent protein (BFP) from the use of an mRNA comprising a poly(A) tail that was transcribed from a linear vector encoding a poly(A) tract in which a BsaI endonuclease cleavage site resides within the poly (A) encoding region for the translation of a reporter protein (BFP) (**Figures 1A and 1B**) at 30°C in human primary T cells. The mRNA produced from a plasmid comprising a poly(A) tract encoding region was compared to an mRNA produced from a standard method of adding a poly(A) tract by a transcription vector and a polyadenylation enzyme. In order to determine the efficiency of the transcript mRNA for translation for both methods, BFP was used as the reporter protein. As shown in the figure, the shaded region is the %BFP+ and the hatched regions are the BFP MFI.

Figure 5 is a bar graph that illustrates the expression of blue fluorescent protein (BFP) from the use of an mRNA comprising a poly(A) tail that was transcribed from a linear vector encoding a poly(A) tract in which a BsaI endonuclease cleavage site resides within the poly (A) encoding region for the translation of a reporter protein (BFP). RNA was generated from a linear vector encoding a poly(A) tract in which a BsaI endonuclease cleavage site resides within the poly (A) encoding region for the translation of a reporter protein (BFP) and the RNA was used to transfect human primary T cells. The RNA generated from pEVL300 (300 polyA encoded tail) and pWNY (enzymatic tailing) transfected into cells were assayed by looking at % BFP+ cells and the brightness (MFI) of those BFP+ cells over time, with and without the 30°C 24 hour incubation and were then compared.

Figure 6 shows the schematic of pEVL (extended variable length) vector (Panel A) and the quantification of TCR α knockout using pEVL and other vectors encoding a T7 promoter that were enzymatically poly-adenylated (panel B). This vector encodes a T7 promoter, necessary for RNA polymerase, an MCS for ease-of-cloning, an encoded poly-A tail of multiple lengths, ranging from 60bp (pEVL 100) to 500bp (pEVL 500), and a unique cleavage site, BsaI, to define the end of the polyA tail and terminate transcription. Panel B: Quantification of TCR α knockout using pEVL and other vectors encoding a T7 promoter that were enzymatically poly-adenylated. TCR α knockout was achieved using TALEN, electroporating primary human CD4+ T cells using the amount of RNA as indicated in the figure. pEVL constructs are able to achieve 90% knockout when cells are cultured at 37 degrees Celsius, and when incubated at 30°C for 24hours following electroporation, the lowest dose of TALEN provided by pEVL performs better than both enzymatically tailed mRNA.

Figure 7 shows that the TCR α CRISPR guide 4 (**SEQ ID NO. 5**) generates the highest TCR α -Knock out (KO) when used with a Cas9 protein expressed on a linear vector for a polyadenylated mRNA encoding Cas9.

Figure 8 shows GFP fluorescence of primary human T cells measured by a BD LSRII 24 or 48 hours following mRNA electroporation of mRNAs comprising poly(A) tails generated from a pEVL vector and AAV transduction of primary human T cells.

Figure 9 shows GFP mean fluorescence intensity (MFI) in primary human T cells 24 or 48 hours following mRNA

electroporation of Cas9 and Ad5 proteins (wildtype or mutants, as indicated, see e.g., **SEQ ID NOS: 1-4**) and transduction of AAV. mRNA from Cas9 was generated from a pEVL construct for generating a poly(A) tail of 200 covalently linked adenosines.

Figure 10 shows the shortening of poly(A) tracts upon cloning into standard circular plasmid cloning vector at 25 °C.

Figure 11 shows the generation and characterization of pEVL: a linear plasmid vector for generation of mRNA with extended encoded polyA tracts. A) Schematic of linear plasmid and conversion to pEVL B) Schematic of pEVL and method used for generation of extended poly(A) tracts in pEVL C) Shortening of poly(A) tracts upon cloning into standard circular or linear plasmid cloning vectors at 30 °C. BFP followed by Poly(A) tracts inserts of 72, 164, and 325 base pairs bounded by restriction enzyme sites.

Figure 12 shows the generation and characterization of mRNA from pEVL-encoded templates A) IVT mRNA encoding blue fluorescent protein (BFP) generated from pWNY with enzymatic tailing and pEVL-100 through pEVL-500 BFP-pEVL-100 to -500 were digested with XbaI and BsaI, and pWNY with ScaI and BsiWI, to generate template for IVT. IVT was carried out with ARCA capping, and for pWNY, enzymatic tailing with EPAP. After purification, 200ng of each transcript was imaged on a FlashGel (Lonza) system. Typically, pEVL produces a single band of defined length, whereas pWNY with enzymatic tailing produces transcripts of a more heterogeneous length. B) Relative potency and representative flow plots generated by mRNA encoding BFP generated from a circular plasmid vector with enzymatic poly-adenylation or from pEVL-100/pEVL-200. 1µg of IVT mRNA from the indicated template was electroporated into pre-stimulated primary human T-cells. After 24hr at 30 °C, the cells were analyzed by flow cytometry for the percentage of cells expressing BFP as well as the MFI of the BFP. C) Relative potency and representative flow plots from generated by mRNA encoding BFP generated from pEVL-100 through pEVL-500 Equimolar amounts of IVT mRNA from BFP-pEVL-100 to 500 was electroporated into pre-stimulated primary human T-cells. After 24hr at 30C, the percentage of cells expressing BFP and the BFI MFI was analyzed by flow cytometry.

Figure 13 shows the application of pEVL-encoded mRNA for gene editing in primary human T-cells A) pEVL-mediated editing of the TCRA locus with TALENs in primary human T-cells A TALEN pair targeting the constant region of the TCRA locus was cloned into pEVL-300. After IVT mRNA production, the indicated amounts of each TALEN half were electroporated into pre-stimulated primary human T-cells. After 72 hours, successful TCR knockout was assayed by flow cytometry for a lack of CD3 expression. B) pEVL-mediated editing of the TCRA locus with CRISPR/Cas9 in primary human T-cells A Cas9-T2A-mCherry construct was cloned into pEVL-200. After IVT mRNA production, Cas9 was electroporated into pre-stimulated primary human T-cells. 4 hr post-electroporation, a CRISPR guide targeting the TCRA constant region was delivered via AAV6 transduction. At 24 and 72hr post- electroporation, Cas9 electroporation efficiency was determined by FACS analysis of mCherry fluorescence. After 7 days, successful TCR knockout was assayed by flow cytometry for a lack of CD3 expression.

Figure 14 shows sequencing electropherograms covering the poly(A) tract in pEVL-300 Forward and reverse sequencing electropherograms generated using standard sanger sequencing chemistry over the poly(A) template in pEVL-300, demonstrating the existence of a homo-polymeric tract of at least 300 base pairs. Note that with a homopolymeric tract of this size, clear 3' boundaries are not expected due to polymerase slippage, thus explaining the gradual diminishment of the sequence and wavy appearance of the processed electropherogram that occur around the end of the tract.

Figure 15 shows the potency of mRNA generated from pEVL-300 variants with encoded terminal U, -UU, G-, - GG residues BFP-pEVL variants were generated for which the mRNA poly(A) tract was terminated by -U, -UU, -G, or -GG residues following BsaI digestion. mRNA was generated from these pEVL variants, and 0.5 microgram was transfected into primary human T-cells. BFP fluorescence was monitored by flow cytometry over a 7 day period. Plots represent relative MFI vs. time for each variant mRNA.

Figure 16 shows H9 T-cell line growth after transfection with pEVL encoded mRNA.

Figure 17 shows K562 cells line growth after transfection with pEVL encoded mRNA.

Figure 18 shows Jurkat cells growth after transfection with the pEVL-encoded mRNA.

Figure 19 shows that HepG2 liver cells growth after transfection with the pEVL-encoded mRNA.

Figure 20 shows CD34 cells that were transfected with BFP mRNA

Definitions

[0046] "Template strand" as defined herein, refers to the one strand of DNA that is used as a template for RNA synthesis and can also be referred to as the "noncoding strand." During the process of transcription, the RNA polymerase traverses the template strand and uses base pairing complementarity with the template strand to create an RNA copy. The RNA polymerase traverses the template strand in a 3' to 5' direction producing an RNA molecule from 5' to 3' as an exact copy of the coding strand, in the exception that the thymines are replaced with uracils in the RNA strand. In some alternatives, a template strand is provided, wherein the template strand can comprise a plurality of thymine nucleotides. In some alternatives, a nucleic acid is provided, wherein the nucleic acid is a DNA, wherein the DNA is a double stranded DNA comprising the template strand. a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0047] "Coding strand" as defined herein, refers to the DNA strand which has the same base sequence as the RNA transcript (mRNA) that is produced during transcription. The coding strand is the strand used when displaying a DNA sequence in the 5' to the 3' direction.

[0048] As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (e.g., enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded. As referenced above, in some alternatives, the CRISPR/Cas9 system described herein, whereby the polynucleotide encoding the Cas9 nuclease or a derivative or functional fragment thereof (e.g., **SEQ ID NOs. 6-14**) is provided with a poly(T) or poly(A) tail of a desired length and prepared in accordance with the teachings described herein, is provided with a guide RNA that comprises one or more modified bases, such as any one or more

of the modified bases described above. Exemplary guide RNAs useful with the alternatives described herein, which may contain one or more of the modified bases set forth above are provided in **SEQ ID Nos. 5, 15, 16, and 17.**

[0049] A nucleotide is composed of a nucleobase, a five-carbon sugar (i.e., either ribose or 2-deoxyribose), and one or more than one phosphate groups (i.e., two or three phosphate groups). Thus, the term "nucleotide" generally refers to a nucleoside monophosphate, but a nucleoside diphosphate or nucleoside triphosphate can be considered a nucleotide as well.

[0050] Without the phosphate group, the nucleobase and sugar compose a nucleoside. The phosphate groups form bonds with either the 2, 3, or 5-carbon of the sugar, with the 5-carbon site most common. Cyclic nucleotides form when the phosphate group is bound to two of the sugar's hydroxyl groups. Nucleotides contain either a purine or a pyrimidine base. Ribonucleotides are nucleotides in which the sugar is ribose. Deoxyribonucleotides are nucleotides which has deoxyribose as the sugar. Nucleic acids are polymeric macromolecules made from nucleotide monomers. In DNA, the purine bases are adenine (A) and guanine (G), while the pyrimidines are thymine (T) and cytosine (C). RNA uses uracil (U) in place of thymine (T). Adenine always pairs with thymine by two hydrogen bonds, while guanine always pairs with cytosine through three hydrogen bonds, each due to their unique structures.

[0051] "Nucleosides" as described herein, are glycosylamines that can be thought of as nucleotides without a phosphate group. A nucleotide is composed of a nucleobase, also referred to a nitrogenous base, a five-carbon sugar (i.e., ribose or deoxyribose), and one or more phosphate groups while a nucleoside consists simply of a nucleobase and a 5-carbon sugar. However, in a nucleoside, the base is bound to either ribose or deoxyribose via a beta-glycosidic linkage. Examples of nucleosides include cytidine, uridine, adenosine, guanosine, thymidine and inosine.

[0052] "Adenine" is a nucleobase, and is a chemical component of DNA and RNA. As used herein, adenines can refer to polyadenosine ribonucleotides, polyadenosine monophosphates or polyadenylyls. The shape of adenine is complementary to either thymine in DNA or uracil in RNA. Thymine, is one of the four nucleobases in the nucleic acid of DNA. As used herein, thymines can refer to polythymidine ribonucleotides, polythymidine monophosphates or polythymidylys. As used herein, the term "covalently linked adenines," as referred to in the term "poly(A) tract" in reference to a polynucleotide comprising DNA or RNA, can refer to a polymer of covalently linked deoxyadenosine nucleotides comprising moieties of polyadenosine ribonucleotides, polyadenosine monophosphates, or polyadenylyls. As used herein the term "covalently linked thymines," as referred to in a poly(T) tract, can refer to a polymer of covalently linked deoxythymidine nucleotides comprising moieties of polythymidine ribonucleotides, polythymidine monophosphates, or polythymidylys.

[0053] "Polyadenylation" as described herein, refers to the addition of covalently linked adenines at the 3'-end of a primary, or pre-messenger RNA (pre-mRNA). The addition of a stretch of covalently linked adenine residues (poly(A) tail) is a process that produces a mature messenger RNA (mRNA) for translation. Polyadenylation, a eukaryotic mechanism, is initiated *in vivo* when transcription of a gene is terminated and the pre-mRNA is cleaved by a protein, Cleavage and Polyadenylation Specificity Factor (CPSF). Following cleavage, polyadenylate polymerase polymerizes the poly(A) tail and can add up to 200-250 covalently linked adenine residues.

[0054] A "telomere" as described herein is a region of repetitive nucleotide sequences at each end of a nucleic acid, which protects the ends from deterioration. In some alternatives, a polynucleotide is provided wherein the polynucleotide comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide comprises a first protelomerase target site in the left arm and a second protelomerase target site in the right arm.

[0055] An untranslated region (or UTR) refers to either of two sections, one on each side of a coding sequence on a strand of mRNA. If it is found on the 5' side, it is called the 5' UTR (or leader sequence), or if it is found on the 3' side, it is called the 3' UTR (or trailer sequence). UTRs are found in naturally occurring mRNAs. In some alternatives, the RNA does not have a 3' UTR or a 5' UTR. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII).

[0056] "Nuclease" or "endonuclease" as described herein, refers to an enzyme capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. In some alternatives a polynucleotide encoding a nuclease is provided. Without being limiting, examples of endonucleases can include for example, a CRISPR enzyme or a restriction endonuclease.

[0057] A "restriction enzyme" or "restriction endonuclease" is an enzyme that recognize and bind DNA at or near specific recognition nucleotide sequences so that it can cut at a restriction cleavage sites. In some alternatives, a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. In some alternatives the endonuclease recognition site is specific for a restriction endonuclease. Without being limiting, examples of Type II restriction endonucleases can include, for example, AatII, AbaSI, Acc65I, Accl, Acil, AclI, Afel, AfIII, AfIII, Agel, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, Apol, AscI, AseI, AsiSI, Aval, Avail, AvrII, BaeGI, Bael, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, Bccl, BceAI, BcgI, BciVI, BclI, BcoDI, BfaI, BfuAI, BfuCI, BglI, BglII, BglIII, BlnI, BmgBI, Bmrl, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiWI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, BsrI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Eco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinPI, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, NruI, NsiI, NspI, Nt.AlwI, Nt.BbvCI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.CviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SspI, StuI, StyD4I, Styl, SwaI, TaqI, TfiI, TliI, TseI, Tsp45I, Tsp509I, TspMI TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, and ZraI. In some alternatives, the restriction endonuclease is AatII, AbaSI, Acc65I, Accl, Acil, AclI, Afel, AfIII, AfIII, Agel, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, Apol, AscI, AseI, AsiSI, Aval, Avail, AvrII, BaeGI, Bael, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, Bccl, BceAI, BcgI, BciVI, BclI, BcoDI, BfaI, BfuAI, BfuCI, BglI, BglII, BglIII, BlnI, BmgBI, Bmrl, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiWI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, BsrI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Eco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinPI, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, NruI, NsiI, NspI, Nt.AlwI, Nt.BbvCI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.CviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SspI, StuI, StyD4I, Styl, SwaI, TaqI, TfiI, TliI, TseI, Tsp45I, Tsp509I, TspMI TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, or ZraI. In some alternatives, the restriction endonuclease is BsaI. In some alternatives, the restriction endonuclease is StuI. In some alternatives, the restriction site is inverted and the restriction endonuclease cleavage site is inverted.

[0058] A type II restriction endonuclease as described herein, refers to an endonuclease that form homodimers, with recognition sites that are usually undivided and palindromic and 4-8 nucleotides in length. They recognize and cleave DNA at the same site, and they do not use ATP or AdoMet for their activity.

[0059] A "Type IIS restriction endonuclease," as described herein, refers an endonuclease that cleaves DNA at a defined distance from their palindromic or a non-palindromic asymmetric recognition sites. Examples of Type IIS restriction endonucleases include but are not limited to Acil, MnlI, AlwI, BbvI, Bccl, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, Bael, BsaXI, CspCI, Afal, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI,

Mael, Maell, Mvnl, Pall, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefl, BcnI, Bisl, Blsl, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, Cdil, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Ital, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, Aaul, Acc113I, Acc16I, AccB1I, AceIII, Acsl, Acvl, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvallI, Avill, BanIII, Baul, Bbel, BbrPI, Bul, Bbv12I, BbvII, Bce83I, Bcol, Bcul, Bfml, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, Dsal, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, Fbal, FblI, FriOI, FunI, FunII, GdII, Gsal, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MflI, MhII, MIsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvrl, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, Pcel, Pff23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, Rcal, Sdul, Sfr274I, Sfr303I, Sful, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, Aocl, Axyl, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, Cspl, Drail, Eco065I, Eco81I, Eco91I, Espl, KflI, Lgul, Mabl, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, Mrel, Mssl, Rgal, Sdal, SfaAI, Sgfl, Smil, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNI, Maml, MroXI, OilI, Pdml, Rsel, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMWM, Cjel, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PflBI, UbaF14I, Xagl, Aasl, Bdal, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi and UbaF13I. The Type II restriction endonuclease cleavage site and recognition sites are shown in **Table 1** (SEQ ID Nos: 18-632, 634-636). a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides. Some polynucleotides comprise a first nucleic acid sequence comprising at least one restriction endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides, wherein the second nucleic acid comprises the restriction endonuclease cleavage site, and wherein the restriction endonuclease cleavage site is within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said restriction endonuclease recognition site is located within 1-55 base pairs such as, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs, of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs, of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the Type IIS restriction endonuclease is Acil, MnlI, AlwI, BbvI, Bccl, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MbolI, MlyI, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, Bsal, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, Mmel, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, Bael, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, Mael, Maell, Mvnl, Pall, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefl, BcnI, Bisl, Blsl, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, Cdil, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Ital, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, Aaul, Acc113I, Acc16I, AccB1I, AceIII, Acsl, Acvl, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvallI, Avill, BanIII, Baul, Bbel, BbrPI, Bul, Bbv12I, BbvII, Bce83I, Bcol, Bcul, Bfml, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I,

BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FblI, FriOI, FunI, FunII, GdIII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, NsbI, NspIII, NspV, PaeI, PagI, Paul, PceI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CeiI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, said endonuclease recognition site is located within 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 55 base pairs of the endonuclease cleavage site for the endonuclease or any number of base pairs between two aforementioned values, and said endonuclease cleavage site is within 1, 5, 10, 15, 20, 25, 30, 35 or 40 base pairs of the most 5' thymine of the plurality of thymine nucleotides or any number of base pairs between any two aforementioned values. In some alternatives, the endonuclease recognition site is inverted and the endonuclease cleavage site is inverted. In some alternatives, the endonuclease recognition site and endonuclease cleavage site comprise a sequence set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636), wherein the endonuclease recognition site and endonuclease cleavage site are specific for the Type II restriction endonuclease Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PleI, SfaNI, AclI, BciVI, BfuAI, BmgBI, Bmri, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, Afal, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaiI, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, Mvni, PalI, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AfiI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, BcnI, Bisl, Blsl, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, CdiI, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, Acsi, Acvi, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvilI, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, Bcol, Bcul, Bfml, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FblI, FriOI, FunI, FunII, GdIII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, NsbI, NspIII, NspV, PaeI, PagI, Paul, PceI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CeiI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. As shown in **Table 1**, the endonuclease, or restriction endonuclease can cleave between 1 and 50 bases away from a recognition site (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs from the endonuclease cleavage site of from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0060] A "recognition site" as described herein, refers to a sequence on a nucleic acid site that is recognized and bound by a restriction enzyme, nuclease, or a restriction endonuclease.

[0061] A "cleavage site" as described herein, is a site on a nucleic acid that is cleaved by a restriction endonuclease or endonuclease. In some alternatives, the endonuclease is a Type IIS endonuclease. In some alternatives, the recognition site and cleavage site are inverted.

[0062] A "palindromic sequence" as described herein, refers to a nucleic acid sequence on double-stranded DNA or RNA wherein reading 5' (five-prime) to 3' (three prime) forward on one strand matches the sequence reading backward 5' to 3' on the complementary strand or on itself. Complimentary strands can bind to the compliment in a palindromic sequence leading to a "hairpin" structure.

[0063] "A promoter" refers to a region of DNA that initiates replication. Promoters can be 100-1000 base pairs long. In some alternatives, of the polynucleotide, the polynucleotide further comprises a DNA polymerase promoter.

[0064] "Tertiary structure" is the 3D structure formed by a double stranded or a single stranded nucleic acid and is dependent on the sequences. Examples of tertiary structures seen in nucleic acid include but are not limited to a double helix, stem loops, hairpins, major and minor groove triplexes (RNA), quadruplexes, coaxial stacking, tetraloops, A-minor motifs and ribose zippers. In some alternatives of the polynucleotide, the polynucleotide further comprises a sixth sequence, wherein the sixth sequence is capable of forming a tertiary structure. In some alternatives, the tertiary structure comprises a double helix, stem loops, hairpins, major and minor groove triplexes (RNA), quadruplexes, coaxial stacking, tetraloops, A-minor motifs and/or ribose zippers.

[0065] "Specific" or "Specificity" as described herein, refers to the ability of a protein to bind to a defined binding site. As described herein, an enzyme can be specific for binding to a specific sequence, for example, a specific sequence on a nucleic acid.

[0066] "Upstream" and "downstream" as used herein, refers to relative positions in either DNA or RNA. Each strand of DNA or RNA has a 5' end and a 3' end, so named for the carbon position on the deoxyribose (or ribose) ring. Upstream and downstream relate to the 5' to 3' direction in which RNA transcription takes place. Upstream is toward the 5' end of the RNA molecule and downstream is toward the 3' end. When considering double-stranded DNA, upstream is toward the 5' end of the coding strand for the gene in question and downstream is toward the 3' end. Due to the anti-parallel nature of DNA, this means the 3' end of the template strand is upstream of the gene and the 5' end is downstream.

[0067] "Sticky end" as referred herein refers to an overhang, which is a stretch of unpaired nucleotides in the end of a DNA molecule. Sticky ends can be created with an endonuclease, such as a restriction endonuclease. For example, some endonucleases cleave a palindromic sequence and can leave an overhang, or a sticky end.

[0068] "Blunt end" as referred herein, refers to a blunt-ended molecule in which both strands of in a nucleic acid terminate in a base pair. Some alternatives relate to a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the endonuclease produces a blunt end during cleavage. In some alternatives, the endonuclease is Stul.

DETAILED DESCRIPTION

[0069] *In vitro* transcribed (IVT) mRNA has emerged as an important modality of transient heterologous protein expression for therapeutic applications. Electroporation of IVT mRNA's is presently being applied in translational contexts ranging from *in vivo* expression of antigenic proteins in dendritic cells for cancer and infectious disease vaccine purposes, to *ex vivo* expression of nucleases in primary hematopoietic stem cells and T-cells for gene editing, and expression of chimeric antigen receptors in primary human T- cells for cancer immunotherapy. Nano-particle based IVT mRNA-mediated transient gene expression is under development for *in vivo* administration as a novel modality for protein replacement therapies.

[0070] An important issue in any IVT mRNA application is to obtain efficient protein expression. Increased efficiency of expression reduces costs by directly reducing the quantity of mRNA necessary for a required level of expression, and can also reduce the potential for antigenicity of the mRNA due to the reduced exposure of a patient's cells to a foreign nucleic acid. Numerous determinants of the efficiency of expression of an mRNA have been identified, including the nature of the 5' cap, the efficiency of capping, nature of 5' and 3' UTR's, codon optimization of protein coding sequence, presence of miRNA target sequences in the protein coding sequence and UTR's, and the length of the polyadenosine (poly(A)) tail. Of these determinants, the length of the poly(A) tail is most problematic to optimize: although poly(A) tail length is known to be one of the most important physiological determinants of translational efficiency and mRNA stability, existing technologies do not allow for production of IVT mRNA with defined length poly(A) tails in the physiological (>200 base pairs) range.

[0071] The alternatives described herein demonstrate a novel method for generation of homopolymeric tracts of arbitrary length and content, and demonstrate that a previously described linear plasmid, linear plasmid, is capable of propagating poly(A) tracts up to at least 500 base pairs in length. Further, in an exemplary alternative, linear plasmid is modified by removing extraneous BsaI sites while incorporating a unique BsaI site downstream from the poly(A) tract to create pEVL, a linear plasmid that allows for facile generation of IVT mRNA's with defined, extended poly(A) tails using standard T7 phage RNA polymerase chemistry.

[0072] Described herein are methods for making a linear polynucleotide having a polymer of covalently linked deoxyadenosine nucleotides, which can be extended to a desired length by virtue of the presence of an endonuclease site within or upstream of said polymer of covalently linked deoxyadenosine nucleotides such that a poly(A) insert of a desired length can be ligated to the polynucleotide having the polymer of covalently linked deoxyadenosine nucleotides and endonuclease site thereby extending the poly(A) tract to the desired length. The polynucleotide having a polymer of covalently linked deoxyadenosine nucleotides can also be referred to as a polymer of covalently linked deoxythymidines or a polymer of covalently linked deoxythymidine monophosphates, when referencing to the antisense or template strand. The polynucleotide strand can be digested and ligated to extend the length further to 500 thymine nucleotides or more. This polynucleotide can serve as the "anti-sense" or template strand that can then be transcribed so as to generate an RNA having a poly(A) tract of a desired length, e.g., the length of the poly(T) polymer after ligation with the poly(T) insert. The polynucleotide as described herein can serve as a DNA template for transcription with increased stability, and can improve gene transcription with fewer steps leading to improved protein translation due to a longer poly(A) tract in the transcribed RNA which would be a more pure product, e.g., the lengths of the transcribed RNA would all be one size. Accordingly, several compositions and methods for generating nucleic acids having a poly(T) tract and poly(A) tract nucleic acids are described herein. As used herein, in some aspects, poly(A) and poly(T) are used interchangeably in the same context depending on reference to a sense or anti-sense strand. The use of these compositions and methods to increase the stability of a nucleic acid transcript, such as a DNA template used for transcription, enhance and improve gene transcription such as allowing fewer steps for generating a homogeneous RNA product, and/or improve protein translation such as a more efficacious product, are contemplated. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Endonucleases can be an enzyme that can enzymes that cleave the phosphodiester bond within a polynucleotide. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the

endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, translation is performed for at least, greater than, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times.

[0073] The polynucleotide described herein, can further comprise genes for expression. Examples of genes can include but are not limited to transcriptional regulators (e.g., eukaryotic histone remodeling enzymes, transcription factors, enhancers and repressors), chimeric antigen receptors (CARs), T-cell receptors (TCR), nucleases (e.g., Cas9 or a derivative thereof or active fragment thereof, see e.g., **SEQ ID. Nos. 6-14**, MegaTAL, TALEN), epigenetic modulators (e.g., DNA methylation proteins, histone modification proteins, chromatin remodeling proteins, methyl-CpG binding proteins) and vaccine antigens (e.g., cancer vaccine antigens and infectious disease antigens). Accordingly, in some alternatives, the polynucleotide encodes a gene. In some alternatives, the gene encodes eukaryotic histone remodeling enzymes, transcription factors, enhancers, repressors, CARs, TCR's, Cas9 or derivatives thereof or active fragments thereof see e.g., **SEQ ID. Nos. 6-14**, MegaTAL, TALEN, DNA methylation proteins, histone modification proteins, chromatin remodeling proteins, methyl-CpG binding proteins, or a vaccine antigen. In many of the alternatives described herein, the polynucleotide described herein having a poly(T) or poly(A) tail encodes a Cas9 nuclease and said polynucleotide is delivered to a cell with a guide RNA so as to allow for CRISPR/Cas9 gene editing. The basic components of CRISPR/Cas9 system comprise a target gene, a guide RNA, and a Cas9 endonuclease. An important aspect of applying CRISPR/Cas9 for gene editing is the need for a system to deliver the guide RNAs efficiently to a wide variety of cell types. This could for example involve delivery of an *in vitro* generated guide RNA as a nucleic acid (the guide RNA generated by *in vitro* transcription or chemical synthesis). In some alternatives the nucleic acid encoding the guide RNA is rendered nuclease resistant by incorporation of modified bases, such as 2'-O-methyl bases. An important system for expressing guide RNAs in this context is based on the use of adeno-associated virus (AAV) vectors because AAV vectors are able to transduce a wide range of primary cells. AAV vectors do not cause infection and are not known to integrate into the genome. Therefore, the use of AAV vectors has the benefits of being both safe and efficacious.

[0074] The 3'-termini of eukaryotic RNAs reflects their regulatory status and plays an important role in determining the fate of RNAs. The function of the poly(A) tail in mRNA is important for nuclear export, facilitating translation initiation and efficiency, and ensuring the stability of the mRNA, all of which is dependent on the length of the poly-A tail. In eukaryotes, the polyadenylation protects the mRNA molecule from exonucleases and is important for transcription termination, for export of the mRNA from the nucleus, and for translation efficiency. Some prokaryotic mRNAs are also polyadenylated, although the poly(A) tail function is different from that in eukaryotes. For example, in prokaryotes, polyadenylation promotes the degradation of a regulatory RNA that inhibits the replication of bacterial plasmids and may play a similar role in the degradation of mRNA.

[0075] Due to the benefits of adding a poly(A) tail to an mRNA, techniques have been reported in which poly(A) tails have been added through a commercial polyadenylate enzyme (e.g., E-PAP), and PCR methods using a specific 5'-specific primer and a poly(A) tail-specific primer. However, there are many undesirable aspects to these techniques. Use of a polyadenylate enzyme, such as E-PAP, will generate poly(A) tails of varying lengths, for example, which is not desired in some applications. Furthermore, enzymatic approaches are an additional costly step and usually require extensive purification procedures, which can lead to a significant loss of the product. Although the addition of a poly (A) tail to the mRNA by PCR avoids the extra enzymatic step, PCR methodologies can also bring additional problems, such as length variation, as well as amplification abnormalities, which can stem from specific genes that are not readily amplified by PCR. PCR is also restricted by the number of adenine residues that can be contained in a primer (~120). More importantly, it is also limited to genes that can be amplified by PCR. Genes that are refractory to PCR include but are not limited to genes with repetitive elements (including TAL effector nucleases, which are currently used in genome editing), secondary structure, skewed GC ratios, and other recalcitrant features. Still further, the specific PCR

conditions required for certain genes can also affect amplification (e.g., TAL Effector Nucleases).

[0076] The addition of short poly(A) sequences can also be performed through commercially available circular *Escherichia coli* plasmids that contain poly-thymine (poly (T)) tracts for transcribing an mRNA with a poly(A) tract. However, commercially available plasmids only contain poly(T) tracts of 30 bases in length, which are not effective for *in vivo* use (only *in vitro*). Plasmids can be custom-ordered to have up to 100 poly(T) tracts, but the poly(T) tracts can rapidly shorten to about 30-40 bases under normal growth conditions. Another undesired aspect can arise from the use of circular plasmids required by these methodologies, such as supercoiling and the consequent inability to clone large or "difficult" polynucleotide sequences that are not easily maintained or unstable in the supercoiled plasmid form. Further complicating these approaches, is that the template is very unstable during the propagation in *E. coli*, leading to tracts that can only transcribe poly(A) tails of about 30 adenines, which can spontaneously shorten again leading to the problem of different poly(A) tail lengths in the product mRNA. Sustaining a much longer length of poly(A) tract is correlated with translational efficiency, and in order to maintain a poly(A) tail length greater than 30 adenines requires a 25 °C growth condition so as to prevent shortening. However, low temperatures can also extend the propagation time significantly.

[0077] In the present disclosure, the addition of a poly(A) tract of a desired length has been made possible by generating a poly(T) containing polynucleotide having one or more endonuclease sites (e.g., within the poly(T) domain itself or juxtaposed to the poly(T) domain, such as within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the most 5' thymine residue of the poly(T) domain in the template strand, but configured or spaced to permit enzymatic cleavage within the poly(T) domain) thereby allowing the introduction of an insert comprising an additional poly(T) tract and ligation, which when transcribed, allows one to control the length of the poly(A) tract in a resultant RNA. New approaches to add a poly(A) tail to a "difficult" or large gene are also described herein. Endonucleases can be an enzyme that can enzymes that cleave the phosphodiester bond within a polynucleotide. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0078] For quite some time the field has desired the ability to control the length of a poly(A) tract so as to increase the polynucleotide stability during replication, increase transcriptional efficiency, increase half-life or stability of an RNA molecule, as well as to increase the translational efficiency of a gene. An increased half-life of RNA can also allow for the use of RNA for increased protein production. RNA can also be used as a therapeutic mRNA in which its increased half-life can lead to production of a protein that is needed in therapy, and the breakdown of the therapeutic mRNA can be controlled by using a determined poly(A) tail length in order to lengthen the time of treatment.

[0079] Poly(A) tails with alternative termination bases can also be achieved by the methods described herein. To date, there are no methods described in which a poly(A) tail can end in an alternative nucleotide base such as guanine, cytosine, thymine, or uracil by use of an enzyme or an available plasmid. Creating a poly(A) tail with alternative terminal sequences can allow one to test the influence of various tails. In some alternatives, a polynucleotide having a plurality of thymine nucleotides is made and an endonuclease recognition site is inserted therein or juxtaposed to the poly(T) tract (e.g., within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the most 5' thymine residue of the poly(T) domain in the template strand, but configured or spaced to permit enzymatic cleavage within the poly(T) domain) thereby allowing the introduction of an insert comprising an additional poly(T) tract and ligation, which when transcribed, allows one to control the length of the poly(A) tract in a resultant RNA. In some alternatives, the polynucleotide is extended by insertion and ligation of a

polymer of covalently linked thymine residues terminating in a guanine, cytosine, or an adenine. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0080] Important factors for mRNA production can include, but are not limited to, the stability of the tail length, the purity and uniformity of the mRNA, and the cost and time factors. Described herein are methods, wherein the addition of a poly(A) tract to an mRNA is realized in a one step process. A one step process is highly desirable over the multi-step processes that involve the use of PCR and enzymes to add on a poly(A) tract. Additionally, the mRNA produced from a polynucleotide comprising a poly(T) tract having one or more endonuclease cleavage sites allowing the introduction of an insert, can be used in both biotechnology and therapeutic approaches. The polynucleotide that generates the resultant polypeptide, e.g., a vector, can also have a consensus sequence for initiating the translation process. Without being limiting, such consensus sequences can include Kozak, T3, SP6, and/or T7 consensus sequences. The polynucleotide that generates the resultant nucleic acid can also include stop codons and/or mammalian expression sequences, such as activation sequences or enhancer sequences. Without being limiting, the stop codons can include TAG, TAA, and TGA sequences. Without being limiting, stop codons in RNA can include UAG, UAA, and UGA sequences. In some alternatives of the polynucleotide, the polynucleotide comprises stop codons. In some alternatives, the stop codons are TAG, TAA, or TGA sequences. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0081] By one approach, a linear polynucleotide having a plurality of thymine nucleotides is made and an endonuclease recognition site is inserted therein or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the most 5' thymine residue of the poly(T) domain of the template strand, but configured or spaced to permit enzymatic cleavage within the poly(T) domain) thereby allowing the introduction of an insert comprising an additional poly(T) tract and ligation, which when transcribed, allows one to control the length of the poly(A) tract in a resultant RNA. Accordingly, methods for making a nucleic acid from a polynucleotide having a plurality of thymine nucleotides and an endonuclease recognition site disposed within or juxtaposed to (e.g., 1-55 nucleotides downstream from the most downstream or most 5' thymine of the poly(T) tract) said plurality of thymine nucleotides of the template strand) is provided herein. Methods for enhancing transcription and translation utilizing said compositions are also alternatives. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG.

In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0082] In some alternatives, a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides is provided. The polynucleotide can comprise a first nucleic acid sequence comprising at least one endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 1 to 55 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs) of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs) of a most 5' thymine (or 5' terminal thymine) of the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises a T3, SP6, and/or a T7 sequence. In some alternatives, the polynucleotide may further comprise a Kozak sequence. In some alternatives, the polynucleotide may further comprise a stop codon. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the covalently linked thymines terminate in a guanine, adenine, or cytosine after cleavage by the endonuclease. In some alternatives, there exists a sequence that is complimentary to an endonuclease site (on the single strand), wherein the sequence is complimentary to any one of the restriction endonuclease recognition sites set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636) and SEQ ID NO: 633. In some alternatives, the endonuclease recognition sites and the complimentary sequence is inverted. Endonucleases can be an enzyme that can enzymes that cleave the phosphodiester bond within a polynucleotide. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0083] In some alternatives, the resultant linear polynucleotide comprising said template strand, comprises a first and second nucleic acid. The second nucleic acid comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides or any number of thymine nucleotides between any two aforementioned values, and the second nucleic acid further comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides. The first nucleic acid comprises the endonuclease recognition site, wherein the first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 55 base pairs of the endonuclease cleavage site for the endonuclease or any number of base pairs between any two aforementioned values, and said endonuclease cleavage site is within 1 to 40 base pairs of the most 5' thymine of the plurality of thymine nucleotides. In some alternatives, this allows enzymatic cleavage within the plurality of thymine nucleotides (poly(T) domain) leaving a poly (T) insert of a desired length comprising a final polymer of covalently linked thymine residues that is at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275,

300, 325, 350, 375, 400, 425, 450, 475 or 500 thymine residues or any number of thymine residues between any two aforementioned values. As shown in **Table 1** (SEQ ID Nos: 18-632, 634-636) and SEQ ID NO: 633, an endonuclease recognition site can reside between 1 and 50 nucleic acids away from a cleavage site, therefore in order to achieve a poly(A) tail of 500 adenines, the poly(T) must be longer than 500 thymines in length, depending on the endonuclease used. In some alternatives, the resultant polynucleotide having a polymer of covalently linked thymine nucleotides, an endonuclease recognition site within said polymer of covalently linked thymine nucleotides or within 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the most 5' thymine residue of the poly(T) domain but configured or spaced to permit enzymatic cleavage within the poly(T) domain), and a poly (T) insert of a desired length comprises a final polymer of covalently linked thymine residues that is at least, greater than, equal to, or any number in between 200 or 550 thymine residues, is provided. In some alternatives, the resultant polynucleotide having a polymer of covalently linked thymine nucleotides, an endonuclease site within said polymer of covalently linked thymine nucleotides or within 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from most 5' thymine residue of the poly(T) domain in the template strand, but configured or spaced to permit enzymatic cleavage within the poly(T) domain), and a poly (T) insert of a desired length comprises a final polymer of covalently linked thymine residues that is at least, greater than, equal to, or any number in between 200, 250, 300, 350, 400, 450, 500 or 550 thymine residues. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the covalently linked thymines terminate in a guanine, adenine, or cytosine after cleavage by the endonuclease. In some alternatives, the polynucleotide can further encode a gene. In some alternatives, the gene is an endonuclease. In some alternatives, the gene is Cas9 or a derivative thereof or active fragment thereof, see e.g., **SEQ ID. Nos. 6-14**. In some alternatives, the gene is a TALEN. In some alternatives, the gene is a MegaTAL. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the covalently linked thymines terminate in a guanine, adenine, or cytosine after cleavage by the endonuclease. In some alternatives, the endonuclease recognition sites and the complimentary sequence is inverted. In some alternatives, the endonuclease recognition site is a sequence set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease recognition site set forth in **Table 1** is inverted. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

[0084] Disclosed herein are methods of making such a nucleic acid, such as RNA, as well. By some approaches, the method comprises providing the polynucleotide of any of the alternatives described above, and contacting said polynucleotide with a polymerase in the presence of nucleotides. In some alternatives, the polynucleotide further comprises a promoter for a DNA or RNA polymerase or both. In some alternatives, the nucleotides that are included in the reaction with the polymerase are adenine, cytosine, guanine, thymine, and/or uracil. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PfiI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI,

BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1I, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, Bcni, Bisi, Blsi, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BsiFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Itai, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, Sati, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, Acsl, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, Bcol, Bcul, Bfml, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, Fbal, FbII, FriOI, FunI, FunII, GdiII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, Misi, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvri, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, Pcel, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, Rcal, Sdul, Sfr274I, Sfr303I, Sful, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, Tati, Vha464I, Vnei, Xapi, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, Axyl, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, Cspi, Drail, Eco065I, Eco81I, Eco91I, Espl, KfiI, Lgul, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, Mrei, MssI, Rgal, Sdal, SfaAI, Sgfi, Smil, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNI, Mami, MroXI, Olil, Pdml, Rsel, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, Cjel, Cjul, Cjull, Dril, Eam1105I, EdHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, Xagl, Aasl, Bdal, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. In some alternatives, the resultant RNA obtained after transcription of the aforementioned poly(T) containing constructs comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked adenine residues and said RNA can contain a sequence complimentary to an endonuclease recognition site (on the single strand), such as StuI or BsaI, within said poly(A) tail or juxtaposed to said poly(A) tail (e.g., within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values upstream (5' in orientation) from the first adenine residue of the poly(A) domain), when the polynucleotide is not cleaved by the endonuclease. In some alternatives, wherein the polynucleotide is not cleaved by the endonuclease, the resulting nucleic acid (RNA) comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 or 500 covalently linked adenine residues. In some alternatives, the resultant RNA (mRNA) terminates in a guanine, cytosine or a uracil. In some alternatives, the mRNA comprises a poly(A) tail, wherein within said poly(A) tail there exists a sequence that is complimentary to an endonuclease site (on the single strand), such as StuI or BsaI site. In some alternatives, there exists a sequence that is complimentary to an endonuclease site (on the single strand), wherein the sequence is complimentary to any one of the endonuclease recognition sites set forth in Table 1 (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease recognition sites and the complimentary sequence is inverted. In some alternatives, the mRNA comprises a first sequence encoding an RNA compliment to at least one endonuclease site, such as StuI or BsaI site, wherein the sequence encoding the RNA compliment is covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further comprises or encodes a gene. In some alternatives, the gene is an endonuclease gene. In some alternatives, the gene encodes Cas9 or a derivative thereof or active fragment thereof, see e.g., SEQ ID. Nos. 6-14, TALEN or a MegaTAL. In some alternatives, the at least one endonuclease recognition site is inverted, and the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site (on the single strand). In some alternatives, the endonuclease recognition site is a sequence set forth in Table 1 (SEQ ID Nos: 18-632, 634-636). In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site, such as StuI or BsaI site. In some alternatives, the one endonuclease is a Type IIS restriction endonuclease recognition site. In some alternatives, a pharmaceutical composition is provided, wherein the pharmaceutical composition comprises a pharmaceutical vehicle and a nucleic acid. In some alternatives, methods are provided for delivering a pharmaceutical composition to a subject in need. In some alternatives, methods are provided for introducing an mRNA into a cell for delivery to a subject in need. In these alternatives, the cell is provided for translation of a protein for therapy in the subject in need. In some alternatives, the protein is a nuclease,

such as Cas 9 or a derivative thereof, see e.g., SEQ ID. Nos. 6-14, TALEN or MegaTAL. In some alternatives, a method of delivering a pharmaceutical composition comprising the nucleic acid is provided, wherein the method comprises delivering the pharmaceutical composition to a subject in need. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

Endonuclease sites, Restriction Endonuclease Sites and Enzymes (Endonucleases).

[0085] A gene can be added to a plasmid by the amplification of the gene utilizing PCR methods, wherein endonuclease recognition sites are added to the gene by way of primers and said gene is then inserted into the plasmid by molecular cloning techniques, e.g., ligation reactions. "Restriction sites," "endonuclease recognition sites," or "endonuclease cleavage sites" or "restriction enzyme binding domains" are locations on a nucleic acid that contain specific sequences of nucleotides that are recognized and cleaved by restriction enzymes and such restriction recognition sites can vary from 4 to 10 bases in length. Restriction sites can be palindromic, and depending on the type of restriction enzyme the restriction enzyme can cut the sequence between two nucleotides within the recognition site, or it can cut upstream or downstream from the recognition site, such that the endonuclease cleavage site is a specific distance away from an endonuclease recognition site. "Restriction enzyme" as described herein, refers to an enzyme that can cut nucleic acid at or near specific recognition nucleotide sequences known as restriction sites. Restriction enzymes are commonly classified into three types, which differ in their structure and whether they cut their nucleic acid substrate at their recognition site, or if the recognition and cleavage sites are separate from one another. To cut a nucleic acid, all restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of a double stranded nucleic acid sequence. There are over 3000 restriction enzymes, of which over 600 are commercially available. There are 5 types of restriction enzymes (Type I, II, III, IV, and V). Type II enzymes are homodimers, in which the recognition sites are usually undivided and palindromic and 4-8 nucleotides in length. They recognize and cleave DNA at the same site, and they do not use ATP or AdoMet for their activity and many require only Mg^{2+} as a cofactor. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

[0086] Additionally, there are also subgroups under the Type II, such as Type IIS restriction endonucleases. Type IIS

restriction endonucleases can cleave DNA at a defined distance from their non-palindromic asymmetric recognition sites. Accordingly, in some alternatives provided herein, the endonuclease recognition site or binding site for the enzyme is within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50 or 55 nucleotides (or a number of nucleotides within a range defined by any two of the aforementioned values) from the most 5' thymine on the plurality of thymine residues in the template strand, which then becomes the plurality of adenine residues of the poly(A) tail upon transcription of the sequence, and upon binding of the endonuclease to said endonuclease recognition site or binding site for the enzyme, cleavage within the poly(T) domain occurs. Cleavage within the plurality of thymine residues can occur when the endonuclease recognition site is inverted. Additionally, the enzymes can also function as dimers. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

[0087] Without being limiting, examples of endonuclease sites that can be incorporated into one or more of the nucleic acids described herein, which have the capacity to generate a poly(T) region of a desired size or have the capacity to generate an RNA having a poly(A) region of a desired length after transcription include: AatII, AbaSI, Acc65I, AccI, Acil, AcII, AfeI, AflII, AflIII, AgeI, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, Apol, AscI, AseI, AsiSI, Aval, Avail, AvrII, BaeGI, BaeI, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, BccI, BceAI, BcgI, BciVI, BclI, BcoDI, BfaI, BfuAI, BfuCI, BglI, BglII, BglIII, BlnI, BmgBI, Bmrl, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiVI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, BsrI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Eco53KI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinPI, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, Nrul, NsiI, NspI, NtAlwI, NtBbvCI, NtBsmAI, NtBspQI, NtBstNBI, NtCviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, Pvul, Pvull, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, Sfil, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SspI, Stul, StyD4I, Styl, SwaI, TaqI, TfiI, TliI, TseI, Tsp45I, Tsp509I, TspMI, TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, and/or ZraI restriction sites.

[0088] Examples of Type IIS restriction enzymes can include but are not limited to Acil, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PleI, SfaNI, Acul, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHII, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1I, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefl, BcnI, Bisl, BslI, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CauII, CdiI, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Itai, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, Aaul, Acc113I, Acc16I, AccB1I, AceIII, Acsl, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvailI, AvilI, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, BcoI, Bcul, Bfml, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, Bsbl, BscBI, BscCI, Bse118I, BseAI, BseCI,

BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, Ehel, ErhI, FauNDI, FbaI, FbII, FriOI, FunI, FunII, GdiII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, NsbI, NspIII, NspV, PaeI, PagI, Paul, PciI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, PysI, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, CjuI, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, Aasi, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi and UbaF13I. As shown in **Table 1**, these enzymes have a recognition site that is several base pairs away from the enzyme cleavage site. As shown in **Table 1**, below, the Type IIS restriction will recognize a sequence recognition site and can cut away from the sequence recognition site. In alternatives described herein, these sequence recognition sites can be inverted so that the cleavage site will be within the plurality of thymine residues.

[0089] As shown for MnlI for example, in **Table 1**, MnlI will recognize CCTC bound to its complement on a double stranded nucleic acid and will cleave 7 nucleotides away from the recognition site. As shown in **Table 1**, there are enzymes that can also cleave about 40 or more nucleic acid residues away from the recognition site. In some alternatives described herein, the sequence recognition site comprises a sequence recognition site of any one of the sequence recognition sites described in **Table 1**. In some alternatives, the sequence recognition site is inverted for a Type IIS restriction endonuclease. In some alternatives, the cut site, or endonuclease cleavage site is inverted. In some alternatives, the endonuclease recognition site comprises any one of the endonuclease recognition sites described in **Table 1**. In some alternatives, the endonuclease is Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PfiI, SfaNI, AclI, BciVI, BfuAI, BmgBI, BmrI, Bpml, BpuEI, Bsal, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENI, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaiI, FnuDII, FspBI, Glai, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, Mvni, Pali, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AfiI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, BcnI, Bisl, BlsI, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BsiFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, CdiI, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, GluI, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, Acsi, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, Bbel, BbrPI, BulI, Bbv12I, BbvII, Bce83I, BcoI, Bcul, BfmI, BfrBI, BfrI, BlnI, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, Ehel, ErhI, FauNDI, FbaI, FbII, FriOI, FunI, FunII, GdiII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, NsbI, NspIII, NspV, PaeI, PagI, Paul, Peel, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, Real, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, Sial, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, PysI, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, CjuI, Cjull, Dni, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, Aasi, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or

UbaF13I.

[0090] Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
Acil	CCGC (SEQ ID No. 18)	C / C G C (SEQ ID No. 18) G G C / G (SEQ ID No. 19)
MnII	CCTC (SEQ ID No. 20)	C C T C N N N N N N / (SEQ ID No. 21) G G A G N N N N N N / N (SEQ ID No. 22)
AlwI	GGATC (SEQ ID No. 23)	G G A T C N N N N / N (SEQ ID No. 24) C C T A G N N N N N / (SEQ ID No. 25)
BbvI	GCAGC (SEQ ID No. 26)	G C A G C N N N N N N N N N N I N N N N (SEQ ID No. 27) C G T C G N N N N N N N N N N N N N N I (SEQ ID No. 28)
BccI	CCATC (SEQ ID No. 29)	G C A G C N N N N N N N N N N I N N N N (SEQ ID No. 30) C G T C G N N N N N N N N N N N N N N / (SEQ ID No. 31)
BceAI	ACGGC (SEQ ID No. 32)	A C G G C N N N N N N N N N N N N N N I N N (SEQ ID No. 33) T G C C G N N N N N N N N N N N N N N N I (SEQ ID No. 34)
BsmAI	GTCTC (SEQ ID No. 35)	G T C T C N / N N N N (SEQ ID No. 36) C A G A G N N N N N / (SEQ ID No. 37)
BsmFI	GGGAC (SEQ ID No. 38)	G G G A C N N N N N N N N N N N N N N I N N N N (SEQ ID No. 39) C C C T G N N N N N N N N N N N N N N N I (SEQ ID No. 40)
BspCNI	CTCAG (SEQ ID No. 41)	C T C A G N N N N N N N N N N / (SEQ ID No. 42) G A G T C N N N N N N N N N N / N N (SEQ ID No. 43)
BsrI	ACTGG (SEQ ID No. 44)	A C T G G N / (SEQ ID No. 45) T G A C / C N (SEQ ID No. 46)
BtsCI	GGATG (SEQ ID No. 47)	G G A T G N N / (SEQ ID No. 48) C C T A C / N N (SEQ ID No. 49)
FokI	GGATG (SEQ ID No. 50)	G G A T G N N N N N N N N N N N N N N N (SEQ ID No. 51) C C T A C N N N N N N N N N N N N N N N I (SEQ ID No. 52)
HgaI	GACGC (SEQ ID No. 53)	G A C G C N N N N N I N N N N N (SEQ ID No. 54) C T G C G N N N N N N N N N N N I (SEQ ID No. 55)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
HphI	GGTGA (SEQ ID No. 56)	G G T G A N N N N N N N N / (SEQ ID No. 57)
		C C A C T N N N N N N N / N (SEQ ID No. 58)
HpyAV	CCTTC (SEQ ID No. 59)	C C T T C N N N N N N / (SEQ ID No. 60)
		G G A A G N N N N N I N (SEQ ID No. 61)
MboII	GAAGA (SEQ ID No. 62)	G A A G A N N N N N N N N / (SEQ ID No. 63)
		C T T C T N N N N N N N / N (SEQ ID No. 64)
MlyI	GAGTC (SEQ ID No. 65)	G A G T C N N N N N / (SEQ ID No. 66)
		C T C A G N N N N N / (SEQ ID No. 67)
PleI	GAGTC (SEQ ID No. 68)	G A G T C N N N N / N (SEQ ID No. 69)
		C T C A G N N N N N / (SEQ ID No. 70)
SfaNI	GCATC (SEQ ID No. 71)	G C A T C N N N N N I N N N N (SEQ ID No. 72)
		C G T A G N N N N N N N N N / (SEQ ID No. 73)
AcuI	CTGAAG (SEQ ID No. 74)	C T G A A G N N N N N N N N N N N N N N N N N / (SEQ ID No. 75)
		G A C T T C N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 76)
BciVI	GTATCC (SEQ ID No. 77)	G T A T C C N N N N N N N / (SEQ ID No. 78)
		C A T A G G N N N N N / N (SEQ ID No. 79)
BfuAI	ACCTGC (SEQ ID No. 80)	A C C T G C N N N N / N N N N (SEQ ID No. 81)
		T G G A C G N N N N N N N N I (SEQ ID No. 82)
BmgBI	CACGTC (SEQ ID No. 83)	C A C / G T C (SEQ ID No. 84)
		G T G / C A G (SEQ ID No. 85)
BmrI	ACTGGG (SEQ ID No. 86)	A C T G G G N N N N N / (SEQ ID No. 87)
		T G A C C C N N N N / N (SEQ ID No. 88)
Bpml	CTGGAG (SEQ ID No. 89)	C T G G A G N N N N N N N N N N N N N N N N N / (SEQ ID No. 90)
		G A C C T C N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 91)
BpuEI	CTTGAG (SEQ ID No. 92)	C T T G A G N N N N N N N N N N N N N N N N N / (SEQ ID No. 93)
		G A A C T C N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 94)
BsaI	GGTCTC (SEQ ID No. 95)	G G T C T C N / N N N N (SEQ ID No. 96)
		C C A G A G N N N N N / (SEQ ID No. 97)
BseRI	GAGGAG (SEQ ID No. 98)	G A G G A G N N N N N N N N N N N N / (SEQ ID No. 99)
		C T C C T C N N N N N N N N N N N N / N N (SEQ ID No. 100)
BsgI	GTGCAG (SEQ ID No. 101)	G T G C A G N N N N N N N N N N N N N N N N N / (SEQ ID No. 102)
		C A C G T C N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 103)
BsmI	GAATGC (SEQ ID No. 104)	G A A T G C N / (SEQ ID No. 105)
		C T T A C / G N (SEQ ID No. 106)
BspMI	ACCTGC (SEQ ID No. 107)	A C C T G C N N N N / N N N N (SEQ ID No. 108)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	107)	T G G A C G N N N N N N N N / (SEQ ID No.109)
BsrBI	CCGCTC (SEQ ID No. 110)	C C G / C T C (SEQ ID No. 110)
		G G C / G A G (SEQ ID No. 111)
BsrDI	GCAATG (SEQ ID No. 112)	G C A A T G N N / (SEQ ID No. 113)
		C G T T A C / N N (SEQ ID No. 114)
BtgZI	GCGATG (SEQ ID No. 115)	G C G A T G N N N N N N N N N N / N N N N (SEQ ID No. 116)
		C G C T A C N N N N N N N N N N N N N N / (SEQ ID No. 117)
BtsI	GCAGTG (SEQ ID No. 118)	G C A G T G N N / (SEQ ID No. 119)
		C G T C A C / N N (SEQ ID No. 120)
EarI	CTCTTC (SEQ ID No. 121)	C T C T T C N / N N N (SEQ ID No. 122)
		G A G A A G N N N N / (SEQ ID No. 123)
Ecil	GGCGGA (SEQ ID No. 124)	G G C G G A N N N N N N N N N N N N / (SEQ ID No. 125)
		C C G C C T N N N N N N N N N N / N N (SEQ ID No. 126)
MmeI	TCCRAC (SEQ ID No. 127)	T C C R A C N N N N N N N N N N N N N N N N N N N N / (SEQ ID No. 128)
		A G G Y T G N N N N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 129)
NmeAIII	GCCGAG (SEQ ID No. 130)	G C C G A G N N N N N N N N N N N N N N N N N N N N / (SEQ ID No. 131)
		C G G C T C N N N N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 132)
BbvCI	CCTCAGC (SEQ ID No. 133)	C C / T C A G C (SEQ ID No. 133)
		G G A G T / C G (SEQ ID No. 134)
Bpu10I	CCTNAGC (SEQ ID No. 135)	C C / T N A G C (SEQ ID No. 135)
		G G A N T / C G (SEQ ID No. 136)
BspQI	GCTCTTC (SEQ ID No. 137)	G C T C T T C N / N N N (SEQ ID No. 138)
		C G A G A A G N N N N / (SEQ ID No. 139)
SapI	GCTCTTC (SEQ ID No. 140)	G C T C T T C N / N N N (SEQ ID No. 141)
		C G A G A A G N N N N / (SEQ ID No. 142)
BaeI	ACNNNGTAYC (SEQ ID No. 143)	NN/NNNNNNNNNNNCAANNNNNGTGGNNNNNNNNNNNN/ (SEQ ID No. 144)
		/NNNNNNNNNNNGTTNNNNNCACNNNNNNNNNNNN/NN (SEQ ID No. 145)
BsaXI	ACNNNNCTCC (SEQ ID No. 146)	NNN/NNNNNNNNNACNNNNNCTCCNNNNNNNNNN/ (SEQ ID No. 147)
		/NNNNNNNNNNNTGNNNNNGAGGNNNNNNNN/NNN (SEQ ID No. 148)
CspCI	CAANNNGTGG (SEQ ID No. 149)	NN/NNNNNNNNNNNCAANNNNNGTGGNNNNNNNNNNNN/ (SEQ ID No. 150)
		/NNNNNNNNNNNNNGTTNNNNNCACNNNNNNNNNNNN/NN (SEQ ID No. 151)
AccII	CGCG (SEQ ID No. 152)	C G / C G (SEQ ID No. 152)
		G C / G C (SEQ ID No. 152)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
AfaI	GTAC (SEQ ID No. 153)	G T / A C (SEQ ID No. 153)
		C A / T G (SEQ ID No. 153)
AluBI	AGCT (SEQ ID No. 154)	A G / C T (SEQ ID No. 154)
		T C / G A (SEQ ID No. 154)
AspLEI	GCGC (SEQ ID No. 155)	G C G / C (SEQ ID No. 155)
		C / G C G (SEQ ID No. 155)
BscFI	GATC (SEQ ID No. 156)	/ G A T C (SEQ ID No. 156)
		C T A G / (SEQ ID No. 156)
Bsh1236I	CGCG (SEQ ID No. 157)	C G / C G (SEQ ID No. 157)
		G C / G C (SEQ ID No. 157)
BshFI	GGCC (SEQ ID No. 158)	G G / C C (SEQ ID No. 158)
		C C / G G (SEQ ID No. 158)
BshI	GGCC (SEQ ID No. 159)	G G / C C (SEQ ID No. 159)
		C C / G G (SEQ ID No. 159)
BsiSI	CCGG (SEQ ID No. 160)	C / C G G (SEQ ID No. 160)
		G G C / C (SEQ ID No. 160)
BsnI	GGCC (SEQ ID No. 161)	G G / C C (SEQ ID No. 161)
		C C / G G (SEQ ID No. 161)
Bsp143I	GATC (SEQ ID No. 162)	/ G A T C (SEQ ID No. 162)
		C T A G / (SEQ ID No. 162)
BspACI	CCGC (SEQ ID No. 163)	C / C G C (SEQ ID No. 163)
		G G C / G (SEQ ID No. 163)
BspANI	GGCC (SEQ ID No. 164)	G G / C C (SEQ ID No. 164)
		C C / G G (SEQ ID No. 164)
BspFNI	CGCG (SEQ ID No. 165)	C G / C G (SEQ ID No. 165)
		G C / G C (SEQ ID No. 165)
BssMI	GATC (SEQ ID No. 166)	/ G A T C (SEQ ID No. 166)
		C T A G / (SEQ ID No. 166)
BstENII	GATC (SEQ ID No. 167)	/ G A T C (SEQ ID No. 167)
		C T A G / (SEQ ID No. 167)
BstFNI	CGCG (SEQ ID No. 168)	C G / C G (SEQ ID No. 168)
		G C / G C (SEQ ID No. 168)
BstHHI	GCGC (SEQ ID No. 169)	G C G / C (SEQ ID No. 169)
		C / G C G (SEQ ID No. 169)
BstKTI	GATC (SEQ ID No. 170)	G A T / C (SEQ ID No. 170)
		C / T A G (SEQ ID No. 170)
BstMBI	GATC (SEQ ID No. 171)	/ G A T C (SEQ ID No. 171)
		C T A G / (SEQ ID No. 171)
BsuRI	GGCC (SEQ ID No. 172)	G G / C C (SEQ ID No. 172)
		C C / G G (SEQ ID No. 172)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
CfoI	GCGC (SEQ ID No. 173)	G C G / C (SEQ ID No. 173)
		C / G C G (SEQ ID No. 173)
Csp6I	GTAC (SEQ ID No. 174)	G / T A C (SEQ ID No. 174)
		C A T / G (SEQ ID No. 174)
FaeI	CATG (SEQ ID No. 175)	C A T G / (SEQ ID No. 175)
		/ G T A C (SEQ ID No. 175)
Fail	YATR (SEQ ID No. 176)	Y A / T R (SEQ ID No. 176)
		R T / A Y (SEQ ID No. 176)
FnuDII	CGCG (SEQ ID No. 177)	C G / C G (SEQ ID No. 177)
		G C / G C (SEQ ID No. 177)
FspBI	CTAG (SEQ ID No. 178)	C / T A G (SEQ ID No. 178)
		G A T / C (SEQ ID No. 178)
GlaI	GCGC (SEQ ID No. 179)	G C / G C (SEQ ID No. 179)
		C G / C G (SEQ ID No. 179)
HapII	CCGG (SEQ ID No. 180)	C / C G G (SEQ ID No. 180)
		G G C / C (SEQ ID No. 180)
Hin1II	CATG (SEQ ID No. 181)	C A T G / (SEQ ID No. 181)
		/ G T A C (SEQ ID No. 181)
R9529	GCGC (SEQ ID No. 182)	G / C G C (SEQ ID No. 182)
		C G C / G (SEQ ID No. 182)
Hsp92II	CATG (SEQ ID No. 183)	C A T G / (SEQ ID No. 183)
		/ G T A C (SEQ ID No. 183)
HspAI	GCGC (SEQ ID No. 184)	G / C G C (SEQ ID No. 184)
		C G C / G (SEQ ID No. 184)
MaeI	CTAG (SEQ ID No. 185)	C / T A G (SEQ ID No. 185)
		G A T / C (SEQ ID No. 185)
Maell	ACGT (SEQ ID No. 186)	A / C G T (SEQ ID No. 186)
		T G C / A (SEQ ID No. 186)
MvnI	CGCG (SEQ ID No. 187)	C G / C G (SEQ ID No. 187)
		G C / G C (SEQ ID No. 187)
Pall	GGCC (SEQ ID No. 188)	G G / C C (SEQ ID No. 188)
		C C / G G (SEQ ID No. 188)
RsaNI	GTAC (SEQ ID No. 189)	G / T A C (SEQ ID No. 189)
		C A T / G (SEQ ID No. 189)
SetI	ASST (SEQ ID No. 190)	A S S T / (SEQ ID No. 190)
		/ T S S A (SEQ ID No. 190)
SgeI	CNNG (SEQ ID No. 191)	C N N G N N N N N N N N / N N N N (SEQ ID No. 192)
		G N N C N N N N N N N N N N N N N I (SEQ ID No. 193)
Sse9I	AATT (SEQ ID No. 194)	/ A A T T (SEQ ID No. 194)
		T T A A / (SEQ ID No. 194)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
Thai	CGCG (SEQ ID No. 195)	C G / C G (SEQ ID No. 195)
		G C / G C (SEQ ID No. 195)
Tru1I	TTAA (SEQ ID No. 196)	T / T A A (SEQ ID No. 196)
		A A T / T (SEQ ID No. 196)
Tru9I	TTAA (SEQ ID No. 197)	T / T A A (SEQ ID No. 197)
		A A T / T (SEQ ID No. 197)
Tscl	ACGT (SEQ ID No. 198)	A C G T / (SEQ ID No. 198)
		/ T G C A (SEQ ID No. 198)
TspEI	AATT (SEQ ID No. 199)	/ A A T T (SEQ ID No. 199)
		T T A A / (SEQ ID No. 199)
TthHB8I	TCGA (SEQ ID No. 200)	T / C G A (SEQ ID No. 200)
		A G C / T (SEQ ID No. 200)
Xspl	CTAG (SEQ ID No. 201)	C / T A G (SEQ ID No. 201)
		G A T / C (SEQ ID No. 201)
AflI	GGWCC (SEQ ID No. 202)	G / G W C C (SEQ ID No. 202)
		C C W G / G (SEQ ID No. 202)
AgsI	TTTAA (SEQ ID No. 203)	T T S / A A (SEQ ID No. 203)
		A / A S T T (SEQ ID No. 203)
AspS9I	GGNCC (SEQ ID No. 204)	G / G N C C (SEQ ID No. 204)
		C C N G / G (SEQ ID No. 204)
AsuC2I	CCSGG (SEQ ID No. 205)	C C / S G G (SEQ ID No. 205)
		G G S / C C (SEQ ID No. 205)
AsuI	GGNCC (SEQ ID No. 206)	G / G N C C (SEQ ID No. 206)
		C C N G / G (SEQ ID No. 206)
Bcefl	ACGGC (SEQ ID No. 207)	A C G G C N N N N N N N N N N / N (SEQ ID No. 208)
		T G C C G N N N N N N N N N N / (SEQ ID No. 209)
BcnI	CCSGG (SEQ ID No. 230)	C C / S G G (SEQ ID No. 230)
		G G S / C C (SEQ ID No. 230)
BisI	GCNGC (SEQ ID No. 231)	G C / N G C (SEQ ID No. 231)
		C G N / C G (SEQ ID No. 231)
BlsI	GCNGC (SEQ ID No. 232)	G C N / G C (SEQ ID No. 232)
		C G / N C G (SEQ ID No. 232)
Bme1390I	CCNGG (SEQ ID No. 233)	C C / N G G (SEQ ID No. 233)
		G G N / C C (SEQ ID No. 233)
Bme18I	GGWCC (SEQ ID No. 234)	G / G W C C (SEQ ID No. 234)
		C C W G / G (SEQ ID No. 234)
BmrFI	CCNGG (SEQ ID No. 235)	C C / N G G (SEQ ID No. 235)
		G G N / C C (SEQ ID No. 235)
BscGI	CCCGT (SEQ ID No. 236)	C C C G T N (SEQ ID No. 237)
		G G G C A / N (SEQ ID No. 238)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
BseBI	CCWGG (SEQ ID No. 239)	C C / W G G (SEQ ID No. 239) G G W / C C (SEQ ID No. 239)
BsiLI	CCWGG (SEQ ID No. 240)	C C / W G G (SEQ ID No. 240) G G W / C C (SEQ ID No. 240)
BsiZI	GGNCC (SEQ ID No. 241)	G / G N C C (SEQ ID No. 241) C C N G / G (SEQ ID No. 241)
BsiFI	GGGAC (SEQ ID No. 242)	G G G A C N N N N N N N N N / N N N N (SEQ ID No. 243) C C C T G N N N N N N N N N N N N / (SEQ ID No. 244)
BsoMAI	GTCTC (SEQ ID No. 245)	G T C T C N / N N N N (SEQ ID No. 246) C A G A G N N N N N / (SEQ ID No. 247)
BspNCI	CCAGA (SEQ ID No. 248)	C C A G A N (SEQ ID No. 249) G G T C T / N (SEQ ID No. 250)
Bst2UI	CCWGG (SEQ ID No. 251)	C C / W G G (SEQ ID No. 251) G G W / C C (SEQ ID No. 251)
Bst71I	GCAGC (SEQ ID No. 252)	G C A G C N N N N N N N N N / N N N N (SEQ ID No. 253) C G T C G N N N N N N N N N N / (SEQ ID No. 254)
BstDEI	CTNAG (SEQ ID No. 255)	C / T N A G (SEQ ID No. 255) G A N T / C (SEQ ID No. 255)
BstOI	CCWGG (SEQ ID No. 356)	C C / W G G (SEQ ID No. 356) G G W / C C (SEQ ID No. 356)
BstSCI	CCNGG (SEQ ID No. 257)	/ C C N G G (SEQ ID No. 257) G G N C C / (SEQ ID No. 257)
Caull	CCSGG (SEQ ID No. 258)	C C / S G G (SEQ ID No. 258) G G S / C C (SEQ ID No. 258)
CdiI	CATCG (SEQ ID No. 259)	C A T C / G (SEQ ID No. 259) G T A G / C (SEQ ID No. 260)
Cfr13I	GGNCC (SEQ ID No. 261)	G / G N C C (SEQ ID No. 261) C C N G / G (SEQ ID No. 261)
Eco47I	GGWCC (SEQ ID No. 262)	G / G W C C (SEQ ID No. 262) C C W G / G (SEQ ID No. 262)
EcoRII	CCWGG (SEQ ID No. 263)	/ C C W G G (SEQ ID No. 263) G G W C C / (SEQ ID No. 263)
FaqI	GGGAC (SEQ ID No. 264)	G G G A C N N N N N N N N N / N N N N (SEQ ID No. 265) C C C T G N N N N N N N N N N N N I (SEQ ID No. 266)
FinI	GGGAC (SEQ ID No. 267)	G G G A C N (SEQ ID No. 268) C C C T G / N (SEQ ID No. 269)
Fsp4HI	GCNGC (SEQ ID No. 270)	G C / N G C (SEQ ID No. 270) C G N / C G (SEQ ID No.270)
Glul	GCNGC (SEQ ID No.	G C / N G C (SEQ ID No. 271)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	271)	C G N / C G (SEQ ID No. 271)
Hin4II	CCTTC (SEQ ID No. 272)	C C T T C N N N N N / (SEQ ID No. 273)
		G G A A G N N N N N / N (SEQ ID No. 274)
HpyF3I	CTNAG (SEQ ID No. 275)	C / T N A G (SEQ ID No. 275)
		G A N T / C (SEQ ID No. 275)
Ital	GCNGC (SEQ ID No. 276)	G C / N G C (SEQ ID No. 276)
		C G N / C G (SEQ ID No. 276)
MaellI	GTNAC (SEQ ID No. 277)	/ G T N A C (SEQ ID No. 277)
		C A N T G / (SEQ ID No. 277)
MspR9I	CCNGG (SEQ ID No. 278)	C C / N G G (SEQ ID No. 278)
		G G N / C C (SEQ ID No. 278)
Mval	CCWGG (SEQ ID No. 279)	C C / W G G (SEQ ID No. 279)
		G G W / C C (SEQ ID No. 279)
NmuCI	GTSAC (SEQ ID No. 280)	/ G T S A C (SEQ ID No. 280)
		C A S T G / (SEQ ID No. 280)
Psp6I	CCWGG (SEQ ID	/ C C W G G (SEQ ID No. 281)
	No. 281)	
		G G W C C / (SEQ ID No. 281)
PspPI	GGNCC (SEQ ID No. 282)	G / G N C C (SEQ ID No. 282)
		C C N G / G (SEQ ID No. 282)
SatI	GCNGC (SEQ ID No. 283)	G C / N G C (SEQ ID No. 283)
		C G N / C G (SEQ ID No. 283)
SinI	GGWCC (SEQ ID No. 284)	G / G W C C (SEQ ID No. 284)
		C C W G / G (SEQ ID No. 284)
TscAI	CASTG (SEQ ID No. 285)	N N C A S T G N N / (SEQ ID No. 286)
		/ N N G T S A C N N (SEQ ID No. 286)
VpaK11 BI	GGWCC (SEQ ID No. 287)	G / G W C C (SEQ ID No. 287)
		C C W G / G (SEQ ID No. 287)
AanI	TTATAA (SEQ ID No. 288)	T T A / T A A (SEQ ID No. 288)
		A A T / A T T (SEQ ID No. 288)
AatI	AGGCCT (SEQ ID No. 289)	A G G / C C T (SEQ ID No. 289)
		T C C / G G A (SEQ ID No. 289)
AauI	TGTACA (SEQ ID No. 290)	T / G T A C A (SEQ ID No. 290)
		A C A T G / T (SEQ ID No. 290)
Acc113I	AGTACT (SEQ ID	A G T / A C T (SEQ ID No. 291)
	No. 291)	
		T C A / T G A (SEQ ID No. 291)
Acc16I	TGCGCA (SEQ ID No. 292)	T G C / G C A (SEQ ID No. 292)
		A C G / C G T (SEQ ID No. 292)
AccB1I	GGYRCC (SEQ ID No. 293)	G / G Y R C C (SEQ ID No. 293)
		C C R Y G / G (SEQ ID No. 293)
AceII	CAGCTC (SEQ ID No.	C A G C T C N N N N N N / N N N N (SEQ ID No. 295)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	294)	G T C G A G N N N N N N N N N / (SEQ ID No. 296)
AcsI	RAATTY (SEQ ID No. 297)	R / A A T T Y (SEQ ID No. 298)
		Y T T A A / R (SEQ ID No. 298)
AcvI	CACGTG (SEQ ID No. 299)	C A C / G T G (SEQ ID No. 299)
		G T G / C A C (SEQ ID No. 299)
Acyl	GRCGYC (SEQ ID No. 300)	G R / C G Y C (SEQ ID No. 300)
		C Y G C / R G (SEQ ID No. 300)
AhlI	ACTAGT (SEQ ID No. 301)	A / C T A G T (SEQ ID No. 301)
		T G A T C / A (SEQ ID No. 301)
Alw21I	GWGCWC (SEQ ID No. 302)	G W G C W / C (SEQ ID No. 302)
		C / W C G W G (SEQ ID No. 302)
Alw44I	GTGCAC (SEQ ID	G / T G C A C (SEQ ID No. 303)
	No. 303	C A C G T / G (SEQ ID No. 303)
Ama87I	CYCGRG (SEQ ID No. 304)	C / Y C G R G (SEQ ID No. 304)
		G R G C Y / C (SEQ ID No. 304)
Aor51HI	AGCGCT (SEQ ID No. 305)	A G C / G C T (SEQ ID No. 305)
		T C G / C G A (SEQ ID No. 305)
AsiAI	ACCGGT (SEQ ID No. 306)	A / C C G G T (SEQ ID No. 306)
		T G G C C / A (SEQ ID No. 306)
AsnI	ATTAAT (SEQ ID No. 307)	A T / T A A T (SEQ ID No. 307)
		T A A T / T A (SEQ ID No. 307)
Asp718I	GGTACC (SEQ ID No. 308)	G / G T A C C (SEQ ID No. 308)
		C C A T G / G (SEQ ID No. 308)
AspHI	GWGCWC (SEQ ID No. 309)	G W G C W / C (SEQ ID No. 309)
		C / W C G W G (SEQ ID No. 309)
AsuII	TTCGAA (SEQ ID No. 310)	T T / C G A A (SEQ ID No. 310)
		A A G C / T T (SEQ ID No. 310)
AsuNHI	GCTAGC (SEQ ID No. 311)	G / C T A G C (SEQ ID No. 311)
		C G A T C / G (SEQ ID No. 311)
AvallI	ATGCAT (SEQ ID	A T G C A T (SEQ ID No. 312)
	No. 312)	T A C G T A (SEQ ID No. 312)
Avill	TGCGCA (SEQ ID No. 313)	T G C / G C A (SEQ ID No. 313)
		A C G / C G T (SEQ ID No. 313)
Ball	TGGCCA (SEQ ID No. 314)	T G G / C C A (SEQ ID No. 314)
		A C C / G G T (SEQ ID No. 314)
BanIII	ATCGAT (SEQ ID No. 315)	A T / C G A T (SEQ ID No. 315)
		T A G C / T A (SEQ ID No. 315)
BauI	CACGAG (SEQ ID No. 316)	C / A C G A G (SEQ ID No. 317)
		G T G C T / C (SEQ ID No. 318)
BbeI	GGCGCC (SEQ ID No.	G G C G C / C (SEQ ID No. 320)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	319)	C / C G C G G (SEQ ID No. 320)
BbrPI	CACGTG (SEQ ID No. 321)	C A C / G T G (SEQ ID No. 321)
		G T G / C A C (SEQ ID No. 321)
Bbul	GCATGC (SEQ ID No. 322)	G C A T G / C (SEQ ID No. 322)
		C / G T A C G (SEQ ID No. 322)
Bbv12I	GWGCWC (SEQ ID No. 323)	G W G C W / C (SEQ ID No. 323)
		C / W C G W G (SEQ ID No. 323)
BbvII	GAAGAC (SEQ ID	G A A G A C N N / N N N N (SEQ ID No. 325)
	No. 324)	C T T C T G N N N N N N / (SEQ ID No. 326)
Bce83I	CTTGAG (SEQ ID No. 327)	C T T G A G N N N N N N N N N N N N N N N N N N / (SEQ ID No. 328)
		G A A C T C N N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 329)
BcoI	CYCGRG (SEQ ID No. 330)	C / Y C G R G (SEQ ID No. 330)
		G R G C Y / C (SEQ ID No. 330)
BcuI	ACTAGT (SEQ ID No. 331)	A / C T A G T (SEQ ID No. 331)
		T G A T C / A (SEQ ID No. 331)
BfmI	CTRYAG (SEQ ID No. 332)	C / T R Y A G (SEQ ID No. 332)
		G A Y R T / C (SEQ ID No. 332)
BfrBI	ATGCAT (SEQ ID No. 333)	A T G C A / T (SEQ ID No. 333)
		T / A C G T A (SEQ ID No. 333)
BfrI	CTTAAG (SEQ ID No. 334)	C / T T A A G (SEQ ID No. 334)
		G A A T T / C (SEQ ID No. 334)
BlnI	CCTAGG (SEQ ID No. 335)	C / C T A G G (SEQ ID No. 335)
		G G A T C / C (SEQ ID No. 335)
BmcAI	AGTACT (SEQ ID No. 336)	A G T / A C T (SEQ ID No. 336)
		T C A / T G A (SEQ ID No. 336)
BmeT111	CYCGRG (SEQ ID	C / Y C G R G (SEQ ID No. 337)
	No. 337)	G R G C Y / C (SEQ ID No. 337)
BmiI	GGNNCC (SEQ ID No. 338)	G G N / N C C (SEQ ID No. 338)
		C C N / N G G (SEQ ID No. 338)
Bmul	ACTGGG (SEQ ID No. 339)	A C T G G G N N N N N N / (SEQ ID No. 339)
		T G A C C C N N N N / N (SEQ ID No. 339)
BmyI	GDGCHC (SEQ ID No. 340)	G D G C H / C (SEQ ID No. 340)
		C / H C G D G (SEQ ID No. 340)
Bpu14I	TTCGAA (SEQ ID No. 341)	T T / C G A A (SEQ ID No. 341)
		A A G C / T T (SEQ ID No. 341)
BpvUI	CGATCG (SEQ ID No. 342)	C G A T / C G (SEQ ID No. 342)
		G C / T A G C (SEQ ID No. 342)
Bsa29I	ATCGAT (SEQ ID No. 343)	A T / C G A T (SEQ ID No. 343)
		T A G C / T A (SEQ ID No. 343)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
BsaOI	CGRYCG (SEQ ID No. 344)	C G R Y / C G (SEQ ID No. 344)
		G C / Y R G C (SEQ ID No. 344)
Bsbl	CAACAC (SEQ ID No. 345)	C A A C A C N N N N N N N N N N N N N N N N N N N N N N / (SEQ ID No. 346)
		G T T G T G N N N N N N N N N N N N N N N N N N N N N / N N (SEQ ID No. 347))
BscBI	GGNNCC (SEQ ID	G G N / N C C (SEQ ID No. 348)
	No. 348)	C C N / N G G (SEQ ID No. 348)
BscCI	GAATGC (SEQ ID No. 349)	G A A T G C N / (SEQ ID No. 350)
		C T T A C / G N (SEQ ID No. 351)
Bse118I	RCCGGY (SEQ ID No. 352)	R / C C G G Y (SEQ ID No. 352)
		Y G G C C / R (SEQ ID No. 352)
BseAI	TCCGGA (SEQ ID No. 353)	T / C C G G A (SEQ ID No. 353)
		A G G C C / T (SEQ ID No. 353)
BseCI	ATCGAT (SEQ ID No. 354)	A T / C G A T (SEQ ID No. 354)
		T A G C / T A (SEQ ID No. 354)
BseDI	CCNNGG (SEQ ID No. 355)	C / C N N G G (SEQ ID No. 355)
		G G N N C / C (SEQ ID No. 355)
BsePI	GCGCGC (SEQ ID No. 356)	G / C G C G C (SEQ ID No. 356)
		C G C G C / G (SEQ ID No. 356)
BseSI	GKGCMC (SEQ ID No. 357)	G K G C M / C (SEQ ID No. 357)
		C / M C G K G (SEQ ID No. 357)
BseX3I	CGGCCG (SEQ ID No. 358)	C / G G C C G (SEQ ID No. 358)
		G C C G G / C (SEQ ID No. 358)
Bsh1285I	CGRYCG (SEQ ID	C G R Y / C G (SEQ ID No. 359)
	No. 359)	G C / Y R G C (SEQ ID No. 359)
BshNI	GGYRCC (SEQ ID No. 360)	G / G Y R C C (SEQ ID No. 360)
		C C R Y G / G (SEQ ID No. 360)
BshTI	ACCGGT (SEQ ID No. 361)	A / C C G G T (SEQ ID No. 361)
		T G G C C / A (SEQ ID No. 361)
BshVI	ATCGAT (SEQ ID No. 362)	A T / C G A T (SEQ ID No. 362)
		T A G C / T A (SEQ ID No. 362)
BsiCI	TTCGAA (SEQ ID No. 363)	T T / C G A A (SEQ ID No. 363)
		A A G C / T T (SEQ ID No. 363)
BsiHKCI	CYCGRG (SEQ ID No. 364)	C / Y C G R G (SEQ ID No. 364)
		G R G C Y / C (SEQ ID No. 364)
BsiMI	TCCGGA (SEQ ID No. 365)	T / C C G G A (SEQ ID No. 365)
		A G G C C / T (SEQ ID No. 365)
BsiQI	TGATCA (SEQ ID No. 366)	T / G A T C A (SEQ ID No. 366)
		A C T A G / T (SEQ ID No. 366)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
BsiXI	ATCGAT (SEQ ID No. 367)	A T / C G A T (SEQ ID No. 367)
		T A G C / T A (SEQ ID No. 367)
Bsp106I	ATCGAT (SEQ ID No. 368)	A T / C G A T (SEQ ID No. 368)
	No. 368)	T A G C / T A (SEQ ID No. 368)
Bsp119I	TTCGAA (SEQ ID No. 369)	T T / C G A A (SEQ ID No. 369)
		A A G C / T T (SEQ ID No. 369)
Bsp120I	GGGCCC (SEQ ID No. 370)	G / G G C C C (SEQ ID No. 370)
		C C C G G / G (SEQ ID No. 370)
Bsp13I	TCCGGA (SEQ ID No. 371)	T / C C G G A (SEQ ID No. 371)
		A G G C C / T (SEQ ID No. 371)
Bsp1407I	TGTACA (SEQ ID No. 372)	T / G T A C A (SEQ ID No. 372)
		A C A T G / T (SEQ ID No. 372)
Bsp1431I	RGC GCY (SEQ ID No. 373)	R G C G C / Y (SEQ ID No. 373)
		Y / C G C G R (SEQ ID No. 373)
Bsp19I	CCATGG (SEQ ID No. 374)	C / C A T G G (SEQ ID No. 374)
		G G T A C / C (SEQ ID No. 374)
Bsp68I	TCGCGA (SEQ ID No. 375)	T C G / C G A (SEQ ID No. 375)
		A G C / G C T (SEQ ID No. 375)
BspA2I	CCTAGG (SEQ ID No. 376)	C / C T A G G (SEQ ID No. 376)
		G G A T C / C (SEQ ID No. 376)
BspCI	CGATCG (SEQ ID No. 377)	C G A T / C G (SEQ ID No. 377)
	No. 377)	G C / T A G C (SEQ ID No. 377)
BspGI	CTGGAC (SEQ ID No. 378)	C T G G A C N (SEQ ID No. 379)
		G A C C T G / N (SEQ ID No. 380)
BspLU11I	ACATGT (SEQ ID No. 381)	A / C A T G T (SEQ ID No. 381)
		T G T A C / A (SEQ ID No. 381)
BspMAI	CTGCAG (SEQ ID No. 382)	C T G C A / G (SEQ ID No. 382)
		G / A C G T C (SEQ ID No. 382)
BspMII	TCCGGA (SEQ ID No. 383)	T / C C G G A (SEQ ID No. 383)
		A G G C C / T (SEQ ID No. 383)
BspOI	GCTAGC (SEQ ID No. 384)	G C T A G / C (SEQ ID No. 384)
		C / G A T C G (SEQ ID No. 384)
BspT104I	TTCGAA (SEQ ID No. 385)	T T / C G A A (SEQ ID No. 385)
		A A G C / T T (SEQ ID No. 385)
BspT107I	GGYRCC (SEQ ID No. 386)	G / G Y R C C (SEQ ID No. 386)
		C C R Y G / G (SEQ ID No. 386)
BspTI	CTTAAG (SEQ ID No. 387)	C / T T A A G (SEQ ID No. 387)
		G A A T T / C (SEQ ID No. 387)
BspXI	ATCGAT (SEQ ID No. 388)	A T / C G A T (SEQ ID No. 388)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	No. 388)	T A G C / T A (SEQ ID No. 388)
BssAI	RCCGGY (SEQ ID No. 389)	R / C C G G Y (SEQ ID No. 389)
		Y G G C C / R (SEQ ID No. 389)
BssHI	CTCGAG (SEQ ID No. 390)	C / T C G A G (SEQ ID No. 390)
		G A G C T / C (SEQ ID No. 390)
BssNAI	GTATAC (SEQ ID No. 391)	G T A / T A C (SEQ ID No. 391)
		C A T / A T G (SEQ ID No. 391)
BssNI	GRCGYC (SEQ ID No. 392)	G R / C G Y C (SEQ ID No. 392)
		C Y G C / R G (SEQ ID No. 392)
BssT1I	CCWWGG (SEQ ID No. 393)	C / C W W G G (SEQ ID No. 393)
		G G W W C / C (SEQ ID No. 393)
Bst1107I	GTATAC (SEQ ID No. 394)	G T A / T A C (SEQ ID No. 394)
		C A T / A T G (SEQ ID No. 394)
Bst98I	CTTAAG (SEQ ID No. 395)	C / T T A A G (SEQ ID No. 395)
		G A A T T / C (SEQ ID No. 395)
BstACI	GRCGYC (SEQ ID No. 396)	G R / C G Y C (SEQ ID No. 396)
		C Y G C / R G (SEQ ID No. 396)
BstAFI	CTTAAG (SEQ ID No. 397)	C / T T A A G (SEQ ID No. 397)
		G A A T T / C (SEQ ID No. 397)
BstAUI	TGTACA (SEQ ID No. 398)	T / G T A C A (SEQ ID No. 398)
		A C A T G / T (SEQ ID No. 398)
BstBAI	YACGTR (SEQ ID No. 399)	Y A C / G T R (SEQ ID No. 399)
		R T G / C A Y (SEQ ID No. 399)
BstC8I	GCNNGC (SEQ ID No. 400)	G C N / N G C (SEQ ID No. 400)
		C G N / N C G (SEQ ID No. 400)
BstDSI	CCRYGG (SEQ ID No. 401)	C / C R Y G G (SEQ ID No. 401)
		G G Y R C / C (SEQ ID No. 401)
BstH2I	RGCGCY (SEQ ID No. 402)	R G C G C / Y (SEQ ID No. 402)
		Y / C G C G R (SEQ ID No. 402)
BstHPI	GTAAAC (SEQ ID No. 403)	G T T / A A C (SEQ ID No. 403)
		C A A / T T G (SEQ ID No. 403)
BstNSI	RCATGY (SEQ ID No. 404)	R C A T G / Y (SEQ ID No. 404)
		Y / G T A C R (SEQ ID No. 404)
BstSFI	CTRYAG (SEQ ID No. 405)	C / T R Y A G (SEQ ID No. 405)
		G A Y R T / C (SEQ ID No. 405)
BstSLI	GKGCMC (SEQ ID No. 406)	G K G C M / C (SEQ ID No. 406)
		C / M C G K G (SEQ ID No. 406)
BstSNI	TACGTA (SEQ ID No. 407)	T A C / G T A (SEQ ID No. 407)
		A T G / C A T (SEQ ID No. 407)
BstX2I	RGATCY (SEQ ID No. 408)	R / G A T C Y (SEQ ID No. 408)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	408)	Y C T A G / R (SEQ ID No. 408)
BstZI	CGGCCG (SEQ ID No. 409)	C / G G C C G (SEQ ID No. 409)
		G C C G G / C (SEQ ID No. 409)
BsuTUI	ATCGAT (SEQ ID No. 410)	A T / C G A T (SEQ ID No. 410)
		T A G C / T A (SEQ ID No. 410)
BtuMI	TCGCGA (SEQ ID No. 411)	T C G / C G A (SEQ ID No. 411)
		A G C / G C T (SEQ ID No. 411)
BveI	ACCTGC (SEQ ID No. 412)	A C C T G C N N N N / N N N N (SEQ ID No. 413)
		T G G A C G N N N N N N N N / (SEQ ID No. 414)
CeiI	TCATGA (SEQ ID No. 415)	T / C A T G A (SEQ ID No. 415)
		A G T A C / T (SEQ ID No. 415)
Cfr10I	RCCGGY (SEQ ID No. 416)	R / C C G G Y (SEQ ID No. 416)
		Y G G C C / R (SEQ ID No. 416)
Cfr42I	CCGCGG (SEQ ID	C C G C / G G (SEQ ID No. 417)
	No. 417)	
		G G / C G C C (SEQ ID No. 417)
Cfr9I	CCCGGG (SEQ ID No. 418)	C / C C G G G (SEQ ID No. 418)
		G G G C C / C (SEQ ID No. 418)
CfrI	YGGCCR (SEQ ID No. 419)	Y / G G C C R (SEQ ID No. 419)
		R C C G G / Y (SEQ ID No. 419)
Csp45I	TTCGAA (SEQ ID No. 420)	T T / C G A A (SEQ ID No. 420)
		A A G C / T T (SEQ ID No. 420)
CspAI	ACCGGT (SEQ ID No. 421)	A / C C G G T (SEQ ID No. 421)
		T G G C C / A (SEQ ID No. 421)
DinI	GGCGCC (SEQ ID No. 422)	G G C / G C C (SEQ ID No. 422)
		C C G / C G G (SEQ ID No. 422)
DrdII	GAACCA (SEQ ID No. 423)	G A A C C A N (SEQ ID No. 424)
		C T T G G T / N (SEQ ID No. 425)
DsaI	CCRYGG (SEQ ID No. 426)	C / C R Y G G (SEQ ID No. 426)
		G G Y R C / C (SEQ ID No. 426)
Ecl136II	GAGCTC (SEQ ID No. 427)	G A G / C T C (SEQ ID No. 427)
		C T C / G A G (SEQ ID No. 427)
EclXI	CGGCCG (SEQ ID	C / G G C C G (SEQ ID No. 428)
	No. 428)	
		G C C G G / C (SEQ ID No. 428)
Eco105I	TACGTA (SEQ ID No. 429)	T A C / G T A (SEQ ID No. 429)
		A T G / C A T (SEQ ID No. 429)
Eco130I	CCWWGG (SEQ ID No. 430)	C / C W W G G (SEQ ID No. 430)
		G G W W C / C (SEQ ID No. 430)
Eco147I	AGGCCT (SEQ ID No. 431)	A G G / C C T (SEQ ID No. 431)
		T C C / G G A (SEQ ID No. 431)
Eco24I	GRGCYC (SEQ ID No.	G R G C Y / C (SEQ ID No. 432)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	432)	C / Y C G R G (SEQ ID No.432)
Eco32I	GATATC (SEQ ID No. 433)	G A T / A T C (SEQ ID No. 433)
		C T A / T A G (SEQ ID No. 433)
Eco47III	AGCGCT (SEQ ID No. 434)	A G C / G C T (SEQ ID No. 434)
		T C G / C G A (SEQ ID No. 434)
Eco52I	CGGCCG (SEQ ID No. 435)	C / G G C C G (SEQ ID No. 435)
		G C C G G / C (SEQ ID No. 435)
Eco72I	CACGTG (SEQ ID No. 436)	C A C / G T G (SEQ ID No. 436)
		G T G / C A C (SEQ ID No. 436)
Eco88I	CYCGRG (SEQ ID No. 437)	C / Y C G R G (SEQ ID No. 437)
	437)	G R G C Y / C (SEQ ID No. 437)
EcoICRI	GAGCTC (SEQ ID No. 438)	G A G / C T C (SEQ ID No. 438)
		C T C / G A G (SEQ ID No. 438)
EcoT14I	CCWWGG (SEQ ID No. 439)	C / C W W G G (SEQ ID No. 439)
		G G W W C / C (SEQ ID No. 439)
EcoT22I	ATGCAT (SEQ ID No. 440)	A T G C A / T (SEQ ID No. 440)
		T / A C G T A (SEQ ID No. 440)
EcoT38I	GRGCTC (SEQ ID No. 441)	G R G C Y / C (SEQ ID No. 441)
		C / Y C G R G (SEQ ID No. 441)
EgeI	GGCGCC (SEQ ID No. 442)	G G C / G C C (SEQ ID No. 442)
		C C G / C G G (SEQ ID No. 442)
EheI	GGCGCC (SEQ ID No. 443)	G G C / G C C (SEQ ID No. 443)
		C C G / C G G (SEQ ID No. 443)
ErhI	CCWWGG (SEQ ID No. 444)	C / C W W G G (SEQ ID No. 444)
		G G W W C / C (SEQ ID No. 444)
FauNDI	CATATG (SEQ ID No. 445)	C A / T A T G (SEQ ID No. 445)
		G T A T / A C (SEQ ID No. 445)
FbaI	TGATCA (SEQ ID No. 446)	T / G A T C A (SEQ ID No. 446)
	446)	A C T A G / T (SEQ ID No. 446)
FblI	GTMKAC (SEQ ID No. 447)	G T / M K A C (SEQ ID No. 447)
		C A K M / T G (SEQ ID No. 447)
FriOI	GRGCTC (SEQ ID No. 448)	G R G C Y / C (SEQ ID No. 448)
		C / Y C G R G (SEQ ID No. 448)
FunI	AGCGCT (SEQ ID No. 449)	A G C / G C T (SEQ ID No. 449)
		T C G / C G A (SEQ ID No. 449)
FunII	GAATTC (SEQ ID No. 450)	G / A A T T C (SEQ ID No. 450)
		C T T A A / G (SEQ ID No. 450)
GdII	CGGCCR (SEQ ID No. 451)	C / G G C C R (SEQ ID No. 451)
		G C C G G / Y (SEQ ID No. 452)
GsaI	CCCAGC (SEQ ID No. 453)	C C C A G / C (SEQ ID No. 453)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	453)	G / G G T C G (SEQ ID No. 454)
HaeI	WGGCCW (SEQ ID No. 455)	W G G / C C W (SEQ ID No. 455)
		W C C / G G W (SEQ ID No. 455)
HgiAI	GWGCWC (SEQ ID No. 456)	G W G C W / C (SEQ ID No. 456)
		C / W C G W G (SEQ ID No. 456)
Hin1I	GRCGYC (SEQ ID No. 457)	G R / C G Y C (SEQ ID No. 457)
		C Y G C / R G (SEQ ID No. 457)
HindII	GTyrAC (SEQ ID No. 458)	G T Y / R A C (SEQ ID No. 458)
		C A R / Y T G (SEQ ID No. 458)
Hpy178III	TCNNGA (SEQ ID No. 459)	T C / N N G A (SEQ ID No. 459)
		A G N N / C T (SEQ ID No. 459)
Hpy8I	GTNNAC (SEQ ID No. 460)	G T N / N A C (SEQ ID No. 460)
		C A N / N T G (SEQ ID No. 460)
Hsp92I	GRCGYC (SEQ ID No. 461)	G R / C G Y C (SEQ ID No. 461)
		C Y G C / R G (SEQ ID No. 461)
Kpn2I	TCCGGA (SEQ ID No. 462)	T / C C G G A (SEQ ID No. 462)
		A G G C C / T (SEQ ID No. 462)
Ksp22I	TGATCA (SEQ ID No. 463)	T / G A T C A (SEQ ID No. 463)
		A C T A G / T (SEQ ID No. 463)
KspAI	GTAAAC (SEQ ID No. 464)	G T T / A A C SEQ ID No. 464)
		C A A / T T G SEQ ID No. 464)
KspI	CCGCGG SEQ ID No. 631)	C C G C / G G SEQ ID No. 631)
		G G / C G C C SEQ ID No. 631)
MfiI	RGATCY (SEQ ID No. 465)	R / G A T C Y (SEQ ID No. 465)
		Y C T A G / R (SEQ ID No. 465)
MhII	GDGCHC (SEQ ID No. 466)	G D G C H / C (SEQ ID No. 466)
		C / H C G D G (SEQ ID No. 466)
MisI	TGGCCA (SEQ ID No. 467)	T G G / C C A (SEQ ID No. 467)
		A C C / G G T (SEQ ID No. 467)
MluNI	TGGCCA (SEQ ID No. 468)	T G G / C C A (SEQ ID No. 468)
		A C C / G G T (SEQ ID No. 468)
Mly113I	GGCGCC (SEQ ID No. 469)	G G / C G C C (SEQ ID No. 469)
		C C G C / G G (SEQ ID No. 469)
Mph1103I	ATGCAT (SEQ ID No. 470)	A T G C A / T (SEQ ID No. 470)
		T / A C G T A (SEQ ID No. 470)
MroI	TCCGGA (SEQ ID No. 471)	T / C C G G A SEQ ID No. 471)
		A G G C C / T SEQ ID No. 471)
MroNI	GCCGGC (SEQ ID No. 472)	G / C C G G C (SEQ ID No. 472)
		C G G C C / G (SEQ ID No. 472)
Msp20I	TGGCCA (SEQ ID No. 473)	T G G / C C A (SEQ ID No. 473)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	473)	A C C / G G T (SEQ ID No. 473)
MspCI	CTTAAG (SEQ ID No. 474)	C / T T A A G (SEQ ID No. 474)
		G A A T T / C (SEQ ID No. 474)
MstI	TGCGCA (SEQ ID No. 475)	T G C / G C A (SEQ ID No. 475)
		A C G / C G T (SEQ ID No. 475)
MunI	CAATTG (SEQ ID No. 476)	C / A A T T G (SEQ ID No. 476)
		G T T A A / C (SEQ ID No. 476)
MvrI	CGATCG (SEQ ID No. 477)	C G A T / C G (SEQ ID No. 477)
		G C / T A G C (SEQ ID No. 477)
NgoAIV	GCCGGC (SEQ ID No. 478)	G / C C G G C (SEQ ID No. 478)
		C G G C C / G (SEQ ID No. 478)
Nsbl	TGCGCA (SEQ ID No. 479)	T G C / G C A (SEQ ID No. 479)
		A C G / C G T (SEQ ID No. 479)
NspIII	CYCGRG (SEQ ID No. 480)	C / Y C G R G (SEQ ID No. 480)
		G R G C Y / C (SEQ ID No. 480)
NspV	TTCGAA (SEQ ID No. 481)	T T / C G A A (SEQ ID No. 481)
		A A G C / T T (SEQ ID No. 481)
PaeI	GCATGC (SEQ ID No. 632)	G C A T G / C (SEQ ID No. 632)
		C / G T A C G (SEQ ID No. 632)
PagI	TCATGA (SEQ ID No. 482)	T / C A T G A (SEQ ID No. 482)
		A G T A C / T (SEQ ID No. 482)
Paul	GCGCGC (SEQ ID No. 483)	G / C G C G C (SEQ ID No. 483)
		C G C G C / G (SEQ ID No. 483)
PceI	AGGCCT (SEQ ID No. 484)	A G G / C C T (SEQ ID No. 484)
		T C C / G G A (SEQ ID No. 484)
PdII	GCCGGC (SEQ ID No. 485)	G C C / G G C (SEQ ID No. 485)
		C G G / C C G (SEQ ID No. 485)
Pfi23II	CGTACG (SEQ ID No. 486)	C / G T A C G (SEQ ID No. 486)
		G C A T G / C (SEQ ID No. 486)
PinAI	ACCGGT (SEQ ID No. 487)	A / C C G G T (SEQ ID No. 487)
		T G G C C / A (SEQ ID No. 487)
Ple19I	CGATCG (SEQ ID No. 488)	C G A T / C G (SEQ ID No. 488)
		G C / T A G C (SEQ ID No. 488)
PmaCI	CACGTG (SEQ ID No. 489)	C A C / G T G (SEQ ID No. 489)
		G T G / C A C (SEQ ID No. 489)
PshBI	ATTAAT (SEQ ID No. 490)	A T / T A A T (SEQ ID No. 490)
		T A A T / T A (SEQ ID No. 490)
Psp124BI	GAGCTC (SEQ ID No. 491)	G A G C T / C (SEQ ID No. 491)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	491)	C / T C G A G (SEQ ID No. 491)
Psp1406I	AACGTT (SEQ ID No. 492)	A A / C G T T (SEQ ID No. 492)
		T T G C / A A (SEQ ID No. 492)
PspAI	CCCGGG (SEQ ID No. 493)	C / C C G G G (SEQ ID No. 493)
		G G G C C / C (SEQ ID No. 493)
PspLI	CGTACG (SEQ ID No. 494)	C / G T A C G (SEQ ID No. 494)
		G C A T G / C (SEQ ID No. 494)
PspN4I	GGNNCC (SEQ ID No. 495)	G G N / N C C (SEQ ID No. 495)
		C C N / N G G (SEQ ID No. 495)
Psul	RGATCY (SEQ ID No. 496)	R / G A T C Y (SEQ ID No. 496)
		Y C T A G / R (SEQ ID No. 496)
Rcal	TCATGA (SEQ ID No. 497)	T / C A T G A (SEQ ID No. 497)
		A G T A C / T (SEQ ID No. 497)
Sdul	GDGCHC (SEQ ID No. 498)	G D G C H / C (SEQ ID No. 498)
		C / H C G D G (SEQ ID No. 498)
Sfr274I	CTCGAG (SEQ ID No. 499)	C / T C G A G (SEQ ID No. 499)
		G A G C T / C (SEQ ID No. 499)
Sfr303I	CCGCGG (SEQ ID No. 500)	C C G C / G G (SEQ ID No. 500)
		G G / C G C C (SEQ ID No. 500)
Sful	TTCGAA (SEQ ID No. 501)	T T / C G A A (SEQ ID No. 501)
		A A G C / T T (SEQ ID No. 501)
SgrBI	CCGCGG (SEQ ID No. 502)	C C G C / G G (SEQ ID No. 502)
		G G / C G C C (SEQ ID No. 502)
Sial	CTCGAG (SEQ ID No. 503)	C / T C G A G (SEQ ID No. 503)
		G A G C T / C (SEQ ID No. 503)
SpaHI	GCATGC (SEQ ID No. 504)	G C A T G / C (SEQ ID No. 504)
		C / G T A C G (SEQ ID No. 504)
SseBI	AGGCCT (SEQ ID No. 505)	A G G / C C T (SEQ ID No. 505)
		T C C / G G A (SEQ ID No. 505)
SspBI	TGTACA (SEQ ID No. 506)	T / G T A C A (SEQ ID No. 506)
		A C A T G / T (SEQ ID No. 506)
SstI	GAGCTC (SEQ ID No. 507)	G A G C T / C (SEQ ID No. 507)
		C / T C G A G (SEQ ID No. 507)
SstII	CCGCGG (SEQ ID No. 508)	C C G C / G G (SEQ ID No. 508)
		G G / C G C C (SEQ ID No. 508)
SunI	CGTACG (SEQ ID No. 509)	C / G T A C G (SEQ ID No. 509)
		G C A T G / C (SEQ ID No. 509)
TatI	WGTACW (SEQ ID No. 510)	W / G T A C W (SEQ ID No. 510)
		W C A T G / W (SEQ ID No. 510)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
Vha464I	CTTAAG (SEQ ID No. 511)	C I T T A A G (SEQ ID No. 511)
		G A A T T I C (SEQ ID No. 511)
VneI	GTGCAC (SEQ ID No. 512)	G / T G C A C (SEQ ID No. 512)
		C A C G T / G (SEQ ID No. 512)
Vspl	ATTAAT (SEQ ID No. 513)	A T I T A A T (SEQ ID No. 513)
		T A A T / T A (SEQ ID No. 513)
XapI	RAATTY (SEQ ID No. 514)	R / A A T T Y (SEQ ID No. 514)
		Y T T A A / R (SEQ ID No. 514)
XhoII	RGATCY (SEQ ID No. 515)	R / G A T C Y (SEQ ID No. 515)
		Y C T A G / R (SEQ ID No. 515)
XmaCI	CCCGGG (SEQ ID No. 516)	C / C C G G G (SEQ ID No. 516)
		G G G C C / C (SEQ ID No. 516)
XmaIII	CGGCCG (SEQ ID No. 517)	C / G G C C G (SEQ ID No. 517)
		G C C G G / C (SEQ ID No. 517)
XmaJI	CCTAGG (SEQ ID No. 518)	C / C T A G G (SEQ ID No. 518)
		G G A T C / C (SEQ ID No. 518)
XmiI	GTMKAC (SEQ ID No. 519)	G T / M K A C (SEQ ID No. 519)
		C A K M / T G (SEQ ID No. 519)
ZhoI	ATCGAT (SEQ ID No. 520)	A T / C G A T (SEQ ID No. 520)
		T A G C / T A (SEQ ID No. 520)
Zsp2I	ATGCAT (SEQ ID No. 521)	A T G C A / T (SEQ ID No. 521)
		T / A C G T A (SEQ ID No. 521)
AocI	CCTNAGG (SEQ ID No. 522)	C C / T N A G G (SEQ ID No. 522)
		G G A N T / C C (SEQ ID No. 522)
AxyI	CCTNAGG (SEQ ID No. 523)	C C / T N A G G (SEQ ID No. 523)
		G G A N T / C C (SEQ ID No. 523)
Bpu1102I	GCTNAGC (SEQ ID No. 524)	G C / T N A G C (SEQ ID No. 524)
		C G A N T / C G (SEQ ID No. 524)
Bse21I	CCTNAGG (SEQ ID No. 525)	C C / T N A G G (SEQ ID No. 525)
		G G A N T / C C (SEQ ID No. 525)
Bsp1720I	GCTNAGC (SEQ ID No. 526)	G C / T N A G C (SEQ ID No. 526)
		C G A N T / C G (SEQ ID No. 526)
BstPI	GGTNACC (SEQ ID No. 527)	G / G T N A C C (SEQ ID No. 527)
		C C A N T G / G (SEQ ID No. 527)
CelII	GCTNAGC (SEQ ID No. 528)	G C / T N A G C (SEQ ID No. 528)
		C G A N T / C G (SEQ ID No. 528)
CpoI	CGGWCCG (SEQ ID No. 529)	C G / G W C C G (SEQ ID No. 529)
		G C C W G / G C (SEQ ID No. 529)
Cspl	CGGWCCG (SEQ ID No. 530)	C G / G W C C G (SEQ ID No. 530)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	No. 530)	G C C W G / G C (SEQ ID No. 530)
Drall	RGGNCCY (SEQ ID No. 531)	R G / G N C C Y (SEQ ID No. 531) Y C C N G / G R (SEQ ID No. 531)
Eco065I	GGTNACC (SEQ ID No. 532)	G / G T N A C C (SEQ ID No. 532) C C A N T G / G (SEQ ID No. 532)
Eco81I	CCTNAGG (SEQ ID No. 533)	C C / T N A G G (SEQ ID No. 533) G G A N T / C C (SEQ ID No. 533)
Eco91I	GGTNACC (SEQ ID No. 534)	G / G T N A C C (SEQ ID No. 534) C C A N T G / G (SEQ ID No. 534)
Espl	GCTNAGC (SEQ ID No. 535)	G C / T N A G C (SEQ ID No. 535) C G A N T / C G (SEQ ID No. 535)
KflI	GGGWCCC (SEQ ID No. 536)	G G / G W C C C (SEQ ID No. 536) C C C W G / G G (SEQ ID No. 536)
Lgul	GCTCTTC (SEQ ID No. 634)	G C T C T T C N / N N N (SEQ ID No. 635) C G A G A A G N N N / (SEQ ID No. 636)
MabI	ACCWGGT (SEQ ID No. 539)	A / C C W G G T (SEQ ID No. 539) T G G W C C / A (SEQ ID No. 539)
PpuXI	RGGWCCY (SEQ ID No. 540)	R G / G W C C Y (SEQ ID No. 540) Y C C W G / G R (SEQ ID No. 540)
Psp5II	RGGWCCY (SEQ ID No. 541)	R G / G W C C Y (SEQ ID No. 541) Y C C W G / G R (SEQ ID No. 541)
PspEI	GGTNACC (SEQ ID No. 542)	G / G T N A C C (SEQ ID No. 542) C C A N T G / G (SEQ ID No. 542)
Rsr2I	CGGWCCG (SEQ ID No. 543)	C G / G W C C G (SEQ ID No. 543) G C C W G / G C (SEQ ID No. 543)
SauI	CCTNAGG (SEQ ID No. 544)	C C / T N A G G (SEQ ID No. 544) G G A N T / C C (SEQ ID No. 544)
AbsI	CCTCGAGG (SEQ ID No. 545)	C C / T C G A G G (SEQ ID No. 545) G G A G C T / C C (SEQ ID No. 545)
CciNI	GCGGCCGC (SEQ ID No. 546)	G C / G G C C G C (SEQ ID No. 546) C G C C G G / C G (SEQ ID No. 546)
FspAI	RTGCGCAY (SEQ ID No. 547)	R T G C / G C A Y (SEQ ID No. 547) Y A C G / C G T R (SEQ ID No. 547)
MauBI	CGCGCGCG (SEQ ID No. 548)	C G / C G C G C G (SEQ ID No. 548) G C G C G C / G C (SEQ ID No. 548)
MreI	CGCCGGCG (SEQ ID No. 549)	C G / C C G G C G (SEQ ID No. 549) G C G G C C / G C (SEQ ID No. 549)
MssI	GTTTAAAC (SEQ ID No. 550)	G T T T / A A A C (SEQ ID No. 550) C A A A / T T T G (SEQ ID No. 550)
RgaI	GCGATCGC (SEQ ID	G C G A T / C G C (SEQ ID No. 551)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	No. 551)	C G C / T A G C G (SEQ ID No. 551)
SdaI	CCTGCAGG (SEQ ID No. 552)	C C T G C A / G G (SEQ ID No. 552)
		G G / A C G T C C (SEQ ID No. 552)
SfaAI	GCGATCGC (SEQ ID No. 553)	G C G A T / C G C (SEQ ID No. 553)
		C G C / A G C G (SEQ ID No. 553)
Sgfl	GCGATCGC (SEQ ID No. 554)	G C G A T / C G C (SEQ ID No. 554)
	554)	C G C / A G C G (SEQ ID No. 554)
Smil	ATTAAAT (SEQ ID No. 555)	A T T T / A A A T (SEQ ID No. 555)
		T A A A T T T A (SEQ ID No. 555)
Sse232I	CGCCGGCG (SEQ ID No. 556)	C G / C C G G C G (SEQ ID No. 556)
		G C G G C C / G C (SEQ ID No. 556)
Adel	CACNNNGTG (SEQ ID No. 557)	C A C N N N / G T G (SEQ ID No. 557)
		G T G / N N N C A C (SEQ ID No. 557)
Aspl	GACNNNGTC (SEQ ID No. 558)	G A C N / N N G T C (SEQ ID No. 558)
		C T G N N / N C A G (SEQ ID No. 558)
Cail	CAGNNNCTG (SEQ ID No. 559)	C A G N N N / C T G (SEQ ID No. 559)
		G T C / N N N G A C (SEQ ID No. 559)
Psyl	GACNNNGTC (SEQ ID No. 560)	G A C N / N N G T C (SEQ ID No. 560)
		C T G N N / N C A G (SEQ ID No. 560)
Tell	GACNNNGTC (SEQ ID No. 561)	G A C N / N N G T C (SEQ ID No. 561)
		C T G N N / N C A G (SEQ ID No. 561)
Asp700I	GAANNNTTC (SEQ ID No. 562)	G A A N N I N N T T C (SEQ ID No. 562)
		C T T N N / N N A A G (SEQ ID No. 562)
BoxI	GACNNNGTC (SEQ ID No. 563)	G A C N N / N N G T C (SEQ ID No. 563)
		C T G N N / N N C A G (SEQ ID No. 563)
Bse8I	GATNNNNATC (SEQ ID No. 564)	G A T N N / N N A T C (SEQ ID No. 564)
		C T A N N / N N T A G (SEQ ID No. 564)
BseJI	GATNNNNATC (SEQ ID No. 565)	G A T N N / N N A T C (SEQ ID No. 565)
		C T A N N / N N T A G (SEQ ID No. 565)
BsiBI	GATNNNNATC (SEQ ID No. 566)	G A T N N / N N A T C (SEQ ID No. 566)
		C T A N N / N N T A G (SEQ ID No. 566)
BsrBRI	GATNNNNATC (SEQ ID No. 567)	G A T N N / N N A T C (SEQ ID No. 567)
		C T A N N / N N T A G (SEQ ID No. 567)
BstPAI	GACNNNGTC (SEQ ID No. 568)	G A C N N / N N G T C (SEQ ID No. 568)
		C T G N N / N N C A G (SEQ ID No. 568)
CjeNII	GAGNNNNNGT (SEQ ID No. 569)	G A G N N N N N G T (SEQ ID No. 569)
		C T C N N N N N C A (SEQ ID No. 569)
MamI	GATNNNNATC (SEQ ID No. 570)	G A T N N / N N A T C (SEQ ID No. 570)
		C T A N N / N N T A G (SEQ ID No. 570)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
MroXI	GAANNNNTTC (SEQ ID No. 571)	G A A N N / N N T T C (SEQ ID No. 571)
		C T T N N / N N A A G (SEQ ID No. 571)
OliI	CACNNNGTG (SEQ ID No. 572)	C A C N N / N N G T G (SEQ ID No. 572)
		G T G N N / N N C A C (SEQ ID No. 572)
Pdml	GAANNNNTTC (SEQ ID No. 573)	G A A N N / N N T T C (SEQ ID No. 573)
		C T T N N / N N A A G (SEQ ID No. 573)
RseI	CAYNNNRTG (SEQ ID No. 574)	C A Y N N / N N R T G (SEQ ID No. 574)
		G T R N N / N N Y A C (SEQ ID No. 574)
SmiI	CAYNNNRTG (SEQ ID No. 575)	C A Y N N / N N R T G (SEQ ID No. 575)
		G T R N N / N N Y A C (SEQ ID No. 575)
AccB7I	CCANNNNTGG (SEQ ID No. 576)	C C A N N N N / N T G G (SEQ ID No. 576)
		G G T N / N N N N A C C (SEQ ID No. 576)
AspEI	GACNNNGTC (SEQ ID No. 577)	G A C N N N / N N G T C (SEQ ID No. 577)
		C T G N N / N N N C A G (SEQ ID No. 577)
BasI	CCANNNNTGG (SEQ ID No. 578)	C C A N N N N / N T G G (SEQ ID No. 578)
		G G T N / N N N N A C C (SEQ ID No. 578)
BmeRI	GACNNNGTC (SEQ ID No. 579)	G A C N N N / N N G T C (SEQ ID No. 579)
		C T G N N / N N N C A G (SEQ ID No. 579)
BpII	GAGNNNNCTC (SEQ ID No. 580)	NNNNN/NNNNNNNNGAGNNNNNCTC (SEQ ID No. 581)
		/NNNNNNNNNNNNNCTC (SEQ ID No. 582)
Bsc4I	CCNNNNNNNGG (SEQ ID No. 583)	C C N N N N N / N N G G (SEQ ID No. 583)
		G G N N / N N N N N C C (SEQ ID No. 583)
BseLI	CCNNNNNNNGG (SEQ ID No. 584)	C C N N N N N / N N G G (SEQ ID No. 584)
		G G N N / N N N N N C C (SEQ ID No. 584)
BsiYI	CCNNNNNNNGG (SEQ ID No. 585)	C C N N N N N / N N G G (SEQ ID No. 585)
		G G N N / N N N N N C C (SEQ ID No. 585)
BstENI	CCTNNNNNAGG (SEQ ID No. 586)	C C T N N / N N N A G G (SEQ ID No. 586)
		G G A N N N / N N T C C (SEQ ID No. 586)
BstMWI	GCNNNNNNNGC (SEQ ID No. 587)	G C N N N N N / N N G C (SEQ ID No. 587)
		C G N N / N N N N N C G (SEQ ID No. 587)
CjeI	CCANNNNNGT (SEQ ID No. 588)	NNNNNN/NNNNNNNNCCANNNNNNGT (SEQ ID No. 589)
		/NNNNNNNNNNNNNGGT (SEQ ID No. 590)
Cjul	CAYNNNNRTG (SEQ ID No. 591)	C A Y N N N N N R T G (SEQ ID No. 591)
		G T R N N N N N Y A C (SEQ ID No. 591)
Cjull	CAYNNNNCTC (SEQ ID No. 592)	C A Y N N N N N C T C (SEQ ID No. 592)
		G T R N N N N N G A G (SEQ ID No. 593)
Dril	GACNNNGTC	G A C N N N / N N G T C (SEQ ID No. 594)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
	(SEQ ID No. 594)	C T G N N / N N N C A G (SEQ ID No. 594)
Eam1105I	GACNNNNNGTC (SEQ ID No. 595)	G A C N N N / N N G T C (SEQ ID No. 595) C T G N N / N N N C A G (SEQ ID No. 595)
EclHKI	GACNNNNNGTC (SEQ ID No. 596)	G A C N N N / N N G T C (SEQ ID No. 596) C T G N N / N N N C A G (SEQ ID No. 596)
Fall	AAGNNNNNCTT (SEQ ID No. 597)	NNNNN/NNNNNNNNNAAGNNNNNCTTNNNNNNNNNNNN/ (SEQ ID No. 598) /NNNNNNNNNNNNNTTCNNNNNGAANNNNNNNNNN/NNNNN (SEQ ID No. 599)
HpyF10VI	GCNNNNNNNGC (SEQ ID No. 600)	G C N N N N N / N N G C (SEQ ID No. 600) C G N N / N N N N N C G (SEQ ID No. 600)
NgoAVIII	GACNNNNNTGA (SEQ ID No. 601)	NN/NNNNNNNNNNNGACNNNNNTGANNNNNNNNNNNN/ (SEQ ID No. 602) /NNNNNNNNNNNNNCTGNNNNNACTNNNNNNNNNNNN/NN (SEQ ID No. 603)
NruGI	GACNNNNNGTC (SEQ ID No. 604)	G A C N N N / N N G T C (SEQ ID No. 604) C T G N N / N N N C A G (SEQ ID No. 604)
PfiBI	CCANNNNNTGG (SEQ ID No. 605)	C C A N N N N / N T G G (SEQ ID No. 605) G G T N / N N N N A C C (SEQ ID No. 605)
UbaF14I	CCANNNNNTCG (SEQ ID No. 606)	C C A N N N N N T C G (SEQ ID No. 606) G G T N N N N N A G C (SEQ ID No. 607)
XagI	CCTNNNNNAGG(SEQ ID No. 608)	C C T N N / N N N A G G (SEQ ID No. 608) G G A N N N / N N T C C (SEQ ID No. 608)
AasI	GACNNNNNGTC (SEQ ID No. 609)	G A C N N N N / N N G T C (SEQ ID No. 609) C T G N N / N N N N C A G (SEQ ID No. 609)
BdaI	TGANNNNNNTCA (SEQ ID No. 610)	NN/NNNNNNNNNNNTGANNNNNNTCANNNNNNNNNNNN/ (SEQ ID No. 611) /NNNNNNNNNNNNNACTNNNNNNAGTNNNNNNNNNN/NN (SEQ ID No. 612)
Bsp24I	GACNNNNNNTGG (SEQ ID No. 613)	NNNNN/NNNNNNNNNGACNNNNNNTGGNNNNNNNNNNNN/ (SEQ ID No. 614) /NNNNNNNNNNNNNCTGNNNNNNNACCNNNNNNNN/NNNNN (SEQ ID No. 615)
CjePI	CCANNNNNNNTC (SEQ ID No. 616)	NNNNNN/NNNNNNNCCANNNNNNNTCNNNNNNNNNNNNNN/ (SEQ ID No. 617) /NNNNNNNNNNNNNNGGTNNNNNNNAGNNNNNNNN/NNNNNN (SEQ ID No. 618)
DseDI	GACNNNNNGTC (SEQ ID No. 619)	G A C N N N N / N N G T C (SEQ ID No. 619) C T G N N / N N N N C A G (SEQ ID No. 619)
UbaF9I	TACNNNNNRTGT (SEQ ID No. 620)	T A C N N N N N R T G T (SEQ ID No. 620) A T G N N N N N Y A C A (SEQ ID No. 621)
ArsI	GACNNNNNNTTYG (SEQ ID No. 622)	NNNNN/NNNNNNNNNGACNNNNNNTTYGNNNNNNNNNNNN/ (SEQ ID No. 623)

Type IIS Restriction Enzyme	Sequence recognition site	Cut Site
		/NNNNNNNNNNNNNNCTGNNNNNNNAARCNNNNNN/NNNNN (SEQ ID No. 624)
BalI	GAAGNNNNNTAC (SEQ ID No. 625)	NNNNN/NNNNNNNGAAGNNNNNTACNNNNNNNNNNNN/ (SEQ ID No. 626)
		/NNNNNNNNNNNNCTTCNNNNNNATGNNNNNNN/NNNNN (SEQ ID No. 627)
PciI	WCGNNNNNNNCGW (SEQ ID No. 628)	W C G N N N N / N N N C G W (SEQ ID No. 628)
		W G C N N N / N N N N G C W (SEQ ID No. 628)
UbaF131	GAGNNNNNNCTGG (SEQ ID No. 629)	G A G N N N N N C T G G (SEQ ID No. 629)
		C T C N N N N N G A C C (SEQ ID No. 630)

[0091] In some alternatives, a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides is provided. The polynucleotide can comprise a first nucleic acid sequence comprising at least one endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 1-55 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs) of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs) of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. Some alternatives include a polynucleotide having a plurality of thymine nucleotides and an endonuclease recognition site within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the most 5' thymine residue of the poly(T) domain of the template strand, but configured or spaced to permit enzymatic cleavage within the poly(T) domain. In some alternatives the endonuclease recognition site is specific for an endonuclease. In some alternatives, the endonuclease is AatII, AbaSI, Acc65I, AccI, Acil, AclI, AfeI, AfilI, AfilII, AgeI, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, Apol, Ascl, AseI, AsiSI, Aval, Avail, AvrII, BaeGI, BaeI, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, Bccl, BceAI, Bcgl, BciVI, Bell, BcoDI, Bfal, BfuAI, BfuCI, BglI, BglII, BlnI, BmgBI, Bmrl, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, Bsal, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiVI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, BsrI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, Dpnl, DpnII, DraI, DrdI, EaeI, EagI, EarI, Ecil, Fco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinfPI, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, Nrul, NsiI, NspI, Nt.AIwI, Nt.BbvCI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.CviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, Pvul, Pvull, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, Sspl, Stul, StyD4I, Styl, Swal, Taqql, TfiI, TllI, TseI, Tsp45I, Tsp509I, TspMI, TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, or ZraI. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI. In some alternatives, the endonuclease is Stul. In some alternatives, the restriction site is an inverted Acil, MnlI, AlwI, BbvI, Bccl, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI,

FokI, HgaI, HphI, HpyAV, MboII, MlyI, PstI, SfaNI, AclI, BclI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EclI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaeI, FnuDII, FspBI, GluI, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, PstI, RsaNI, SetI, SgeI, Sse9I, TruI, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, BcnI, Bisl, Bisl, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CauII, CdiI, Cfr13I, Eco47I, EcoRII, FaeI, FinI, Fsp4HI, GluI, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, MvaI, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, Acsi, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, BcoI, Bcul, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FblI, FriOI, FunI, FunII, GdiI, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvrl, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, PceI, Pff23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, RcaI, SduI, Sfr274I, Sfr303I, Sful, SgrBI, SlaI, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, Axyl, Bpu1102I, Bse21I, Bsp1720I, BstPI, CclII, Cpol, CspI, DraII, Eco065I, Eco81I, Eco91I, EspI, KfiI, LglI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Maml, MroXI, Olil, Pdml, Rsel, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMWM, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PflBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I endonuclease restriction recognition site. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, said endonuclease recognition site is located within 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 55 base pairs of the endonuclease cleavage site for the endonuclease or any number of base pairs between two aforementioned values, and said endonuclease cleavage site is within 1, 5, 10, 15, 20, 25, 30, 35 or 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides of the template strand or any number of base pairs between any two aforementioned values. In some alternatives, the endonuclease recognition site is inverted and the endonuclease cleavage site is inverted. In some alternatives, the endonuclease recognition site and endonuclease cleavage site comprise a sequence set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5' side of the guanine, cytosine or adenine.

[0092] In some alternatives, the endonuclease is a Type IIS restriction endonuclease, wherein the Type IIS restriction endonuclease cleaves at a distance from the endonuclease recognition site. In some alternatives, the Type IIS restriction endonuclease recognition site, comprises the sequences for the sequence recognition site as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the Type IIS restriction endonuclease is Acil, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PstI, SfaNI, AclI, BclI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EclI, MmeI, NmeAIII,

BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, CspGI, FaeI, FaiI, FnuDII, FspBI, GaiI, HapII, HinII, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, Tru1I, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, BceI, BclI, BsiI, BslI, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BsiFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CauI, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, GluI, Hin4I, HpyF3I, ItaI, MaeIII, MspR9I, MvaI, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, AclI, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, BbeI, BbrPI, BbuI, Bbv12I, BbvII, Bce83I, BcoI, BcuI, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, BmiI, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, BveI, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FblI, FriOI, FunI, FunII, GdiI, GsaI, HaeI, HgiAI, HinI, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhiI, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, NsbI, NspIII, NspV, PaeI, Pagi, Paul, PceI, Pfi23I, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, SlaI, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, Cspl, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNI, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. In some alternatives, the sequence recognition site is inverted. In some alternatives, wherein the sequence recognition site is inverted, the Type IIS restriction endonuclease can cleave within the second nucleic acid sequence, wherein the endonuclease can cleave within the plurality of thymine nucleotides. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

[0093] In some alternatives, a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The polynucleotide can comprise a first nucleic acid sequence comprising at least one endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 1-55 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 base pairs) of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage

site is within 1 to 40 base pairs (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 base pairs) of a most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises a third nucleic acid sequence encoding a nuclease, such as Cas9, a derivative thereof or active fragment thereof, see e.g., **SEQ ID. Nos. 6-14**, a TALEN, or a MegaTAL, wherein the third nucleic acid is operably linked to the second nucleic acid sequence comprising the plurality of thymine nucleotides at opposite end of the attached first nucleic acid comprising the endonuclease recognition site. In some alternatives the at least one endonuclease recognition site is specific for an endonuclease. In some alternatives, the endonuclease recognition site is inverted. In some alternatives the endonuclease recognition site is specific for a Type IIS restriction endonuclease. In some alternatives, the recognition site is a Type IIS restriction endonuclease recognition site. In some alternatives, the Type IIS restriction endonuclease recognition site is inverted. In some alternatives, the endonuclease recognition site comprises any one of the endonuclease recognition sites described in **Table 1** (SEQ ID Nos: 18-632, 634-636). Some alternatives include a polynucleotide having a plurality of thymine nucleotides, a first nucleic acid sequence encoding a nuclease, such as Cas9, a derivative thereof or active fragment thereof, see e.g., **SEQ ID. Nos. 6-14**, a TALEN, or a MegaTAL, and an endonuclease recognition site within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 or 55 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values upstream (5' in orientation) from the first thymine residue of the poly(T) domain but configured or spaced to permit enzymatic cleavage within the poly(T) domain. In some alternatives, the endonuclease is AatII, AbaSI, Acc65I, AccI, Acil, AclI, AfeI, AflII, AflIII, AgeI, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, ApoI, AscI, AseI, AsiSI, Aval, Avall, AvrII, BaeGI, BaeI, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, BclI, BceAI, BcgI, BciVI, Bell, BcoDI, BfaI, BfuAI, BfuCI, BglI, BglII, BplI, BmgBI, Bmrl, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKA, BsiVI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, Bsrl, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Fco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinPII, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MbolI, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, Nrul, NsiI, Nspl, Nt.AlwI, Nt.BbvCI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.CviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PliI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, Sall, SapI, Sau3AI, Sau961, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, Sspl, Stul, StyD4I, Styl, SwaI, Taqql, TfiI, TllI, TseI, Tsp45I, Tsp509I, TspMI TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, or ZraI. In some alternatives, the endonuclease is BsaI. In some alternatives, the endonuclease is Stul. Endonucleases can be an enzyme that cleaves the phosphodiester bond within a polynucleotide. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5'

thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5'side of the guanine, cytosine or adenine.

[0094] Some alternatives relate to an mRNA that comprises a poly A tail, wherein within said poly A tail or upstream of the first adenine residue of said poly(A) tail is the gene. Some of these mRNAs also include a sequence that encodes a gene, such as a nuclease (e.g., Cas9 or derivative thereof or active fragment thereof, see e.g., **SEQ ID. Nos. 6-14**, TALEN, or MegaTAL). In some alternatives, an mRNA having a plurality of adenine nucleotides is provided, wherein the mRNA comprises a first sequence comprising a gene, a second sequence comprising a plurality of adenine nucleotides, and a third sequence that comprises a compliment sequence to an endonuclease recognition site, wherein the first sequence is covalently linked to the second sequence at the 5' end of said plurality of adenine nucleotides and wherein said mRNA comprises a third sequence that comprises a compliment sequence to an endonuclease recognition site wherein the third sequence is covalently linked to said plurality of adenine nucleotides of said second nucleic acid at the 3' end of said plurality of adenine nucleotides of said second nucleic acid. This type of mRNA can occur if the polynucleotide for transcribing the mRNA was not cleaved with the Type IIS restriction endonuclease. Some alternatives include an mRNA having a plurality of adenine nucleotides and at least one complimentary sequence to an endonuclease recognition site, such as Stul or BsaI (on the single strand) within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values upstream (5' in orientation) from the first adenine residue of the poly(A) domain. In some alternatives, the complimentary sequence is a compliment (on the single strand) to the endonuclease recognition site of AatII, AbaSI, Acc65I, AccI, Acil, AclI, AfeI, AflI, AflII, Agel, AhdI, AleI, AluI, AlwI, AlwNI, ApaI, ApaLI, ApeKI, Apol, AscI, AseI, AsiSI, Aval, Avall, AvrII, BaeGI, BaeI, BamHI, BanI, BanII, BbsI, BbvCI, BbvI, BccI, BceAI, BcgI, BciVI, Bell, BcoDI, BfaI, BfuAI, BfuCI, BglI, BgIII, BlnI, BmgBI, BmrI, BmtI, Bpml, Bpu10I, BpuEI, BsaAI, BsaBI, BsaHI, BsaI, BsaJI, BsaVI, BsaXI, BseRI, BseYI, BsgI, BsiEI, BsiHKAI, BsiVI, BslI, BsmAI, BsmBI, BsmFI, BsmI, BsoBI, Bsp1286I, BspCNI, BspDI, BspEI, BspHI, BspMI, BspQI, BsrBI, BsrDI, BsrFI, BsrGI, BsrI, BssHII, BssKI, BssSI, BstAPI, BstBI, BstEII, BstNI, BstUI, BstXI, BstYI, BstZ17I, Bsu36I, BtgI, BtgZI, BtsCI, BtsI, BtsIMutI, Cac8I, ClaI, CspCI, CviAI, CviKI-1, CviQI, DdeI, DpnI, DpnII, DraI, DrdI, EaeI, EagI, EarI, EciI, Fco53kI, EcoNI, EcoO109I, EcoP15I, EcoRI, EcoRV, FatI, Faul, Fnu4HI, FokI, FseI, FspEI, FspI, HaeII, HaeIII, HgaI, HhaI, HincII, HindIII, HinfI, HinP1I, HpaI, HpaII, HphI, Hpy166II, Hpy188I, Hpy188III, Hpy99I, HpyA, HpyCH4III, HpyCH4IV, HpyCH4V, I-CeuI, I-SceI, KasI, KpnI, LpnPI, MboI, MboII, MfeI, MluCI, MluI, MlyI, MmeI, MnlI, MscI, MseI, MslI, MspA1I, MspI, MspJI, MwoI, NaeI, NarI, BbvCI, BsmI, BsrDI, BtsI, NciI, NcoI, NdeI, NgoMIV, NheI, NlaIII, NlaIV, NmeAIII, NotI, NruI, NsiI, NspI, NtAlwI, Nt.BbvCI, Nt.BsmAI, Nt.BspQI, Nt.BstNBI, Nt.CviPII, PacI, PaeR7I, PciI, PfiFI, PfiMI, PI-PspI, PI-SceI, PleI, PluTI, PmeI, PmlI, PpuMI, PshAI, PsiI, PspGI, PspOMI, PspXI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, SalI, SapI, Sau3AI, Sau96I, SbfI, ScrFI, SexAI, SfaNI, SfcI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI, SpeI, SphI, SspI, Stul, StyD4I, Styl, SwaI, TaqI, TfiI, TllI, TseI, Tsp45I, Tsp509I, TspMI TspRI, Tth111I, XbaI, XcmI, XhoI, XmaI, XmnI, or ZraI. In some alternatives, the sequence of the at least one endonuclease site is inverted, wherein the sequence encoding the RNA compliment is a compliment to the inverted sequence of the at least one endonuclease site (on the single strand). In some alternatives, the sequence encoding the RNA compliment is a compliment to any one of the endonuclease recognition sites described in **Table 1** (Type IIS restriction endonucleases) (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease recognition sites described in **Table 1** is inverted (SEQ ID Nos: 18-632, 634-636). In some alternatives, wherein the endonuclease site is the Type IIS restriction endonuclease recognition site, the Type IIS restriction endonuclease recognition sites are recognition sites for Acil, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, Afal, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, Mvnl, Pali, RsaNI, SetI, SgeI, Sse9I, Trull, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, Asul, Bcefi, Bcni, Bisi, Bisi, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BsiFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CaulI, CdiI, Cfr13I, Eco47I, EcoRII, FaqI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Itai, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, Aatl, Aaul, Acc113I, Acc16I, AccB1I, AceIII, Acsl, Acvl, Acyl, AhI, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsulI, AsuNHI, AvalI, AvilI, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, Bcol, Bcul, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI,

BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, BveI, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FblI, FriOI, FunI, FunII, GdiII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, Mlsl, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, MvrI, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, PciI, Pfl23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, SlaI, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, CpoI, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, MabI, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, SgfI, SmiI, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNI, Mami, MroXI, Olil, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMVI, CjeI, CjuI, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi and UbaF13I. In some alternatives, the recognition sites are inverted, thus the complimentary sequence is also inverted. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, the endonuclease cleaves at the at the 5' side of the guanine, cytosine or adenine.

[0095] BsaI is a Type II restriction endonuclease which also falls in the subgroup for Type IIS endonucleases. Type IIS restriction endonucleases can cleave DNA at a defined distance from their non-palindromic asymmetric recognition sites. This feature can be used, for example, for lengthening a tail for a poly (A) tract. Although a tail, such as a poly (A) tract can be created by insertion of the sequence into any restriction site, only a Type IIS restriction site can be used to lengthen a tail, such as a poly (A) tract once a poly (A) tract is in place. Only a Type II S restriction site can cleave adjacent to and not within the recognition sequence, thereby cleaving in the poly (T) region of the said polynucleotide and ensuring that the poly (A) tail that is transcribed from the said polynucleotide does not terminate in a sequence that is related to the restriction site itself.

[0096] Stul is a Type II restriction endonuclease that leaves a blunt end when it cleaves a double stranded DNA. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted.

[0097] In order for the poly(A) tail to terminate without including additional bases beyond it, a Type IIS restriction recognition site can be placed downstream from the intended cut site and within the poly(A) tail, in which the restriction site is "inverted" or "facing the poly(A) site, such that the restriction cleavage site is within the poly(A) encoding region (the plurality of thymine residues in the template strand). Therefore the restriction site can be used to lengthen the tail

once it is in place. The restriction site can be encoded within the poly(A) encoding region. In some alternatives of the polynucleotide, the polynucleotide comprises a Type IIS endonuclease recognition site downstream from the intended cut site, thereby creating a Type IIS endonuclease cleavage site upstream from the Type IIS endonuclease recognition site. In some alternatives, the Type IIS restriction is inverted. In some alternatives, the Type IIS endonuclease cleavage site is at a distance of 1-55 bases away from the Type IIS endonuclease recognition site. In some alternatives, the Type IIS endonuclease cleavage site is at a distance of 1, 5, 10, 20, 30, 40 or 55 bases away from the Type IIS endonuclease recognition site or a distance in between any two of the aforementioned bases from the Type IIS endonuclease recognition site.

[0098] In some alternatives, a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides is provided, wherein the polynucleotide comprises a first nucleic acid sequence comprising at least one endonuclease recognition site and a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is within the plurality of thymine nucleotides and wherein said first nucleic acid is covalently linked to said second nucleic acid at one end of said second nucleic acid, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides or any number of covalently linked thymine nucleotides between any two aforementioned values. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PstI, SfaNI, AclI, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, Bsal, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, Fail, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, TruI, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefl, BcnI, Bisl, Blsl, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, Itai, MaeIII, MspR9I, Mval, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, Aaul, Acc113I, Acc16I, AccB1I, AceIII, Acsl, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, Bbel, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, BcoI, Bcul, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, Dsal, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, Fbal, FblI, FriOI, FunI, FunII, GdII, Gsal, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, Kspl, Mfil, MhII, Misl, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvrl, NgoAIV, Nsbl, NsplIII, NspV, Pael, PagI, Paul, Pcel, Pfl23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, Rcal, Sdul, Sfr274I, Sfr303I, Sful, SgrBI, Slal, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, Xmil, ZhoI, Zsp2I, Aocl, Axyl, Bpu1102I, Bse21I, Bsp1720I, BstPI, CelII, Cpol, Cspl, Drall, Eco065I, Eco81I, Eco91I, Espl, Kfil, Lgul, Mabl, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, Mrel, Mssl, Rgal, Sdal, SfaAI, Sgfl, Smil, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Maml, MroXI, OilI, Pdml, Rsel, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMVI, Cjel, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PflBI, UbaF14I, Xagl, Aasi, Bdal, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or

UbaF13I. In some alternatives, the sequence recognition site is inverted. In some alternatives, the cut site, or endonuclease cleavage site is inverted. In some alternatives, the endonuclease recognition site comprises any one of the endonuclease recognition sites described in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease is Acil, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MbolI, MlyI, PstI, SfaNI, AclI, BciVI, BfuAI, BmgBI, BmrI, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaeI, FnuDII, FspBI, Glal, HapII, Hin1I, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, PstI, RsaNI, SstI, SgeI, Sse9I, TruI, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, Bcni, Bisi, Bisi, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BsiFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, Caull, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, GluI, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, MvaI, NmuCI, Psp6I, PspPI, Sati, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceIII, Acsi, AcvI, Acyl, AhII, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, Bbel, BbrPI, BulI, Bbv12I, BbvII, Bce83I, BcoI, BcuI, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, BsbI, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, Fbal, FblI, FriOI, FunI, FunII, GdII, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MliI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvri, NgoAIV, Nsbl, NspIII, NspV, PaeI, Pagi, Paul, PceI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, PsuI, RcaI, SduI, Sfr274I, Sfr303I, SfuI, SgrBI, SlaI, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, EspI, KfiI, LguI, Mabi, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, RgaI, SdaI, SfaAI, Sgfi, SmiI, Sse232I, Adel, Aspi, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNI, Mami, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, Xagi, Aasi, Bdal, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a BsaI or a StuI restriction endonuclease recognition site and the endonuclease is BsaI or StuI. In some alternatives, the endonuclease cleavage site is between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50 or 55 nucleotides from the endonuclease recognition site or any number of nucleotides between any two aforementioned values away from the endonuclease recognition site. In some alternatives, the polynucleotide further comprises a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9 or a derivative thereof or active fragment thereof, a TALEN, or MegaTAL, see e.g., SEQ ID. Nos. 6-14. In some alternatives, a method of making a polynucleotide having a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, and wherein said template strand comprises a plurality of thymine nucleotides is provided, wherein the method comprises providing a first nucleic acid sequence comprising at least one endonuclease recognition site, providing a second nucleic acid sequence, wherein the second nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least 5, 10, 20, 50, 100, 200, 300, 400, 500 or 550 covalently linked thymine nucleotides and wherein the second nucleic acid comprises the endonuclease cleavage site and joining said first nucleic acid sequence to said second nucleic acid sequence, wherein said first nucleic acid is covalently linked to said second nucleic acid at one end, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI or a StuI restriction endonuclease recognition site, and the endonuclease is BsaI or StuI. In some alternatives, the endonuclease cleavage site is between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50 or 55 nucleotides from the endonuclease recognition site or any number of nucleotides between any two aforementioned values away from the endonuclease recognition site. In some alternatives, the

polynucleotide further comprises a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9 or a derivative thereof or active fragment thereof, a TALEN, or MegaTAL, see e.g., **SEQ ID. Nos. 6-14**. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0099] Disclosed herein are methods for making a linear polynucleotide having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or attached thereto, as well as, compositions comprising these polynucleotides and methods of use thereof. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site, and the endonuclease is BsaI. In some alternatives, the Type IIS restriction endonuclease recognition site is inverted. In some alternatives, the Type IIS restriction endonuclease site is an inverted BsaI restriction endonuclease restriction site. In some alternatives, the polynucleotide or vector having the plurality of covalently linked thymine residues comprises only one BsaI restriction site such that said BsaI site is unique for a linker domain defined by having 5' and 3' polymers of thymine residues flanking said BsaI site. In some alternatives, the polynucleotide comprises or encodes a gene. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is Cas9 or a derivative thereof or active fragment thereof, a TALEN, or MegaTAL, see e.g., **SEQ ID. Nos. 6-14**. In some alternatives, the Type IIS restriction is inverted. In some alternatives, the Type IIS endonuclease cleavage site is at a distance of 11, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50 or 55 nucleotide bases away from the Type IIS endonuclease recognition site or any distance between any two aforementioned values. In some alternatives, the Type IIS endonuclease cleavage site is at a distance of 1-4 bases away from the Type IIS endonuclease recognition site. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

Linear plasmids

[0100] Molecular cloning requires the incorporation of genetic material into a nucleic acid. Despite advances in sequencing and molecular cloning, complete sequencing and analysis of proteins require cloning of specific regions, such as a binding site or a site that confers catalytic activity. Many of these regions are difficult to clone in current vectors, such as circular vectors, however. Difficult coding regions and large inserts can be the cause of superhelical stress in circular plasmids and can generate secondary structures that are substrates for deletion, particularly in regions that contain numerous tandem or inverted repeats. Transcription from cloned promoters can also interfere with plasmid stability.

[0101] Linear vectors provide several desirable features to the use of circular supercoiled plasmids for cloning. Linear vectors are not subject to the supercoiling found in circular plasmids and can stably maintain inserts that have primary or secondary structures that are unstable when supercoiled. This additional stability may result in improved sequencing data and reductions in the number of sequence gaps and cloning gaps in genomic assemblies. Linear vectors can also exhibit the ability to clone larger inserts using standard methods. Linear vector cloning systems can stably clone a nucleic acid, such as DNA, in the range of 10 to over 100 kb, without the use of packaging systems required with cosmid cloning. The concept of using a linear vector for cloning is similar to that of a linear plasmid vector, which is described, for example, in WO 2007/087478 and in Godiska et al (Nucleic Acids Research (2010), vol. 38, no. 6), which are both hereby expressly incorporated by reference in their entireties.

[0102] In order to create a linear vector from a linear plasmid vector with a unique inverted BsaI restriction site within a multicloning site, extra plurality of BsaI sites had to be removed. Therefore, a linear plasmid vector was used for synthesizing the plasmids with various lengths of poly (A) encoding tracts (pEVL). The linear plasmid vector was used for synthesizing a nucleic acid having a poly (T) tract of a desired length and these experiments are described in greater detail.

[0103] Linear vector cloning systems can be used to clone difficult polynucleotide sequences that cannot be cloned into conventional circular plasmid vectors. For example, the linear vectors can maintain fragments that are unstable in the supercoiled plasmid form. The linear mode of replication can impart high fidelity replication of repeats, large palindromes, AT-rich DNA, and high repeats, for example a thymine rich region. As disclosed herein, a linear vector can be used for making a polynucleotide having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or attached thereto. The use of a circular plasmid is unable to support a template for making a long poly(A) tract and commercially available plasmids for transcribing a poly (A) tract are typically limited to about 30 adenines. The use of a linearized plasmid, or a linear plasmid, can enhance the transcription of difficult genes that are large and have high repeats. A linear cloning vector can include a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. As described herein, the polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide further comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. Telomeres can be used for protection of the termini of the linear polynucleotide from recombination and degradation. Telomeres for linearized plasmid vectors can include telomeres derived from bacteriophages lambda N15, K02, PRD1 and PY54, and from linear chromosomes from *Agrobacterium*, for example. "Selectable markers" as described herein, refers to a gene introduced into a nucleic acid to protect a transformed cell from a specific factor in the growth environment, and can be an antibiotic resistance gene, a metabolic marker or a screenable marker. Examples of antibiotic resistance genes that can reside in a linear vector polynucleotide can include kanamycin, ampicillin, tetracycline, and chloramphenicol, for example. Metabolic markers can include amino acid synthesis genes, for example genes for trp synthesis. In some alternatives, the polynucleotide further comprises a selectable marker. In some alternatives, the selectable marker is an antibiotic resistance gene, a metabolic marker or a screenable marker. In some alternatives, the polynucleotide further comprises a metabolic marker. In some alternatives the metabolic marker comprises amino acid synthesis genes, for example genes for trp synthesis. In some alternatives, the polynucleotide comprises an antibiotic resistance gene. In some alternatives, the antibiotic resistance gene is a kanamycin resistant gene, ampicillin resistant gene, tetracycline resistant gene, or chloramphenicol resistant gene. Protelomerase proteins are enzymes that are responsible for the generation of closed hairpinends in linear DNA. The target sites of protelomerases can be inverted repeats. Protelomerase proteins can also process a target site, in which the target site can have dyad symmetrical DNA. Protelomerases are a class of unique proteins that function to generate covalently closed hairpin ends in DNA. These linear DNA ends are arranged such that one strand turns around and becomes the complementary strand. In this manner, these linear DNA molecules do not have free or open ends, and are expected to be stable and not vulnerable to exonuclease degradations. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has a protelomerase target site. In some alternatives, the target site has a dyad symmetrical DNA site. Protelomerase function and sites for binding of the protelomerase are discussed in Godiska et al. (U.S. 9029134; incorporated by reference in its entirety).

[0104] By one approach, a polynucleotide having a plurality of thymine nucleotides is made within a linear vector. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence

comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Accordingly, methods for making a nucleic acid from a linear polynucleotide having a plurality of thymine nucleotides and an endonuclease disposed within said plurality of thymine nucleotides is provided herein. Methods for enhancing transcription and translation utilizing said compositions are also alternatives. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide is a linear cloning vector, wherein the linear cloning vector comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm.

[0105] In some alternatives, a linear polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides is provided. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between, 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide is a linear cloning vector, wherein the linear cloning vector comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. In some alternatives, a gene is cloned into the cloning region wherein the gene encodes a nuclease. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein *StuI* is provided, the endonuclease cleavage site is a *StuI* cleavage site, AGGCCT (SEQ ID NO: 633), and *StuI* cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *StuI*. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *StuI* cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *BsaI* restriction recognition site, and the endonuclease is *BsaI*. In some alternatives, the *BsaI* restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0106] In some alternatives, the polynucleotide comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 525 or 550 covalently linked thymine residues within the template strand. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some

alternatives the Type II endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is inverted. In some alternatives, the Type IIS restriction endonuclease recognition site is a sequence set forth in **Table 1** and is inverted (SEQ ID Nos: 18-632, 634-636). In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI endonuclease recognition site and endonuclease is BsaI. In some alternatives, the BsaI endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one BsaI restriction site. In some alternatives, the polynucleotide has an inverted BsaI restriction site. In some alternatives, the polynucleotide further comprises a gene. In some alternatives the gene is 10-50 kb. In some alternatives, the gene is 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 kb or any length between any two aforementioned values. In some alternatives, the gene is at least 100 Kb. In some alternatives, the polynucleotide or vector having the plurality of covalently linked thymine residues comprises only one BsaI restriction site such that said BsaI site is unique for a linker domain defined by having 5' and 3' polymers of thymine residues flanking said BsaI site. In some alternatives, the polynucleotide or vector having the plurality of covalently linked thymine residues comprises only one BsaI restriction site, wherein the BsaI restriction site is inverted. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide is a linear cloning vector, wherein the linear cloning vector comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0107] Also described herein are methods of making a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymines or any number of thymines between any two aforementioned values. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine. In some alternatives, wherein the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine, resulting in a plurality of thymine residues that can terminate in a guanine, cytosine or adenine. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site, and the endonuclease is BsaI. In some alternatives, the Type IIS restriction endonuclease recognition site is inverted. In some alternatives, the Type IIS restriction endonuclease site is an

inverted BsaI restriction endonuclease restriction site. In some alternatives, the endonuclease recognition site comprises an endonuclease recognition site sequence set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site and the endonuclease is BsaI. In some alternatives, the at least one Type II restriction endonuclease recognition site is an inverted BsaI restriction endonuclease recognition site. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one BsaI restriction site. In some alternatives, the polynucleotide further comprises a gene. In some alternatives, the gene comprises at least, greater than, equal to, or any number in between 1, 5, 10, 20, 30, 40 or 50 Kb or any number between any two aforementioned values. In some alternatives, the gene is at least 100 Kb. In some alternatives the gene is 1-50 kb. In some alternatives, the polynucleotide or vector having the plurality of covalently linked thymine residues comprises only one BsaI restriction site, wherein the BsaI restriction site is inverted. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide is a linear cloning vector, wherein the linear cloning vector comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

PCR amplification.

[0108] Polymerase chain reaction (PCR) is a technique used for many biological applications and involves the amplification of a single or several copies of a piece of a polynucleotide in order to generate thousands or millions of copies of a specific nucleic acid. PCR can result in multiple copies of DNA. PCR utilizes thermal cycling in which several cycles of heating and cooling of the reaction results in melting the template polynucleotide for replication, enzymatic replication, and amplification of copies of the polynucleotide resulting in thousands or millions of copies of the polynucleotide. The melting (90°C to 105 °C), annealing (5°C below the melting temperature of the primer), and amplification (70°C to 74 °C) cycle can be repeated 12 to 35 times and is dependent on the stability of the polynucleotide template and primers. PCR can require a heat stable DNA polymerase, complimentary primers, and nucleotides. Nucleotides for PCR can be adenine, cytosine, guanine, thymine and/or uracil. DNA polymerases for PCR can be a thermal stable polymerase that can polymerize DNA at temperatures from 90°C to 105 °C. Thermal stable polymerases can include without limitation, Taq polymerases, fusion Taq polymerases comprising processivity enhancing domains, genetically engineered Taq enzymes for high fidelity and fast extension, as well as other heat stable polymerases known to those skilled in the art.

[0109] Fusion PCR can also be used to amplify a nucleic acid from a polynucleotide template. However, fusion PCR is also used to extend the amplified nucleic strand at either the 5' end or the 3'end, or for both ends of the nucleic acid by use of extended primers. Fusion PCR can be performed by using a 5'primer and a 3'primer, in which either the 5'primer, 3'primer or both have an extension of sequences as well as a site that is complimentary to the polynucleotide template for extension. For example, fusion PCR can be used to add an additional plurality of thymine nucleotides at the 3' end of a gene sequence.

[0110] Disclosed herein are methods for making a nucleic acid having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or

attached thereto, as well as, compositions comprising these polynucleotides and methods of use thereof. In some alternatives, the endonuclease recognition sites are inverted. In some alternatives, the endonuclease recognition sites comprise the endonuclease recognition sequences set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease recognition site is for the endonuclease Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PstI, SfaNI, AclI, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaiI, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, TruII, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, BcnI, BsiI, BslI, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CauII, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, MvaI, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceII, Acsi, AcvI, Acyl, AhI, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, BbeI, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, BcoI, Bcul, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, Bsbl, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FbII, FriOI, FunI, FunII, GdiI, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvrl, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, PceI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, Rcal, Sdul, Sfr274I, Sfr303I, Sful, SgrBI, Slal, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, Espl, KfiI, LguI, Mabl, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, Rgal, SdaI, SfaAI, Sgfi, Smil, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, MamI, MroXI, OliI, PdmI, RseI, SmiMI, AccB7I, AspEI, BasI, BmeRI, BpII, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, CjeI, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PfiBI, UbaF14I, XagI, AasI, Bdal, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, Pcsi or UbaF13I. In some alternatives, the endonuclease recognition sites are specific for a Type IIS restriction endonuclease. In some alternatives, the Type IIS restriction endonuclease is Acil, MnlI, AlwI, BbvI, BclI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, FokI, HgaI, HphI, HpyAV, MboII, MlyI, PstI, SfaNI, AclI, BciVI, BfuAI, BmgBI, Bmrl, Bpml, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, AfaI, AluBI, AspLEI, BscFI, Bsh1236I, BshFI, BshI, BsiSI, BsnI, Bsp143I, BspANI, BspFNI, BssMI, BstENII, BstFNI, BstHHI, BstKTI, BstMBI, BsuRI, CfoI, Csp6I, FaeI, FaiI, FnuDII, FspBI, Glal, HapII, Hin1II, R9529, Hsp92II, HspAI, MaeI, MaeII, MvnI, Pali, RsaNI, SetI, SgeI, Sse9I, TruII, Tru9I, TscI, TspEI, TthHB8I, XspI, AflI, AgsI, AspS9I, AsuC2I, AsuI, Bcefi, BcnI, BsiI, BslI, Bme1390I, Bme18I, BmrFI, BscGI, BseBI, BsiLI, BsiZI, BslFI, BsoMAI, BspNCI, Bst2UI, Bst71I, BstDEI, BstOI, BstSCI, CauII, CdiI, Cfr13I, Eco47I, EcoRII, FagI, FinI, Fsp4HI, Glul, Hin4II, HpyF3I, ItaI, MaeIII, MspR9I, MvaI, NmuCI, Psp6I, PspPI, SatI, SinI, TscAI, VpaK11BI, AanI, AatI, AauI, Acc113I, Acc16I, AccB1I, AceII, Acsi, AcvI, Acyl, AhI, Alw21I, Alw44I, Ama87I, Aor51HI, AsiAI, AsnI, Asp718I, AspHI, AsuII, AsuNHI, AvaIII, AvII, BanIII, Baul, BbeI, BbrPI, Bbul, Bbv12I, BbvII, Bce83I, BcoI, Bcul, Bfml, BfrBI, BfrI, Bini, BmcAI, BmeT110I, Bmil, Bmul, BmyI, Bpu14I, BpvUI, Bsa29I, BsaOI, Bsbl, BscBI, BscCI, Bse118I, BseAI, BseCI, BseDI, BsePI, BseSI, BseX3I, Bsh1285I, BshNI, BshTI, BshVI, BsiCI, BsiHKCI, BsiMI, BsiQI, BsiXI, Bsp106I, Bsp119I, Bsp120I, Bsp13I, Bsp1407I, Bsp143II, Bsp19I, Bsp68I, BspA2I, BspCI, BspGI, BspLU11I, BspMAI, BspMII, BspOI, BspT104I, BspT107I, BspTI, BspXI, BssAI, BssHI, BssNAI, BssNI, BssT1I, Bst1107I, Bst98I, BstACI, BstAFI, BstAUI, BstBAI, BstC8I, BstDSI, BstH2I, BstHPI, BstNSI, BstSFI, BstSLI, BstSNI, BstX2I, BstZI, BsuTUI, BtuMI, Bvel, CciI, Cfr10I, Cfr42I, Cfr9I, CfrI, Csp45I, CspAI, DinI, DrdII, DsaI, Ecl136II, EclXI, Eco105I, Eco130I, Eco147I, Eco24I, Eco32I, Eco47III, Eco52I, Eco72I, Eco88I, EcoICRI, EcoT14I, EcoT22I, EcoT38I, EgeI, EheI, ErhI, FauNDI, FbaI, FbII, FriOI, FunI, FunII, GdiI, GsaI, HaeI, HgiAI, Hin1I, HindII, Hpy178III, Hpy8I, Hsp92I, Kpn2I, Ksp22I, KspAI, KspI, MfiI, MhII, MlsI, MluNI, Mly113I, Mph1103I, MroI, MroNI, Msp20I, MspCI, MstI, MunI, Mvrl, NgoAIV, Nsbl, NspIII, NspV, PaeI, PagI, Paul, PceI, Pfi23II, PinAI, Ple19I, PmaCI, PshBI, Psp124BI, Psp1406I, PspAI, PspLI, PspN4I, Psul, Rcal, Sdul, Sfr274I, Sfr303I, Sful, SgrBI, Slal, SpaHI, SseBI, SspBI, SstI, SstII, SunI, TatI, Vha464I, VneI, XapI, XhoII, XmaCI, XmaIII, XmaJI, XmiI, ZhoI, Zsp2I, AocI, AxyI, Bpu1102I, Bse21I, Bsp1720I, BstPI, CellI, Cpol, CspI, DralI, Eco065I, Eco81I, Eco91I, Espl, KfiI, LguI, Mabl, PpuXI, Psp5II, PspEI, Rsr2I, Saul, AbsI, CciNI, FspAI, MauBI, MreI, MssI, Rgal,

SdaI, SfaAI, Sgfl, Smil, Sse232I, Adel, Aspl, Cail, Psyl, Tell, Asp700I, BoxI, Bse8I, BseJI, BsiBI, BsrBRI, BstPAI, CjeNII, Maml, MroXI, OliI, Pdml, Rsel, SmiMI, AccB7I, AspEI, BasI, BmeRI, BplI, Bsc4I, BseLI, BsiYI, BstENI, BstMWI, Cjel, Cjul, Cjull, Dril, Eam1105I, EclHKI, Fall, HpyF10VI, NgoAVIII, NruGI, PflBI, UbaF14I, XagI, AasI, BdaI, Bsp24I, CjePI, DseDI, UbaF9I, Arsl, BarI, PcsI or UbaF13I. In some alternatives the Type IIS restriction endonuclease recognition site is inverted and the cleavage site is inverted. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0111] In some alternatives, the poly (T) tract in the nucleic acid can be extended by fusion PCR. In some alternatives, the poly (T) tract in the nucleic acid can be extended at the 5' end of the template strand by fusion PCR. In some alternatives, the poly (T) tract in the nucleic acid can be extended at the 3'end by fusion PCR. In some alternatives, the nucleic acid can be extended at the 5' and the 3'end by fusion PCR. In some alternatives, the nucleic acid can be extended by a plurality of thymine nucleotides at the 3'end by fusion PCR.

[0112] In more alternatives, a method of making a nucleic acid is contemplated, wherein said method comprises providing any of the polynucleotides described above, and contacting said polynucleotide with a polymerase in the presence of nucleotides. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. The polynucleotide is a linear cloning vector, wherein the linear cloning vector comprises a left telomere and at least one selectable marker, a right arm comprising a right telomere and at least one selectable marker, and a cloning region located between the left arm and the right arm. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the primers can have an extension sequence at the 5' end. In some alternatives, the primers can have an extension sequence at the 3' end. In some alternatives, the nucleic acid is DNA. In some alternatives, the polynucleotide comprises a DNA polymerase. In some alternatives, the polynucleotide further comprises a gene. In some alternatives the gene is 1-50 kb. In some alternatives, the gene comprises at least, greater than, equal to, or any number in between 1, 5, 10, 20, 30, 40 or 50Kb. In some alternatives, the mRNA comprises a poly A tail, wherein within said poly A tail there exists a sequence that is complimentary to an endonuclease site (on the single strand), such as StuI or BsaI site. A sequence

complimentary to the endonuclease recognition site can reside in the mRNA if an endonuclease is not provided to cleave the polynucleotide before transcription. In some alternatives, the sequence is complimentary to an endonuclease recognition site set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, an mRNA comprising a plurality of adenine nucleotides is provided, wherein the mRNA comprises a first sequence comprising at least one compliment sequence to an endonuclease recognition site, a second nucleic acid sequence, wherein the second nucleic acid sequence comprises a plurality of adenine nucleotides, and wherein the first sequence is covalently linked to the second nucleic acid sequence at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9 or a derivative thereof or active fragment thereof, a TALEN, or MegaTAL, see e.g., **SEQ ID. Nos. 6-14**. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site. In some alternatives, the at least one endonuclease site is a Stul recognition site. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the sequence encoding an RNA compliment is a compliment to an endonuclease recognition site set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the endonuclease recognition site as set forth in **Table 1** (SEQ ID Nos: 18-632) is an inverted sequence. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

In vivo and in vitro transcription.

[0113] *In vitro* and *in vivo* transcription can give the ability to synthesize RNA that is critical for many biological techniques. For example, *in vivo* and *in vitro* transcription can generate radiolabeled and nonisotopically labeled RNA probes that can be used in blot hybridizations, nuclease protection assays, as well as RNA that can be used in translation to generate a specific protein.

[0114] *In vitro* transcription requires a purified linear DNA template containing an RNA polymerase promoter, ribonucleotide triphosphates or nucleotides, a buffer system that includes DTT and magnesium ions, and an appropriate RNA polymerase. The exact conditions used in the transcription reaction depend on the amount of RNA needed for a specific application. Basic laboratory protocols for *in vitro* transcription, as well as, commercial kits can be used in order to synthesize nucleic acid, for example RNA. Commercial kits for synthesizing RNA can include, for example, MEGAscript Kits (Life Technologies), TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), HiScribe and MiniV™ *In vitro* Transcription Kit (Epicentre). Other transcription kits can be used for making RNA and are known to those skilled in the art. The protocol for RNA generation can require linear polynucleotide templates of any length that possesses a RNA promoter region and preferably 1 to about 5 moles of nucleic acid or RNA can be produced per mole of polynucleotide template. RNA polymerases can include SP6 RNA polymerase, T7 RNA polymerase, N4 RNA polymerase II, and T3 RNA polymerases. Transcription of an RNA can be performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C

or 37°C. Transcription can also be performed for a time at least, greater than, equal to, or any time in between 0.5, 1, 2, 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 hours. In some alternatives, the RNA transcripts can be at least, greater than, equal to, or any size between 0.5, 1, 2, 3, 4, or 5 Kb. Use of a linear plasmid can stabilize the gene so that it is easily transcribed, especially if the gene has difficulty in cloning into supercoiled circular plasmids. In some alternatives, the RNA transcription can be stabilized at 30°C - 37°C for at least 100 hours using the polynucleotide of any of the alternatives described herein for transcription. The RNA transcript or mRNA with an extended poly(A) tract can be used successfully for transfection, in which the mRNA is protected (e.g., capped) and can be sustained for *in vivo* translation. RNA transcribed from a polynucleotide can comprise a poly(A) tract terminating in a guanine, cytosine, or a uracil. While natural mRNA comprises a polyadenylation sequence in the 5'UTR and/or 3'UTR of every natural mRNA, a portion of which remains in the mRNA, the mRNAs made in accordance with the teachings described herein do not need and do not contain such 5'UTR and/or 3' UTR polyadenylation sequences. Accordingly, the mRNAs made in accordance with the teachings described herein are not naturally occurring mRNAs by virtue of the fact that they lack said 5'UTR and/or 3'UTR polyadenylation sequences. Furthermore, in several alternatives, guanylation or diguanylation is added to the poly(A) sequence, preferably at the terminal ends, which can provide extended stability and duration of expression. Additionally, some alternative mRNAs described herein are codon optimized for expression in humans and the alternative mRNAs described herein contain poly(A) tails that are longer than the naturally occurring mRNAs for the particular sequence. Thus, the mRNAs made in accordance with the teachings described herein are not naturally occurring mRNAs.

[0115] In some alternatives, the mRNAs may comprise a gene. In this aspect, many synthetic genes can be designed to increase their protein expression level. The design process of codon optimization can be to alter rare codons to codons known to increase maximum protein expression efficiency. In some embodiments, codon selection is described, wherein codon selection can be performed by using algorithms that are known to those skilled in the art to create synthetic genetic transcripts optimized for high protein yield. Programs containing algorithms for codon optimization are known to those skilled in the art. Programs can include, for example, OptimumGene™, GeneGPS® algorithms, etc.

[0116] Described herein are methods for making a nucleic acid having a polymer of covalently linked thymine nucleotides, which can be extended to a desired length by virtue of the presence of an endonuclease site within said polymer of covalently linked thymine nucleotides such that a poly(T) insert of a desired length can be ligated to the nucleic acid having the polymer of covalently linked thymine nucleotides and endonuclease site thereby extending the poly(T) tract to the desired length. Some alternatives include methods of making a polynucleotide having a plurality of thymine nucleotides and an endonuclease recognition site within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the first thymine residue of a poly(T) domain but configured or spaced to permit enzymatic cleavage within the poly(T) domain. After enzymatic cleavage and ligation to a second polymer of thymine nucleotides this resultant nucleic acid can then be transcribed so as to generate an RNA having a poly(A) tract of the desired length, e.g., the length of the poly(T) polymer after ligation with a poly(T) insert. Accordingly, in some alternatives, the methods described herein can include the step of contacting a nucleic acid having a polymer of covalently linked thymine nucleotides, including a poly(T) insert positioned at an endonuclease cleavage site within the polymer of covalently linked thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides or a number of nucleotides within a range defined by any two of the aforementioned values from the first thymine residue of a poly(T) domain with a polymerase in the presence of nucleotides. In some alternatives, said nucleic acid or polynucleotide further comprises a promoter for a DNA or RNA polymerase. In some alternatives, an RNA polymerase is used. In some alternatives, the RNA polymerase is SP6 RNA polymerase, T7 RNA polymerase, N4 RNA polymerase II, or T3 RNA polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine, and/or uracil. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 or 500 covalently linked adenines. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a

cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII).

[0117] Disclosed herein are methods for stabilizing an RNA at 30°C - 37°C during translation for at least 100 hours, the RNA generated from a polynucleotide having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or attached thereto, as well as, compositions comprising these polynucleotides. Methods for stabilizing RNA during transcription can be performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, an mRNA comprises a poly A tail, wherein within said poly A tail there exists a sequence that is complimentary to an endonuclease recognition site (on the single strand), such as Stul or Bsal site. In some alternatives, a sequence that is complimentary to an endonuclease recognition site is an endonuclease recognition site set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the recognition site is inverted. In some alternatives, the mRNA comprises a first sequence encoding an RNA compliment to the at least one endonuclease site (on the single strand), wherein the sequence encoding the RNA compliment is covalently linked to a second nucleic acid, wherein the second nucleic acid comprises a plurality of adenine nucleotides, and wherein said first sequence encoding an RNA compliment to at least one endonuclease site of said first nucleic acid is covalently linked to said the said second nucleic acid comprising the plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides of said second nucleic acid. In some alternatives, the mRNA further comprises or encodes a gene. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the gene is an endonuclease gene, such as Cas9, TALEN, or MegaTAL, see e.g., **SEQ ID Nos. 6-14**). In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site (on the single strand). In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Bsal recognition site. In some alternatives, the at least one endonuclease recognition site is a Stul recognition site. In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is BSal. In some alternatives, the Bsal restriction recognition site is inverted.

[0118] The method can comprise providing the linear polynucleotide of any of the alternatives described herein and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30-37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times).. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature equal to 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the RNA comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 350, 375, 400, 425, 450, 475 or 500 covalently linked adenines. In some alternatives, the polynucleotide further comprises a gene. In some alternatives the gene is 1-50 kb. In some alternatives, the gene comprises at least, greater than, equal to, or any number in between 1, 5, 10, 20, 30, 40 or 50Kb. In some alternatives, the mRNA comprises a poly A

tail, wherein within said poly A tail there exists a sequence that is complimentary to an endonuclease site (on the single strand) such as Stul or Bsal site. In some alternatives, within said poly A tail there exists a sequence that is complimentary to an endonuclease recognition site as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the sequence that is complimentary to an endonuclease recognition site is complimentary to the inverted endonuclease recognition site. In some alternatives, the mRNA comprises a first sequence encoding an RNA compliment to the at least one endonuclease site (on the single strand), wherein the sequence encoding the RNA compliment is covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further comprises or encodes a gene. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the gene is an endonuclease gene, such as Cas9 a derivative thereof or active fragment thereof, TALEN, or MegaTAL e.g., **SEQ ID NOs. 6-14**. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease recognition site. In some alternatives, the inverted endonuclease recognition site is an endonuclease recognition site set forth in the sequences in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Bsal recognition site. In some alternatives, the at least one endonuclease site is a Stul recognition site. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is BSal. In some alternatives, the Bsal restriction recognition site is inverted.

Translation.

[0119] Synthesis of proteins can be performed *in vitro* as well as *in vivo*. For *in vivo* synthesis, the proteins can be synthesized in prokaryotic or eukaryotic cells. Synthesis of proteins *in vivo* can be controlled by the length of the poly(A) tail of the mRNA, which can increase the half-life of the mRNA ensuring translation of a higher concentration of protein. An mRNA with a protected 3' end can be sufficient for transfection and sustained *in vivo* translation. In normal growth conditions, an mRNA with a stably maintained poly(A) tract can be sufficient for translation at 30°C to 37 °C for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times).

[0120] The *in vitro* synthesis of proteins in cell-free extracts is an important tool for molecular biologists and has a variety of applications, including the rapid identification of gene products, localization of mutations through synthesis of truncated gene products, protein folding studies, and incorporation of modified or unnatural amino acids for functional studies. The use of *in vitro* translation systems can have advantages over *in vivo* gene expression when the over-expressed product is toxic to the host cell, when the product is insoluble or forms inclusion bodies, or when the protein undergoes rapid proteolytic degradation by intracellular proteases. In principle, it should be possible to prepare a cell-free extract for *in vitro* translation of mRNAs from any type of cells. In practice, only a few cell-free systems have been developed for *in vitro* protein synthesis.

[0121] *In vitro* translation systems can be performed by using treated cellular lysates in which the machinery for translation is used to translate a protein from a purified mRNA, and can include treated bacterial lysate (i.e., *E. coli* cell free systems), wheat germ extract, rabbit reticulocyte lysate, or even a commercially available lysate treated

specifically for *in vitro* translation, for example. Kits for translation can include, Retic Lysate IVT™ (Life Technologies), 1-Step Human *In vitro* Translation System (Thermo Scientific), and other kits that are known to those skilled in the art. Additionally, there are also coupled *in vitro* transcription/translation kits that can be used to produce protein from a starting point of a DNA. Treated cellular lysate for *in vitro* translation must contain essential components of the cellular translational machinery, such as the ribosomes, initiation and elongation factors, tRNAs and other basic components required for protein synthesis, that are known to those skilled in the art. In some alternatives, the translation is performed with an *in vitro* system. The *in vitro* system can comprise ribosomes, initiation and elongation factors, tRNAs and other basic components required for protein synthesis, such as, for example, amino acids. When supplemented with proprietary accessory proteins, ATP, and an energy regenerating system, the treated cellular extracts can sustain synthesis of target proteins from the mRNA templates for up to 6 to 100 hours without the need to remove inhibitory byproducts. The methods for *in vitro* translation can be performed at temperatures between 30°C to 37 °C. However the efficiency of translation can also depend upon the length of the mRNA, the mRNA cap, as well as the poly(A) tail. Efficiency of translation can depend on the poly(A) tail length in which a poly (A) tail of 75 to 300 covalently linked adenines both *in vivo* and *in vitro*.

[0122] Disclosed herein are methods for increasing expression of a protein from an mRNA generated from a polynucleotide having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or attached thereto, as well as, compositions comprising these polynucleotides. The method comprises generating an mRNA from the polynucleotide of any of the alternatives described herein, and translating said mRNA into a peptide. In some alternatives, the mRNA comprises a poly A tail, wherein within said poly A tail there exists a sequence that is complimentary to an endonuclease site (on the single strand), such as Stul or BsaI site. In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the mRNA comprises a first sequence encoding an RNA compliment to the at least one endonuclease site (on the single strand), wherein the sequence encoding the RNA compliment is covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further comprises a gene. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the gene is an endonuclease gene, such as Cas9 a derivative thereof or active fragment thereof, TALEN, or MegaTAL e.g., **SEQ ID NOS. 6-14**. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site. In some alternatives, the at least one endonuclease site is a Stul recognition site. In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the mRNA comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 or 500 covalently linked adenines. In some alternatives, the polynucleotide further comprises a gene. In some alternatives the gene is 1-50 kb. In some alternatives, the method is performed *in vivo*. In some alternatives, the method is performed *in vitro*. In some alternatives, translation is performed in bacterial cells. In some alternatives, translation is performed in eukaryotic cells. In some alternatives, the translation is performed in mammalian cells. In some alternatives, the translation is performed in human cells. In some alternatives, the translation is performed in human CD34+ hematopoietic cells. In some alternatives, the translation is performed in human primary T-cells. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted.

Therapeutic mRNA.

[0123] There are many ways of introducing a nucleic acid into a cell. Transfection is a method of introducing a nucleic acid into a cell. A nucleic acid can be introduced into eukaryotic cells by viral and non-viral methods. Genetic material, such as, supercoiled plasmid DNA, siRNA constructs, and mRNA can be used for transfection. Without being limiting, examples of chemical based transfection methods can be performed using Lipfectamine[®], cyclodextrin, polymers, liposomes, nanoparticles, calcium phosphate co-precipitation, diethylaminoethyl-dextran, DEAE-dextran, polyethylenimine or cationic lipid-based transfection reagents. Without being limiting, examples of non-chemical methods of transfection can include electroporation, cell squeezing, hydrodynamic delivery, optical transfection, and sonoporation. As such, there are numerous ways for introducing a nucleic acid into a cell, however there are limitations to many of these protocols.

[0124] The delivery of naked DNA into a cell can have several drawbacks, such as integration, as well as, being toxic to a cell. The use of viral particles to deliver DNA can also have the disadvantages of integration mutagenesis, as well as, introducing repetitive elements. However the use of RNA for transfection, such as a modified RNA comprising a poly(A) tail of controlled length can have the desired feature of being able to be introduced to a cell for controlled translation of a protein without integrating into the cell genome or having the toxicity effects like DNA. Additionally as the poly(A) tail controls degradation, the mRNA can be used for a controlled duration.

[0125] Messenger RNA (mRNA) plays a fundamental and integral role in every living cell as mRNAs are carriers of genetic information and blueprints for proteins during translation. mRNA allows transient protein expression in all cell types and has many advantages especially over DNA for gene transfer and expression of target molecules. mRNA doesn't require nuclear localization or transcription and is therefore a very safe biomolecule. Additionally, mRNA provides enormous flexibility with respect to production and application because any protein with a known sequence can be encoded and expressed.

[0126] mRNAs are currently being investigated as a novel class of therapeutic in areas such as cancer vaccines, prophylactic vaccinations for infectious diseases, and as a source of therapeutic gene products and protein replacement therapies.

[0127] mRNA can be applied as a gene delivery molecule, or therapeutic mRNA in the field of cancer immunotherapy and stem cell-based biomedical research as an alternative to plasmid DNA. mRNA has several advantages, such as lack of requirement for nuclear entry, which poses a significant barrier to DNA delivery, especially in non-dividing cells. mRNA also does not integrate into the host genome, avoiding aberrant transcription and expression of oncogenes caused by insertional mutagenesis. Delivering DNA into a cell can also be more toxic than the delivery of mRNA.

[0128] However naked mRNA molecules in earlier studies were shown to be not efficient in inducing maturation of antigen-presenting cells or in having an increase in translation of a specific gene. However, mRNA that is protected efficiently can prevent or slow down against RNase-mediated degradation. As described, the use of a stabilized mRNA that can be easily produced can be utilized as a therapeutic mRNA for protein production. As a drug, a stabilized mRNA can supply the biological instructions for producing a protein inside cells, particularly for a person having a genetic disorder. Therapeutic mRNA would also be more efficient than DNA-based gene therapy (which would require the cells to make their own mRNA intermediary before producing a protein) and more effective than recombinant protein therapy.

[0129] Therapeutic mRNA can be used for expression of endonucleases, end-processing enzymes and fusion proteins having endonuclease and end-processing activity. Nucleases are enzymes capable of cleaving the phosphodiester bonds between the nucleotide subunits of nucleic acids. Examples of nucleases can include but are not limited to Cas9 (e.g., **SEQ ID NOs. 6-14**), TALEN and MegaTAL. Cas9 (CRISPR associated protein 9) is an RNA-guided DNA endonuclease enzyme associated with the CRISPR (Clustered Regularly Interspersed Palindromic Repeats) adaptive immunity system in bacteria, for example, *Streptococcus pyogenes*, among other bacteria. *S. pyogenes* can utilize Cas9 to memorize and later interrogate and cleave foreign DNA, such as invading bacteriophage DNA or plasmid DNA. Cas9 performs this interrogation by unwinding foreign DNA and checking for if it is complementary to the 20 basepair

spacer region of the guide RNA. Transcription activator-like effector nucleases (TALENs) are artificial restriction enzymes generated by fusing a TAL effector DNA-binding domain to a DNA cleavage domain. MegaTAL is a fusion of a meganuclease with a TAL effector, is a new class of DNA targeting endonucleases with higher activity and specificity. Therapeutic mRNA can be introduced into a cell and the cell can be administered directly to a patient for targeted cleavage of a DNA sequence and for therapeutic or prophylactic applications, for example, cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, or hemophilia. In some alternatives, not being part of the invention, compositions comprising therapeutic mRNA are provided. In some alternatives, the compositions described herein (e.g., therapeutic mRNA encoding endonucleases, end-processing enzymes and fusion proteins having endonuclease and end-processing activity) can be used in methods of treating, preventing, ameliorating, or inhibiting a disease (e.g., cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, or hemophilia) or ameliorating a disease condition or symptom associated with a disease, such as, cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, or hemophilia. In some alternatives, therapeutic mRNA encoding endonucleases, end-processing enzymes or fusion proteins having endonuclease and end-processing activity, are administered to treat, prevent, ameliorate, or inhibit an autosomal dominant disease, such as achondroplasia, pseudoachondroplasia, the multiple epiphyseal dysplasias, chondrodysplasias, osteogenesis imperfecta, Marfan syndrome, polydactyly, hereditary motor sensory neuropathies I and II (Charcot-Marie-Tooth disease), myotonic dystrophy, or neurofibromatosis or ameliorate a disease condition or symptom associated with an autosomal dominant disease, such as achondroplasia, pseudoachondroplasia, the multiple epiphyseal dysplasias, chondrodysplasias, osteogenesis imperfecta, Marfan syndrome, polydactyly, hereditary motor sensory neuropathies I and II (Charcot-Marie-Tooth disease), myotonic dystrophy, or neurofibromatosis. In some alternatives, therapeutic mRNA encoding endonucleases, end-processing enzymes or fusion proteins having endonuclease and end-processing activity, are introduced into cells. In some alternatives, the cells are administered to treat, prevent, ameliorate, or inhibit a disease caused by misregulation of genes. In some alternatives, therapeutic mRNA encoding endonucleases, end-processing enzymes or fusion proteins having endonuclease and end-processing activity, are administered to treat, prevent, or inhibit a cancer, such as BCL-2, Bcl-XI, and FLIP, or ameliorate a disease condition or symptom associated with a cancer, such as BCL-2, Bcl-XI, and FLIP, by, for example, increasing the mutation rate of genes with anti-apoptotic activity.

[0130] In some alternatives, a composition, not being part of the invention, is provided, wherein the composition comprises a therapeutic RNA of any of the alternatives described herein and a pharmaceutical vehicle. In some alternatives, said composition is delivered to a subject in need.

[0131] In some alternatives, the mRNA comprises a poly A tail, wherein within said poly A tail or juxtaposed thereto there exists a sequence that is complimentary to an endonuclease site (on the single strand), such as Stul or BsaI site. In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the mRNA comprises a first sequence encoding an RNA compliment to the at least one endonuclease recognition site (on the single strand), wherein the sequence encoding the RNA compliment is covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further comprises a gene. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the gene is an endonuclease gene, such as Cas9 or a derivative thereof or active fragment thereof, TALEN, or MegaTAL e.g., **SEQ ID NOs. 6-14**). In some alternatives, the at least one endonuclease recognition site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the at least one endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636), and the recognition site is inverted. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site. In some alternatives, the at least one endonuclease site is a Stul recognition site. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and

a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted.

[0132] Disclosed herein are methods for making a nucleic acid for therapy, the nucleic acid generated from a linear polynucleotide having a plurality of covalently linked thymine nucleotides (a poly(T) tract) and one or more of the aforementioned endonuclease recognition sites inserted therein or attached thereto. In some alternatives, the nucleic acid is suitable or configured for therapy and such constructs are administered to a subject in need. In some alternatives, the nucleic acid comprises a gene. In some alternatives, the gene encodes a protein having therapeutic efficacy. In some alternatives, the subject suffers from a genetic disorder or is in need of a vaccine. In some alternatives, the subject suffers from cancer or a viral disease. In some alternatives, the nucleic acid is administered by a gene delivery molecule, such as a viral plasmid, electroporation or both. In some alternatives, the nucleic acid is mRNA or a DNA vaccine. In some alternatives, the mRNA is a therapeutic mRNA for therapy. In some alternatives, the mRNA comprises a poly A tail, wherein within said poly A tail there exists a sequence that is complimentary to an endonuclease recognition site (on the single strand) such as Stul or Bsal site. In some alternatives, the sequence that is complimentary to an endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the sequence that is complimentary to an endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636) and is inverted. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted.

[0133] In some alternatives, the mRNA comprises a plurality of adenine nucleotides, wherein the mRNA comprises a first sequence comprising at least one compliment sequence to an endonuclease recognition site covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9, a derivative thereof or active fragment thereof, TALEN, or MegaTAL e.g., **SEQ ID NOs. 6-14**. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA compliment is a compliment to the inverted endonuclease site. In some alternatives, the sequence that is complimentary to an endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636). In some alternatives, the sequence that is complimentary to an endonuclease recognition site is an endonuclease recognition site for an endonuclease as set forth in **Table 1** (SEQ ID Nos: 18-632, 634-636) and is inverted. In some alternatives, the at least one endonuclease recognition site is a Type II endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a Bsal recognition site. In some alternatives, the at least one endonuclease recognition site is a Stul recognition site. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted.

[0134] Additional alternatives include a polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a fifth nucleic acid, wherein the fifth nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO: 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the polynucleotide plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked thymines. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type II endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one BsaI restriction endonuclease recognition site. In some alternatives, the polynucleotide further comprises a DNA polymerase promoter region. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter region. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a sixth sequence, wherein the sixth sequence is capable of forming a tertiary structure such as a hairpin. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site, AGGCCT (SEQ ID NO: 633), and StuI cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is StuI. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the StuI cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs from the endonuclease cleavage site or from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs from the endonuclease cleavage site or from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is

located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0135] In some alternatives, a method of making a linear polynucleotide having a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, and wherein said template strand comprises a plurality of thymine nucleotides is provided. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises telomeres. In some alternatives, the polynucleotide comprises a left arm wherein the left arm comprises a left telomere, a right arm wherein the right arm comprises a right telomere and a cloning region located between the left arm and the right arm. In some alternatives, the polynucleotide further comprises a fifth nucleic acid, wherein the fifth nucleic acid comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease is an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked thymines. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site, and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a DNA polymerase promoter. In some alternatives, the polynucleotide further comprises a RNA polymerase promoter. In some alternatives, the plurality of thymine nucleotide comprises at least one guanine, cytosine, or an adenine. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a sixth sequence, wherein the sixth sequence is capable of forming a tertiary structure such as a hairpin. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site,

AGGCCT (SEQ ID NO: 633), and *StuI* cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *StuI*. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *StuI* cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *BsaI* restriction recognition site, and the endonuclease is *BsaI*. In some alternatives, the *BsaI* restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0136] A method of making a nucleic acid comprising providing the linear polynucleotide of any one of the alternatives described herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, a method of making a nucleic acid is provided, wherein the nucleic acid comprises providing the polynucleotide of any one of the alternatives described herein, or the polynucleotide manufactured by of any one of the alternatives described herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises a promoter for a DNA or RNA polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as *Taq* polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a

tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0137] In some alternatives, a nucleic acid manufactured by a method of any one of the alternatives herein is provided. In some alternatives the nucleic acid is a DNA. In some alternatives the nucleic acid is RNA. In some alternatives the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives the number of covalently linked adenines is greater than 120. In some alternatives the number of covalently linked adenines is greater than 200. In some alternatives the number of covalently linked adenines is greater than 300. In some alternatives the nucleic acid further comprises a gene. In some alternatives the gene encodes a protein for therapy. In some alternatives the gene encodes a nuclease. In some alternatives the gene encodes an endonuclease. In some alternatives the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives the covalently linked adenines terminates with a guanine, cytosine, thymine, or a uracil. In some alternatives the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0138] A method of enhancing transcription of a gene, not forming part of the invention, comprising providing the linear polynucleotide of any one of the alternatives described herein, wherein said polynucleotide further comprises a gene, and contacting said polynucleotide with a RNA polymerase in the presence of nucleotides. The method comprises providing the polynucleotide of any one of the alternatives provided herein, or the polynucleotide manufactured by any one of the alternatives described herein and contacting said polynucleotide with a RNA polymerase in the presence of

nucleotides. In some alternatives, the method further comprises providing a Type II restriction endonuclease. In some alternatives, the method further comprises providing a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or Stul. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the method is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0139] Also contemplated is a method of stabilizing an RNA during translation at 30°C - 37°C for at least 100 hours, wherein the method comprises providing the polynucleotide of any one of the alternatives described herein, and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, a method of stabilizing an RNA during *in vitro* translation at 30-37 degrees Celsius for at least 100 hours is provided, wherein the method comprises providing the nucleic acid of any one of the alternatives provided herein or a nucleic acid manufactured by a method of any of the alternatives described herein and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid is an RNA. In some alternatives, the RNA further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature equal to 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene. In some alternatives, the gene encodes a protein for therapy. In some alternatives, the gene encodes an endonuclease. In some alternatives, the gene encodes a nuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the covalently linked adenines terminates with a guanine, cytosine, thymine, or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided,

the endonuclease cleavage site is a *Stu*I cleavage site, AGGCCT (SEQ ID NO: 633), and *Stu*I cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *Stu*I. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *Stu*I cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *Bsa*I restriction recognition site, and the endonuclease is *Bsa*I. In some alternatives, the *Bsa*I restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0140] Also contemplated is a method of stabilizing an RNA during *in vivo* translation at 30- 37 degrees Celsius for at least 100 hours in a cell. The method can comprise transfecting into a cell the nucleic acid of any one of the alternatives described herein or a nucleic acid manufactured by a method of any one of the alternatives described herein placing the cell into a culture vessel with media, supplying the cell with nutrients and amino acids for translation and incubating the cell for at least 100 hours to allow translation. In some alternatives, the mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein *Stu*I is provided, the endonuclease cleavage site is a *Stu*I cleavage site, AGGCCT (SEQ ID NO: 633), and *Stu*I cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *Stu*I. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *Stu*I cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *Bsa*I restriction recognition site, and the endonuclease is *Bsa*I. In some alternatives, the *Bsa*I restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0141] Also contemplated are methods of increasing expression of a protein comprising generating an mRNA from the polynucleotide of any one of the alternatives described herein or from a linear polynucleotide manufactured by a method of any of the alternatives described herein and translating said mRNA into a peptide. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the translating step further comprises providing an RNA polymerase. In some alternatives, the method further comprises providing a Type II restriction endonuclease. In some alternatives, the method further comprises providing a Type IIS restriction endonuclease. In some alternatives, the Type IIS restriction endonuclease is *Bsa*I or *Stu*I. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by

any two of the aforementioned times). In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the RNA further comprises a gene encoding a protein. In some alternatives, the protein is a protein for therapy. In some alternatives, the gene encodes a nuclease. In some alternatives, the gene encodes an endonuclease. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the mRNA comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 350, 375, 400, 425, 450, 475 or 500 covalently linked adenines. In some alternatives, the mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0142] In some alternatives, a method of enhancing stability of a replicating gene is provided, not forming part of the invention, wherein the method comprises providing the linear polynucleotide of any one of the alternatives described herein or a polynucleotide manufactured by any method of any of the alternatives described herein, wherein said polynucleotide further comprises a gene, transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C or any other temperature between any these values. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some

alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0143] In some alternatives, a linear polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides is provided. The linear polynucleotide is as defined in claim 1. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site, and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and wherein the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one Type IIS restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, the polynucleotide further comprises a DNA polymerase promoter. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the polynucleotide further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the polynucleotide further comprises a seventh sequence, wherein the seventh sequence is capable of forming a tertiary structure such as a hairpin. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site, AGGCCT (SEQ ID NO: 633), and StuI cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is StuI. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the StuI cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away the endonuclease cleavage site of from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0144] In some alternatives, a method of making a linear polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5'

terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides.. In some alternatives, the plurality of thymine nucleotides of said third nucleic acid comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked thymines. In some alternatives, the number of covalently linked thymines is greater than 120. In some alternatives, the number of covalently linked thymines is greater than 200. In some alternatives, the number of covalently linked thymines is greater than 300. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site and the endonuclease is a Type II restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site and the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the at least one endonuclease recognition site is a BsaI restriction endonuclease recognition site or a StuI restriction endonuclease recognition site, and wherein the endonuclease is BsaI or StuI. In some alternatives, the endonuclease recognition site is inverted. The polynucleotide is a linear polynucleotide. In some alternatives, the polynucleotide has only one endonuclease recognition site. In some alternatives, wherein the plurality of thymine nucleotides comprises at least one covalently linked guanine, cytosine, or an adenine, wherein the at least one covalently linked guanine, cytosine or adenine is optionally joined to the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site comprises at least one guanine, cytosine, or an adenine, wherein the endonuclease cleaves between a thymine and the at least one guanine, cytosine or adenine. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site, AGGCCT (SEQ ID NO: 633), and StuI cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is StuI. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the StuI cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0145] In some alternatives, a method of making a nucleic acid encoding a nuclease is provided, the method comprising providing the polynucleotide of any of the alternatives described herein or a polynucleotide manufactured by a method of any one of the alternatives described herein and contacting said polynucleotide with a polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises a promoter for a DNA or RNA polymerase. In some alternatives, the polymerase is a DNA polymerase. In some alternatives, the DNA polymerase is a thermal stable polymerase, such as Taq polymerase. In some alternatives, the nucleotides are adenine, cytosine, guanine, thymine and/or uracil. In some alternatives, the method further

comprises providing a set of primers. In some alternatives, the nucleic acid is DNA. In some alternatives, an RNA polymerase is used. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid is RNA. In some alternatives, the RNA comprises a plurality of adenine nucleotides, wherein the plurality of adenine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site, AGGCCT (SEQ ID NO: 633), and StuI cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is StuI. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the StuI cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0146] In some alternatives, a method of enhancing transcription of a gene encoding a nuclease, not being part of the invention, is provided, the method comprising providing polynucleotide of any one of the alternatives provided herein or a polynucleotide manufactured by a method any one of the alternatives provided herein and contacting said polynucleotide with a RNA polymerase in the presence of nucleotides. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is BsaI or StuI. In some alternatives, the contacting is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the method is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours

(e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted. In some alternatives, the endonuclease recognition site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs from the endonuclease cleavage site or from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0147] In some alternatives, a nucleic acid encoding a nuclease manufactured by any one of the methods of any one of the alternatives is provided.

[0148] In some alternatives, a method of stabilizing translation of an RNA encoding a nuclease at 30-37 degrees Celsius for at least 100 hours is provided, wherein the method comprises providing the nucleic acid of any one of the alternatives provided herein or a nucleic acid manufactured by any one of the alternatives provided herein and contacting said nucleic acid with a ribosome, in the presence of amino acids and tRNAs, wherein said RNA is stable at 30- 37 degrees Celsius for at least 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid further comprises an RNA polymerase promoter. In some alternatives, the contacting is performed at a temperature equal to 37°C. In some alternatives, the contacting is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the nucleic acid comprises a plurality of adenines, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. In some alternatives, the number of covalently linked adenines is greater than 120. In some alternatives, the number of covalently linked adenines is greater than 200. In some alternatives, the number of covalently linked adenines is greater than 300. In some alternatives, the nucleic acid is an RNA. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14. In some alternatives, the plurality of adenine nucleotides further comprises a guanine, cytosine or a uracil, and wherein the number of covalently linked adenines terminates with a guanine, cytosine or a uracil. In some alternatives, the nucleic acid further comprises at least one palindromic sequence, wherein the at least one palindromic sequence can form a hairpin. In some alternatives, the nucleic acid further comprises a sequence capable of forming a tertiary structure such as a hairpin. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is

Bsal. In some alternatives, the Bsal restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0149] In some alternatives, a method of expression of a nuclease or increasing expression of a nuclease is provided, wherein the method comprises generating an mRNA from the polynucleotide of any one of the alternatives described herein or a polynucleotide manufactured by a method any one of the alternatives described herein and translating said mRNA into a peptide. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the method further comprises providing an endonuclease. In some alternatives, the endonuclease is a Type II restriction endonuclease. In some alternatives, the endonuclease is a Type IIS restriction endonuclease. In some alternatives, the endonuclease is Bsal or Stul. In some alternatives, the translating is performed at a temperature at least, greater than, equal to, or any temperature in between 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C or 37°C. In some alternatives, the translating is performed for at least, greater than, equal to, or time in between 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours (e.g., at least, or equal to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 hours or within a range defined by any two of the aforementioned times). In some alternatives, the mRNA comprises a plurality of adenines, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0150] In some alternatives, a method of replicating a gene comprising a nuclease or enhancing stability of a replicating gene comprising a nuclease is provided, the method comprising providing the linear polynucleotide of any one of the alternatives provided herein or a linear polynucleotide manufactured by a method of any one of the alternatives provided herein, wherein said polynucleotide comprises a gene encoding a nuclease transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated. In some alternatives, the cell is propagated for 1 day, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, or 14 days, or any other times between these values. In some alternatives, the cell is propagated at 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, or 37°C or any other temperature between any these values. In some alternatives, the nuclease comprises an amino acid sequence set forth in SEQ ID NO. 6 or is encoded by a nucleic acid sequence set forth in SEQ ID NO: 7-14.

[0151] In some alternatives, an RNA having a plurality of adenine nucleotides is provided, the RNA comprising a first sequence comprising at least one complement sequence to an endonuclease recognition site covalently linked to a plurality of adenine nucleotides at the 3' end of said plurality of adenine nucleotides a second sequence that comprises a plurality of adenine nucleotides, wherein the plurality of adenines comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 covalently linked adenines and wherein said complement sequence to an endonuclease recognition site of said first nucleic acid is covalently linked to said plurality of adenine nucleotides of said second nucleic acid at the 3' end of said plurality of adenine nucleotides of said second nucleic acid. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA complement is a complement to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives,

the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site or a StuI recognition site. In some alternatives, the RNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein StuI is provided, the endonuclease cleavage site is a StuI cleavage site, AGGCCT (SEQ ID NO: 633), and StuI cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is StuI. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the StuI cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0152] In some alternatives, a cell manufactured by the methods of any one of any of the alternatives described herein is provided.

[0153] In some alternatives, a pharmaceutical composition, not being part of the invention, is provided wherein the composition comprises a pharmaceutical vehicle and a nucleic acid of any one of the alternatives provided herein or mRNA of any one of the alternatives described herein.

[0154] In some alternatives, not being part of the invention, a nucleic acid for use in treating, ameliorating, or inhibiting a disease in a subject, is provided, wherein the method comprises introducing into a cell the nucleic acid of any one of the alternatives described herein or the mRNA of any one of the alternatives described herein and delivering the cell to the subject. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a human cell.

[0155] In some alternatives, a method of treating, ameliorating, or inhibiting a disease in a subject, not being part of the invention, comprising: delivering the pharmaceutical composition of any of the alternatives described herein to the subject.

[0156] In some alternatives, use of the mRNA in a method of treating, ameliorating, or inhibiting a disease in a subject, not being part of the invention, is provided, wherein the method comprises introducing into a cell the mRNA of any of the alternatives described herein and delivering the cell to the subject. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a human cell.

[0157] In some alternatives, a pharmaceutical composition, not being part of the invention, is provided, wherein the composition comprises a pharmaceutical vehicle and the mRNA of any of the alternatives described herein. In some alternatives, the mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII).

[0158] In some alternatives, not being part of the invention, the pharmaceutical composition is for use in treating, ameliorating, or inhibiting a disease in a subject, not being part of the invention, is provided, wherein the method comprises delivering the pharmaceutical composition of any of the alternatives described herein to the subject.

[0159] In some alternatives, a method of treating, ameliorating, or inhibiting a disease in a subject, not being part of

the invention, is provided, wherein the method comprises delivering the cell of any of the alternatives described herein to the subject.

[0160] In some alternatives, the linear polynucleotide that encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said polynucleotide comprises an endonuclease site at the 3' end of said polynucleotide, preferably outside of the coding region for said protein, and said endonuclease site is joined to at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 covalently linked thymine residues or an amount of covalently linked thymine residues that is within a range defined by any two of the aforementioned values, is provided.

[0161] In some alternatives, an mRNA that encodes a protein, such as a nuclease, for example SEQ ID NOs. 6-14, wherein said mRNA comprises a poly(A) tail that is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, or 500 residues in length or a length that is within a range defined by any two of the aforementioned lengths and, wherein said mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII) is provided.

[0162] In some alternatives, the polynucleotides or nucleic acids for use in treating, ameliorating, or inhibiting a disease in a subject is provided, not being part of the invention, wherein the method comprises introducing into a cell any one or more of the polynucleotides or nucleic acids set forth in any of the aforementioned alternatives; and delivering the cell to the subject. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia.

[0163] In some alternatives, not being part of the invention, the polynucleotides or nucleic acids is for use of treating, ameliorating, or inhibiting a disease in a subject, wherein the method comprises administering any one or more of the polynucleotides or nucleic acids set forth in any of the aforementioned alternatives to a subject. In some alternatives, said disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia.

[0164] In some alternatives, a method of increasing and/or stabilizing expression of a protein is provided, wherein the method comprises generating an mRNA from any one or more of the polynucleotides of any one or more of the aforementioned claims and translating said mRNA into a peptide.

[0165] In some alternatives, not being part of the invention, a method of enhancing transcription or expression of a gene is provided, wherein the method comprises providing any one or more of the polynucleotides or nucleic acids of any one or more of the aforementioned alternatives, wherein said polynucleotide further comprises a gene, such as a nuclease gene, for example SEQ ID NOs. 6-14; and contacting said polynucleotide or nucleic acid with a RNA polymerase in the presence of nucleotides.

[0166] In some alternatives, not being part of the invention, a system for editing at least one target gene in a cell is provided, wherein the system comprises a nucleic acid encoding an endonuclease protein that targets at least one sequence in a cell, and at least one nucleic acid encoding at least one protein which alone or together with other proteins modifies the substrate specificity of at least one ubiquitin ligase enzyme or enzyme complex in the cell and, optionally, a vector that comprises a nucleic acid template for homologous gene targeting.

[0167] In some alternatives, not being part of the invention, a method of editing at least one target gene in a cell is provided, wherein the method comprises introducing into a cell a nucleic acid sequence encoding an endonuclease, introducing into said cell at least one nucleic acid sequence encoding a ubiquitin ligase enzyme/enzyme complex substrate specificity modifying protein and optionally, introducing into said cell a vector that comprises a nucleic acid template capable of homologous gene targeting of at least one genomic sequence in the cell.

[0168] In some alternatives, a linear polynucleotide comprising a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides, is provided. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI restriction endonuclease recognition site. In some alternatives, the at least one Type II restriction endonuclease recognition site is a Stul restriction endonuclease recognition site. The polynucleotide is a linear polynucleotide. In some alternatives, the endonuclease recognition site is inverted. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0169] In some alternatives, an mRNA having a plurality of adenine nucleotides is provided but not forming part of the invention, wherein the mRNA comprises a first sequence comprising at least one complement sequence to an endonuclease recognition site covalently linked to a second sequence, wherein the second sequence comprises a plurality of adenine nucleotides, and wherein said complement sequence to an endonuclease recognition site of said first nucleic acid is covalently linked to said plurality of adenine nucleotides of said second nucleic acid at the 5' end of said plurality of adenine nucleotides of said second nucleic acid. In some alternatives, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9, a derivative thereof or active fragment thereof, TALEN, or MegaTAL. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA complement is a complement to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a BsaI recognition site. In some alternatives, the at least one endonuclease site is a Stul recognition site. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. In some alternatives, the mRNA lacks a 5' and/or 3' UTR or lacks a native 5' and/or 3' UTR and/or lacks one or more sequences required for native polyadenylation such as, a cleavage/polyadenylation specificity factor (CPSF) binding site, a cleavage stimulation factor binding site (CstF), a cleavage factor I binding site (CFI), or a cleavage factor II binding site (CFII). Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the

endonuclease cleavage site is a *Stu*I cleavage site, AGGCCT (SEQ ID NO: 633), and *Stu*I cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *Stu*I. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *Stu*I cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *Bsa*I restriction recognition site, and the endonuclease is *Bsa*I. In some alternatives, the *Bsa*I restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0170] In some alternatives, not being part of the invention, a cell is provided, wherein the cell is manufactured by any of the methods provided herein. The method can comprise providing the linear polynucleotide of any of the alternatives described herein, wherein said polynucleotide further comprises a gene, transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated..

[0171] In some alternatives, not being part of the invention, a method of treating, ameliorating, or inhibiting a disease in a subject is provided. The method comprises introducing into a cell a linear polynucleotide and delivering the cell to the subject. In some alternatives the disease is sickle cell disease, hypercholesterolemia, cancer, autoimmune disease, inherited disorder of metabolism, or immunodeficiency. In some alternatives, the disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome, hemophilia. In some alternatives, the disease is cancer, ischemia, diabetic retinopathy, macular degeneration, rheumatoid arthritis, psoriasis, HIV infection, sickle cell anemia, Alzheimer's disease, muscular dystrophy, neurodegenerative diseases, vascular disease, cystic fibrosis, stroke, hyper IGE syndrome or hemophilia. The linear polynucleotide is defined above. In some alternatives, the endonuclease recognition site is inverted. In some alternatives, the polynucleotide further comprises an RNA polymerase promoter. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a human cell. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein *Stu*I is provided, the endonuclease cleavage site is a *Stu*I cleavage site, AGGCCT (SEQ ID NO: 633), and *Stu*I cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is *Stu*I. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the *Stu*I cleavage site is inverted. In some alternatives, the endonuclease recognition site is a *Bsa*I restriction recognition site, and the endonuclease is *Bsa*I. In some alternatives, the *Bsa*I restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0172] In some alternatives, not being part of the invention, a method of treating, ameliorating, or inhibiting disease in a subject is provided, wherein the method comprises introducing into a cell an mRNA of any and delivering the cell to the subject, the mRNA further encodes a gene. In some alternatives, the gene is an endonuclease gene, such as Cas9, a derivative thereof or active fragment thereof, TALEN, or MegaTAL. In some alternatives, the at least one endonuclease site is inverted, and wherein the sequence encoding an RNA complement is a complement to the inverted endonuclease site. In some alternatives, the at least one endonuclease is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one endonuclease site is a *Bsa*I recognition site. In some alternatives, the at least one endonuclease site is a *Stu*I recognition site. In some alternatives, the cell is a T-cell or a primary cell. In some alternatives, the introducing is performed by electroporation. In some alternatives, the cell is a human cell. In some alternatives, the gene is codon optimized for expression in a mammalian cell, such as a human cell. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a

CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0173] In some alternatives, not being part of the invention, a method of treating, ameliorating, or inhibiting a disease in a subject is provided, wherein the method comprises delivering a cell to the subject. The cell is manufactured by any of the methods provided herein. The method can comprise providing the linear polynucleotide of any of the alternatives described herein, wherein said polynucleotide further comprises a gene, transforming a cell with said polynucleotide and propagating the cell, wherein the polynucleotide is replicated. In some alternatives, the cell is a human cell.

[0174] In some alternatives of treating, ameliorating, or inhibiting a disease in a subject, not being part of the invention, the disease is sickle cell disease, hypercholesterolemia, cancer, autoimmune disease, inherited disorder of metabolism, or immunodeficiency.

[0175] In some alternatives, a method of making a polynucleotide comprising a gene encoding a protein one desires to express is provided, wherein the polynucleotide comprises a plurality of thymine nucleotides and an endonuclease recognition site inserted therein. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a Bsal restriction recognition site, and the endonuclease is Bsal. In some alternatives, the Bsal restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0176] In some alternatives, an mRNA generated by the methods described herein is used as a therapeutic mRNA for treating disease by directly delivering the mRNA into a human, either alone or in conjunction with an encapsulating substance that promotes its deliver to inside a cell. In some alternatives, the mRNA is introduced into a cell. In some alternatives, the cell is a primary cell. In some alternatives, the cell is delivered to a subject in need. In some alternatives the subject is a human.

[0177] In some alternatives of the polynucleotide, the polynucleotide comprises a Type IIS endonuclease recognition site downstream from the intended cut site, thereby creating a Type IIS endonuclease cleavage site upstream from the Type IIS endonuclease recognition site. In some alternatives, the Type IIS restriction is inverted. In some alternatives, the Type IIS endonuclease cleavage site is at a distance of 1-30 bases away from the Type IIS endonuclease recognition site. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0178] In some alternatives, a polynucleotide having a plurality of thymine nucleotides and an endonuclease recognition site inserted therein is provided. The polynucleotide can comprise a first nucleic acid sequence encoding a nuclease, a second nucleic acid sequence comprising at least one restriction endonuclease recognition site and a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the restriction endonuclease cleavage site, wherein the restriction endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides and wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said restriction endonuclease recognition site is located within 55 base pairs of the restriction endonuclease cleavage site for the restriction endonuclease and said restriction endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site. In some alternatives, the at least endonuclease recognition site is a BsaI or a Stul restriction endonuclease recognition site. In some alternatives, the endonuclease cleavage site is between 1-5 nucleotides upstream from the endonuclease recognition site. In some alternatives, the polynucleotide further comprises a gene. In some alternatives, the gene is an endonuclease gene e.g., **SEQ ID NOs. 6-14**. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

[0179] In some alternatives, a method of making a polynucleotide having a plurality of thymine nucleotides and an endonuclease recognition site inserted therein is provided. The method of making a polynucleotide comprising a sequence encoding a nuclease, a template strand, an endonuclease recognition site and an endonuclease cleavage site for an endonuclease inserted therein, wherein said template strand comprises a plurality of thymine nucleotides can comprise providing a first nucleic acid sequence encoding a nuclease, providing a second nucleic acid sequence comprising at least one endonuclease recognition site, providing a third nucleic acid sequence, wherein the third nucleic acid sequence comprises the template strand, wherein the template strand comprises a plurality of thymine nucleotides, wherein said plurality of thymine nucleotides comprises at least, greater than, equal to, or any number in

between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymine nucleotides and wherein the third nucleic acid comprises the endonuclease cleavage site, wherein the endonuclease cleavage site is located within the plurality of thymine nucleotides or within 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides, joining said first nucleic acid to one end of the second nucleic acid sequence and joining said third nucleic acid sequence to one end of the second nucleic acid sequence, wherein said second nucleic acid is covalently linked to one end of said third nucleic acid sequence, and wherein the said endonuclease recognition site is located within 55 base pairs of the endonuclease cleavage site for the endonuclease and said endonuclease cleavage site is within 1 to 40 base pairs of a most 5' thymine in the plurality of thymine nucleotides. In some alternatives, the polynucleotide comprises at least, greater than, equal to, or any number in between 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or 550 covalently linked thymines. In some alternatives, the at least one endonuclease recognition site is a Type II restriction endonuclease recognition site. In some alternatives, the at least one endonuclease recognition site is a Type IIS restriction endonuclease recognition site. In some alternatives, the at least one Type II restriction endonuclease recognition site is a BsaI or a Stul restriction endonuclease recognition site. In some alternatives, the endonuclease cleavage site is between 1-5 nucleotides from the endonuclease recognition site. In some alternatives, the polynucleotide further comprises a gene. In some alternatives, the gene is an endonuclease gene e.g., **SEQ ID NOs. 6-14**. Without being limiting an endonuclease can include, for example, a CRISPR, a restriction endonuclease, a Type II restriction endonuclease and a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, the endonuclease is a CRISPR, a restriction endonuclease, a Type II restriction endonuclease or a Type IIS restriction endonuclease. In some alternatives, wherein Stul is provided, the endonuclease cleavage site is a Stul cleavage site, AGGCCT (SEQ ID NO: 633), and Stul cleaves between the G and C, leaving a blunt end that terminates in a GG. In some alternatives, the endonuclease cleavage site is AGGCCT (SEQ ID NO: 633). In some alternatives, the endonuclease is Stul. In some alternatives, the plurality of adenines terminates in GG. In some alternatives, the Stul cleavage site is inverted. In some alternatives, the endonuclease recognition site is a BsaI restriction recognition site, and the endonuclease is BsaI. In some alternatives, the BsaI restriction recognition site is inverted. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides. In some alternatives, the endonuclease cleavage site is located 1, 2, 3, 4, 5, 6, 7, 7, 8, 9 or 10 base pairs away from the most 5' thymine (or 5' terminal thymine) in the plurality of thymine nucleotides.

Creation of linear plasmid-OK MCS-pA-100, -200, and -300.

[0180] To prepare linear plasmid-OK blunt (Lucigen) for insertion of the poly A tail, an MCS comprising multiple blunt and sticky- ended RE sites as well as a terminal BsaI site [DraIII-FspI-BsiWI-NheI-BsaI] was ligated into linear plasmid-OK blunt to create linear plasmid-OK-MCS. linear plasmid-OK-MCS was digested with NheI, blunted, and dephosphorylated with CIP. To create a long poly(A) tail, oligos of 200 T's and 50 A's were annealed at an equimolar ratio. The annealed oligos were treated with T4 polymerase, Klenow fragment, and T4 PNK to trim flaps, fill gaps, and create blunt, phosphorylated ends. The resulting mixture was run on a 3% agarose gel and fragments in the range of c.a. 200-800bp were gel extracted. After purification, these fragments were ligated into the previously prepared linear plasmid- OK MCS with T4 DNA ligase at room temperature for 2 hours. Resulting colonies were screened for tail insert length by colony PCR using primers that flank the MCS/tail insertion site, and then colonies with inserts of appropriate length were sequenced to confirm correct orientation of the insert. This resulted in the successful identification of tail lengths of 70, 172, and 325bp. These constructs were named linear plasmid-OK-MCS-pA-100, -200, and -300, respectively.

Creation of linear plasmid-OK-MCS-ΔBsaI.

[0181] To create longer tails, it was necessary to mutate the 4 BsaI sites native to linear plasmid-OK to allow for digestion only at the BsaI site in the inserted MCS. One site was removed by Gibson assembly. The remaining 3 sites were removed by restriction digest of a fragment containing the BsaI site to be mutated as well as a pair of flanking RE

sites, blunting and ligation of this fragment into pUC57 digested with EcoRV, site-directed mutagenesis of the BsaI site, and then sub-cloning the mutated fragment back into linear plasmid-OK MCS using the included flanking RE sites. This plasmid was designated linear plasmid-OK-MCS-ΔBsaI.

Creation of pEVL series plasmids.

[0182] Once all native BsaI sites were removed, the fragment containing the polyA tail from linear plasmid-OK-MCS-pA-100, -200, and -300 was cloned into linear plasmid-OK-MCS-ΔBsaI, creating pEVL-100, -200, and -300. pEVL-300 was then digested with BsaI, blunted with Klenow, de-phosphorylated with CIP, and ligated with additional blunt double stranded poly(A) fragment prepared as above. Screening as above resulted in plasmids with 425 and 525bp p(A) tails. These were designated pEVL-400 and pEVL-500.

Cloning of CRISPR associated protein 9 (Cas9) and TCRA guides.

[0183] Cas9 expression construct was obtained from Addgene (plasmid #41815). To ensure nuclear expression of Cas9 in primary human T-cells, Nuclear Localization Signals (NLS's) at both 5' and 3' ends (2X-NLS). To track transfection efficiency, Cas9 was fused in-frame with T2A-mCherry and cloned into an in-house modified version of pUC57 with a T7 promoter called pWNY2.0 to obtain pWNY2.0-2X-NLS-Cas9-T2A-mCherry. Subsequently, 2X-NLS-Cas9-T2A-mCherry was spliced from pWNY2.0 and cloned into pEVL200.

[0184] Guides targeting the constant region of TCR alpha were designed using an online CRISPR design tool. Guides were designed with a U6 promoter and ordered as gblocks (IDT). Subsequently, the gblock comprising U6 promoter and sgRNA (crRNA+tracrRNA) was cloned into a self-complementary (sc) AAV2 vector backbone, and checked for maintenance of intact ITR's. Four different G-block-guide constructs generated in this way were used for AAV6 synthesis, and following a series of pilot experiments, a best-performing guide was chosen further comparative analyses.

Production of IVT mRNA.

[0185] For BFP and TALEN constructs, inserts containing a T7 promoter and Kozak sequence were cloned into pEVL-100 through pEVL-500. These constructs were digested with BsaI to create a free poly(A) tail as well as a RE site upstream of the T7 (typically XbaI or SpeI) for ease of purification. The digested DNA was purified by silica column and eluted in water or 10mM Tris. IVT with ARCA capping was carried out using the mMessage mMachine T7 Ultra kit (Ambion) per the manufacturer's directions with 200-500ng of the T7 to polyA template fragment. IVT reactions were routinely extended to 150min. The DNase step was altered either in time or DNase amount to account for the total amount of template DNA in the reaction. For pEVL constructs, after DNase treatment, the reaction was cleaned up using the RNeasy Mini kit (Qiagen) following the manufacturer's directions. For pWNY constructs, the enzymatic tailing step of the mMessage mMachine T7 Ultra kit was carried out according to the manufacturer's instructions before RNA clean-up. RNA purity was determined by NanoDrop spectrophotometry and gel analysis with the FlashGel RNA system (Lonza).

[0186] For Cas9 mRNA production, pWNY2.0-2X-NLS-Cas9-T2A-mCherry construct was linearized using Adel (DraIII) and Pfl23II (BsiWI) (Fermentas FastDigest) and pEVL200-2X-NLS-Cas9-T2A-mCherry was digested with BsaI and SpeI. Both constructs were checked for completeness of digestion on 1% agarose gels, column purified, and RNA was transcribed in-vitro using the mMessage mMachine T7 ULTRA Transcription kit (Life Technologies) with slight modifications from the manufacturer's protocol. Briefly, the IVT reaction was done for 2.5hrs, DNase treatment for 1 hour. Poly (A) tailing for the pWNY-2.0 construct was performed for 1 hour, and mRNA was subsequently purified using the RNeasy kit (Qiagen), aliquoted and frozen at -80°C.

BFP and TALEN mRNA electroporation of primary human T-cells.

[0187] Freshly isolated primary human T-cells were stimulated with anti-CD3/anti-CD28 microbeads (Dyna) in RPMI with 10% FBS, 2.5% HEPES, and 1% L-glut with 5ng/mL IL-2 and 1ng/mL IL-15. After 2-3 days, the stim beads were removed, and the T-cells were resuspended at 3×10^7 cells/mL in Neon Electroporation system Buffer T (Invitrogen) with 0.25-2µg mRNA per 300,000 cells. Electroporation was carried out on the Neon electroporation system at 1400V, 10ms, and 3 pulses. Immediately following electroporation, cells were resuspended in complete T-cell media with IL-2 and IL-15 (as above). Cells were either placed in a 37 °C incubator or in a 30 °C incubator for 24 hrs and then moved to a 37 °C incubator for a cold- shock step, which has been observed to increase the amount of protein per cell for the period when the mRNA is present (Doyon Y. et al. Transient cold-shock enhances zinc-finger nuclease-mediated gene disruption. *Nature Methods* 7, 459-460 (2010), herein incorporated by reference in its entirety). Expression of the encoded protein was assayed by flow cytometry. For TCR knockout by TALEN or CRISPR, loss of TCR expression was assayed at 72hr-7days after electroporation by staining with anti-CD3 (clone HIT3a, direct FITC or PerCP-Cy5.5 conjugate).

Cas9 mRNA comparison (pWNY2.0 versus pEVL) in primary human T-cells using TCRa-KO as a readout.

[0188] Frozen CD4+ T cells isolated from a donor were stimulated with human T-activator CD3/CD28 Dynabeads (Life Technologies) for 48 hours in antibiotic-free RPMI media with 20% FBS, 100mM HEPES, 2mM Glutamax, 55uM b- ME and cytokines (IL-2 = 50ng/ml, IL-7 = 5ng/ul and IL-15 = 5ng/ul), hereafter referred to as 'RPMI media'. Beads were magnetically removed 48 hours post-stim and cells were rested in RPMI media for 5 hours before prepping them for mRNA transfection. RNA transcribed in-vitro from both pWNY2.0-2X-NLS-Cas9-T2A-mCherry and pEVL200-2X-NLS-Cas9-T2A-mCherry was electroporated into 4.5×10^5 cells using a 10ul tip at two different concentrations - 3ug and 1.5ug (Neon transfection system; Life technologies) at 1400V, 10ms width, pulses =3, after which cells were rested for 3 hours followed by AAV transduction at constant 10% culture volume.

Flow Cytometry.

[0189] Cells were acquired for Cas9-mCherry expression on a LSRII flow cytometer (BD Biosciences) at 24, 48, 72 and 168 hours, respectively. Analysis of TCRa was performed using an anti-CD3 directly conjugated to Alexa 488 (BioLegend) and data was analyzed using FlowJo software (Treestar). Cells were gated on live cells based on the forward and side scatter for downstream analysis of the TCR-Knockout (KO).

Evaluation of transformation-associated tail shortening.

[0190] BFP-pEVL100, 200, and 300 were digested with DraIII (cuts 5' of BFP) and SmaI (cuts in pEVL backbone ~75bp 3' of tail/BsaI site). This fragment was gel purified and ligated back into pEVL digested with DraIII and SmaI and CIP'd as well as pWNY digested with DraIII and SmaI and CIP'd. Following ligation, the ligase was heat-killed and all ligation products were transformed by electroporation into BigEasyTSA electrocompetent cells (Lucigen) for pEVL or Top10 electrocompetent cells (Invitrogen) for pWNY. After recovery at 30°C for 1hr in SOC, aliquots of each transformation were plated in triplicate on Kan plates and grown at 25°C (72hrs), 30°C (24hrs) , or 37°C (20hrs). Colonies were screened for tail length by colony PCR with primers that bind in BFP (5'-cgcaaacgctaaaactacc-3') and 3' of the tail (5'-tcagttctatgtaccagcaagg-3'). For ligations of the 325bp tail into pWNY, recovery of any clones with full-length tail was not possible, so this was excluded from further analysis. For ligations of 70bp and 172bp tails into pWNY, 2 clones of each that screened as full-length by colony PCR were grown overnight in 1ml of TB with carbenicillin in triplicate, with 1 aliquot at 25°C, 1 at 30°C, and one at 37°C. After 24 hours, 1ul of each culture was seeded into a fresh 1ml aliquot of TB+carb. The remaining culture was spun down and the pellet frozen for miniprepping at a later date. This was repeated for a total of 7 full days. At this time, all of the frozen pellets were miniprepped. For each miniprep, 3ug of DNA was digested with BsiVI and BsaI to isolate the tail fragment and the digested product was run on a 3% agarose gel and then stained with GelStar Nucleic acid stain (Lonza). Because there is a BsiVI site in the tail fragment that was inserted into pWNY2.0 as well as one that is present in the pWNY2.0 backbone, there is an additional band of ~95bp on these gels that can be used as a DNA loading control.

Evaluation of propagation-associated tail shortening.

[0191] Freshly transformed colonies of pEVL-200 to -500 were screened for tail length by colony PCR and colonies with the correct tail lengths were seeded into 4 aliquots of 1ml TB+Kan and grown overnight, 2 at 30 °C and 2 at 37 °C. At each temperature, 1 aliquot was grown under arabinose induction and 1 without. After 24 hours, 1ul of each culture without arabinose was seeded into another 1ml of TC+Kan without induction. This was carried out for 2 weeks. At the time of seeding each day, another 1ul was seeded into a 1ml culture of TB+Kan+arabinose. Each induced culture was spun down and the pellet frozen after 24 hours of growth. At the end of 2 weeks, all of these frozen pellets were minipreped and digested with BsaI and BsiVI to isolate the tail fragment. These fragments were run on a 3% agarose gel and stained with GelStar Nucleic Acid stain.

Generation of a linear polynucleotide comprising a plurality of covalently linked thymine nucleotides and an endonuclease recognition site inserted within.

[0192] A polynucleotide comprising a plurality of covalently linked thymine nucleotides (poly(T) tract) and an endonuclease recognition site inserted within the poly(T) tract was constructed in multiple cloning steps. Reference is made to **Figure 1A**, which illustrates the structure of the linear polynucleotide, pEVL, which results from the insertion of the BsaI containing multicloning site (MCS), the removal of the plurality of BsaI sites and the addition of the poly(A) encoding region.

[0193] In order to create a linear polynucleotide comprising a plurality of covalently linked thymine nucleotides and an endonuclease recognition site inserted within, the BsaI sites were located and using site directed PCR mutagenesis and primers for mutations, single base substitutions were made within all four BsaI sites, to result in silent mutations. The unique restriction sites flanking the modifications were used to excise the fragment and clone it into a standard cloning vector (pUC57). Site-directed mutagenesis was used on a pUC57 vector to alter a base in the BsaI site, and the unique restriction sites were used to move the fragment back into cloning vector. This was repeated stepwise for each of the four BsaI sites.

[0194] With the plurality of BsaI sites removed, a new multicloning region, including a now-unique BsaI site, was constructed and used to clone into the linear vector along with a poly(A) sequence. First, a new multicloning site (MCS) was introduced with restriction sites that would support cloning in a Transcription activator-like effector nuclease (TALEN) upstream of a now-unique BsaI site "facing" into where the short poly(A) would be introduced (annotated "MCS" and "BsaI" on the map, respectively, **Figure 1B**). The BsaI site was designed to "face" the poly(A) such that the cut site (annotated "BsaI Cut" on the map, **Figure 1A** and **1B**) would be within the poly(A) sequence, 4 bases upstream of the poly(A) terminus on the coding strand. In this way, RNA transcripts created using this vector as a template would terminate in a poly(A) without any additional bases beyond it. Second, the poly(A) was cloned in directly upstream of the BsaI site and then lengthened to the desired lengths. Synthetic poly(T) and poly(A) oligonucleotides were phosphorylated and annealed to create double stranded poly(A/T) concatamers of various lengths. This mixture was ligated in directly upstream of the BsaI site and clones were selected which had a poly(A)s of desired lengths in the correct orientation. The longest clone obtained from screening had a poly(A) of approximately 300 adenine bases in length, and was designated pEVL300. A variant with a ~100 base poly(A), and another with a ~200 base poly(A) were also saved and designated pEVL100 and pEVL200, respectively. In order to extend the poly(A) beyond 300 bases in length, pEVL300 was digested with BsaI and the same poly(A/T) concatamers used in the previous step were again ligated into the vector. Screening yielded clones with ~400 and ~500 base poly(A)s, designated pEVL400 and pEVL500, respectively. **Figure 1A** shows a final map of the resultant linear vector comprising telomeric ends, a telN open reading frame (ORF1), ORF6, ORF3, ORF5, Poly(A) tract coding region, Kan2 resistance gene, 4 disrupted BsaI sites, and an ORF4 sequence. Reference is made to **Figure 1B**, which shows a zoomed in version of the site of interest comprising the poly(A) encoding tract along with the inverted BsaI restriction site.

Assay to determine the Poly(A) tail stability.

[0195] Polynucleotide vectors comprising a plurality of covalently linked thymine nucleotides and an endonuclease recognition site inserted within were created with the protocol previously described. Four vectors were used to determine Poly(A) tail stability which had 100, 200, 300, or 500 covalently linked thymines for transcribing an mRNA comprising 100, 200, 300, and 500 covalently adenosines (pEVL100, pEVL200, pEVL300, and pEVL500). Once the pEVL vectors were generated, the stability of the mRNA comprising the poly(A) tracts were tested for their dependency on temperature.

[0196] Poly(A) stability in standard cloning vectors for generating a transcript with a poly (A) tail (e.g. pUC57) is extremely limited, shortening to 30-60 bases during overnight propagation, even in chemically competent host cells that are purported to increase stability (e.g. STBL3™). In order to test the stability of the pEVL poly(A) tracts, *E. coli* were grown continuously in liquid culture for a two week period at either 30°C or 37°C. Cultures were split off and induced to produce high copy plasmids for preparation at 1, 6 and 14 days. The plasmids were isolated and digested with BsaI at the terminus of the poly(A), and XbaI upstream of the poly(A) by 151 base pairs. Bands corresponding to the poly(A) tracts were resolved on a 3% agarose gel and run next to size standards.

[0197] As shown in **Figure 2**, the poly(A) tract of pEVL100 and pEVL200 appear to be stable when grown at 30°C or 37°C for at least two weeks; no shortening was detectable. As shown in the gels at day 6 and day 14, the bands remain sharp at one length for the corresponding mRNA at the temperatures of 30°C or 37°C. The poly(A) tract of pEVL300 was less stable when grown at 37°C, with minimal shortening observed at day 6, but notable shortening observed at day 14, in which a smear of mRNA product is shown below the marker of 500 bases. This shortening was largely avoided in cultures grown at 30°C, even at 14 days. Although pEVL500's poly(A) was maintained during a single day of growth at 30°C, the poly(A) tract was significantly unstable with considerable shortening by day 6, even at 30°C. pEVL500 was unstable at 37°C and had shortened in cultures from both days 6 and 14. Any long-term work done with pEVL500 would require poly(A) length screening and validation at each point. However, an end-point pEVL500 plasmid could still be easily maintained in an *E. coli* frozen stock, grown overnight, and purified if required.

[0198] Accordingly from the data it is contemplated that the poly(A) length can dictate the poly(A) tract stability, and is susceptible to shortening at long tract lengths, but the stability is greater than previously achievable by an order of magnitude (30 bases from Promega versus 300 bases). Poly(A) tract lengths that are stable in this system (e.g. pEVL100, pEVL200, pEVL300) are capable of producing mRNA that can be used *in vivo* directly. This is in contrast to the short encoded poly(A) tracts in plasmids currently available which support only the generation of mRNA to be used for *in vitro* translation or would require further enzymatic polyadenylation to be used *in vivo*.

Homogeneous RNA length.

[0199] mRNA production using the pEVL vector, in which the method of creating the pEVL vector was described, as a template produces highly homogenous mRNA, as intended. Reference is made to **Figure 3A**, which show mRNA from a typical pEVL transcription on a Lonza Flashgel (1.2% agarose) while mRNA from a typical standard transcription and enzymatic polyadenylation is shown in **Figure 3B**. To create this mRNA, pEVL200 (lanes 1 & 2) and pEVL300 (lanes 3 & 4) plasmids with inserts were digested with BsaI, column purified, and used as a template for transcription using Ambion's mMessage mMachine kit. The resulting mRNA was purified using Qiagen's Rneasy columns, resulting in transfection-ready mRNA. The transfection-ready mRNA was validated by running it on a Lonza FlashGel with a size marker. The mRNA shown in **Figure 3B** was made using a standard commercial transcription vector as a template followed by enzymatically polyadenylation and then purification using Qiagen's Rneasy columns.

[0200] As shown in **Figure 3A**, a homogeneous poly(A) tail length would result in a single tight band of mRNA on the gel, since all mRNA products would be the same length. **Figure 3A** illustrates the bands of mRNA that were transcribed from pEVL200 and pEVL300 as clean sharp bands. The mRNA produced from the pEVL encoded poly(A) vector was highly homogeneous with only a slight smear below the primary band, indicating minimal byproducts of primarily smaller size **Figure 3B**. The mRNA produced using enzymatic polyadenylation was significantly more varied. Not only did the smear extend above and below the primary band, it was also more pronounced and extended to a far larger range. This is likely due to the Gaussian distribution of the extent of acetylation performed by the enzyme, and is supported by the lower concentration (dimmer) primary band. Accordingly from the data shown, it is contemplated that

the mRNA produced using the encoded poly(A) of pEVL is markedly more homogenous than that produced using enzymatic polyadenylation and is more stable.

Expression of Blue Fluorescent Protein (BFP) from a poly(A) encoding linear vector.

[0201] mRNA produced from the encoded poly(A) linear plasmid was at least as effective as mRNA produced by traditional enzymatic polyadenylation. To test the linear plasmid's effectiveness, linear plasmids comprising a plurality of covalently linked thymine nucleotides (poly(T) tract) and an endonuclease recognition site inserted within the poly(T) tract was made with the protocol previously described. The plasmid further comprised a gene encoding for blue fluorescent protein (BFP), as the reporter protein. The five linear plasmids with the BFP reporter had 100, 200, 300, 400, and 500 poly(T) tracts for transcribing an mRNA transcript with 100, 200, 300, 400, and 500 poly(A) tracts, respectively. The plasmids were used to transfect a constant 1 microgram of different BFP-encoding mRNAs into primary human T-cells and the cells were analyzed 24 hours later by flow cytometry.

[0202] mRNA encoding BFP from two different types of plasmids were produced. The first type of plasmid used was a control plasmid comprising a standard circular cloning vector with a T7 RNA promoter, a ribosome binding site (Kozak sequence), the mTagBFP2 protein coding sequence, and a BsiVI restriction site for linearization (pWNY). The second type of plasmid was the previously described encoded poly(A) vector, either pEVL100, pEVL200, pEVL300, pEVL400, or pEVL500, with the gene encoding BPA cloned directly upstream of the encoded poly(A). Three of the pEVL plasmids in this particular experiment also had a 150 base pair untranslated region after the coding sequence but before the poly(A), noted by 'α', this sequence to have little to no effect on BFP expression. The pWNY plasmids were linearized and purified, transcribed, enzymatically polyadenylated, and purified according to a standard Ambion protocol, known to those skilled in the art. The pEVL plasmids were linearized and purified, transcribed, and purified, skipping the enzymatic polyadenylation step.

[0203] Reference is made to **Figure 4**, in which the data shows that the mRNA from pEVL plasmids transfects at least as efficiently as, and is translated as well or better than, enzymatically encoded poly(A) mRNA. The number of BFP positive cells (left Y-axis) in the pEVL samples were on par with or slightly higher than the pWNY samples, especially for poly(A) tail lengths greater than 100 bases. Translation experiments were performed at 30°C. The level of translation, inferred from the intensity of the blue signal (mean fluorescence intensity, MFI, right Y-axis), was higher in the samples transfected with the mRNA produced from the encoded poly(A) plasmids. Overall, the mRNA produced from the pEVL200 and pEVL300 appeared to give the best results, especially compared to the enzymatically encoded mRNAs. This may be due to the fact that enzymatic polyadenylation only adds approximately 60-100 bases according to the manufacturer. It should however be noted that since a constant 1 microgram of mRNA was transfected, a lower molar amount of the longer poly(A) mRNAs were transfected compared to the shorter poly(A) mRNAs. Accordingly from the data shown, it is contemplated that a poly (A) tail produced from the pEVL200 and pEVL300 can give better results indicating the stability of mRNA with 200 or 300 adenines in the poly(A) tail, and thus can be used for a longer amount of time (24 hours) for translation of a protein. As such, reference is also made to **Figure 3**, which indicates the stability of the mRNA which can be used for translation.

Translation of RNAs generated by enzymatic polyadenylation and linear vectors at 30°C and 37°C.

[0204] Translational efficiency is the rate of mRNA translation into proteins that are within the cells. It can be measured in the amounts of protein per mRNA per hour. As previously described, RNA was generated from a standard method of adding a poly(A) tract by a transcription vector and a polyadenylation enzyme. The previously described linear vector, pEVL, was also transcribed to generate an RNA for translation. The RNAs generated from both methods encoded a BFP reporter for determining translational efficiency. The RNAs were transfected into human primary T-cells.

[0205] Attention is drawn to **Figure 5**, which shows the translational efficiency of the RNAs from enzymatic polyadenylation and the linear vector over time in human primary T-cells. The RNA generated from pEVL300 (300pA encoded tail) and pWNY (enzymatic polyadenylation) transfected into primary T-cells cells were compared by quantifying the percent BFP positive cells and the mean fluorescence intensity (MFI) of the BFP positive cells over time, with and without the 30°C incubation.

[0206] It was discovered that although a 24 hour 30°C incubation post-transfection has been found to increase accumulation of the translated protein, some cell types do not tolerate abnormal culturing for such an extended period of time (for example, CD34+ hematopoietic stem cells fall into this category). It was discovered that mRNA coding for BFP created using the encoded polyA plasmid as a template was more potent compared to mRNA created using standard enzymatic polyadenylation. The cells transfected with pEVL-derived mRNA (pEVL300) had higher percentages of BFP+ cells over time, and the BFP+ cells were significantly brighter (higher MFI, striped bars). Although this effect was seen to some extent when the cells were incubated at 30°C for 24 hours post-transfection (dark bars), the effect was more pronounced when this incubation was removed and the cells were grown continuously at 37°C (light bars). This effect can be due to the fact that the tests were approaching the maximum transfection efficiency when performing the 30°C incubation, and therefore a significant difference due to a reduced experimental dynamic range was not observed.

mRNA production.

[0207] DNA template was linearized/cut with specific restriction enzymes (backbone - pWNY2.0 or pEVL 200) and linearized plasmids were purified using the QiaQuick PCR purification kit (Qiagen). mRNA was made using a kit (mMessage mMachine T7 Ultra; Ambion) with slight modifications from the manufacturer's protocol. Briefly, the *in vitro* translation (IVT) reaction was done for 2.5hrs, DNase treatment for 1 hour. Poly(A) tailing, if required, was done for 1 hour and mRNA was cleaned up using the RNeasy kit (Qiagen), was aliquoted and frozen at -80°C.

Transfection and transduction.

[0208] Primary CD4+ T cells isolated from a donor were stimulated with CD3/CD28 Dynabeads (Life technologies) in the presence of cytokines (IL-2, IL-7 and IL-15) for 60 hours in antibiotic free media. Next, beads were magnetically removed and cells were allowed to rest in fresh media with cytokines for 2.5-5 hours before electroporation. Primary cells were washed with PBS and resuspended in Buffer T (Life Technologies) at 4.5×10^5 cells/10ul and mixed with requisite concentrations of mRNA. Neon (Life Technologies) was used for electroporating mRNA (1400V, 10ms width, pulses =3) using either the 10ul/100ul tip after which the cells were rested for 1-3 hours followed by AAV transduction. AAV was added either based on titer or by volume (not exceeding 20%).

Flow cytometry.

[0209] Analysis of TCRa was performed using the LSRII flow cytometer (BD Biosciences) and data was analyzed using the FlowJo software (Treestar). Briefly, the cells were labeled with a CD3 antibody directly conjugated to Alexa 488 or PerCP-Cy5.5, washed and acquired on LSRII. Cells were gated on live cells based on the forward and side scatter for downstream analysis of the TCR-Knockout (KO).

T7 Endonuclease I (T7EI) assay.

[0210] The cutting efficiency of Cas9 and sgRNA was estimated using the T7EI assay for both TCRa and CCR5 loci. Targeted genomic loci were PCR amplified using specific oligos/primers using either Accuprime Pfx or Hifi Platinum Taq DNA polymerase (Life Technologies), using the manufacturer's instructions. PCR products were checked on gel and cleaned using the QiaQuick PCR purification kit. The DNA concentration of the PCR products was measured and 400ng of the purified product was denatured, subjected to T7EI digestion for 30mins and analyzed on gel.

Cas9 construction into a pEVL vector for generation of an mRNA encoding Cas9 with a poly(A) tail.

[0211] Cas9 was obtained from Addgene (plasmid # 41815), PCR amplified and cloned into a pEVL200 (linear construct) with a T7 promoter and two Nuclear Localization Signals (NLS) - one at both N and C-termini. mCherry was linked to Cas9 with a T2A peptide at 3' end of Cas9. A mCherry only control with a T7 promoter and a single NLS at the 5' end of mCherry was also generated. Generation of a pEVL vector encoding Cas9 was also performed in which an mCherry sequences was not added to the construct. The lengths of the poly(T) region encoding the poly(A) tail can range from 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400 or 500 covalently linked thymines. In some alternatives, the poly(T) region encoding the poly(A) tail can range from 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400 or 500 covalently linked thymines or any length in between any two aforementioned lengths described herein. In some alternatives the poly(A) tail is comprises at least 200 covalently linked thymines.

Expression of TALEN under the control of a pEVL vector.

[0212] As shown in **Figure 6**, panel B, is a schematic of the pEVL (extended variable length) vector. This vector encodes a T7 promoter, necessary for RNA polymerase, an MCS for ease-of-cloning, an encoded poly-A tail of multiple lengths, ranging from 60bp (pEVL 100) to 500bp (pEVL 500), and a unique cleavage site, BsaI, to define the end of the polyA tail and terminate transcription. The gene encoding TALEN was subcloned using standard cloning techniques to incorporate the TALEN gene into the pEVL vector. Quantification of TCR α knockout using pEVL and other vectors encoding a T7 promoter that were enzymatically poly-adenylated was then performed (**Figure 6**, panel B). TCR α knockout was achieved using TALEN, in which primary human CD4⁺ T cells were electroporated using the amount of RNA as indicated in the **Figure 6**, panel B (0.25 μ g, 0.25 μ g and 1 μ g). As shown, pEVL constructs are able to achieve 90% knockout when cells are cultured at 37 °C, and when incubated at 30 °C for 24hours following electroporation, the lowest dose of TALEN provided by pEVL performs better than both enzymatically tailed mRNA.

TCR α CRISPR guide#4 generates the highest TCR α -KO when used with a Cas9 expressed from a linear vector for generation of a polyadenylated mRNA.

[0213] Primary human CD4⁺ T-cells were thawed from a frozen isolate, stimulated with CD3/CD28 Dynabeads (Life Technologies) in the presence of cytokines (IL-2, IL-7 and IL-15) for 60 hours in antibiotic free media. Next, beads were removed and 4.5×10^5 cells per condition were electroporated using a 10uL Neon tip (3×10^5 cells post electroporation) and AAV was added 3 hours post electroporation. Cas9 mRNA expressed from the pEVL construct was used at 1.5ug/sample (1ug post electroporation) and guide-specific AAV's were added at an MOI of 1.33×10^4 /sample. Post electroporation, cells were plated in a 96 well plate in 200ul media with cytokines and left in a 30C CO₂ incubator for 24 hours, after which cells were moved to a 37C CO₂ incubator. (**See Figure 7**).

[0214] At 24 hours post electroporation, cells were checked for Cas9-mCherry expression and subsequently at 72, 96 and 168 hours for both mCherry and TCR-KO with CD3-Alexa488 antibody (Biolegend) using BD LSRII. Voltages were kept same throughout the duration of the experiment. (**See Figure 8**).

Transient expression of Ad5^{wt} proteins and Cas9 expressed from a pEVL vector to increase AAV transduction in human T cells.

[0215] GFP fluorescence of primary human T cells was measured by a BD LSRII 24 or 48 hours following mRNA electroporation of Cas9 generated from a pEVL vector, and AAV transduction of primary human T cells. Cells were treated with 1% culture volume of AAV (2uL equivalent), 1ug Cas9 mRNA, and 0.03ug of each Ad5 protein. AAV was added 4 hours following electroporation, and cells were incubated at 30°C for 24 hours following electroporation, and left at 37°C thereafter. 'Ad5' refers to the combination of E4ORF6 and E1b55k proteins, wildtype or the E1b55k mutants H373A or H354 as indicated. As indicated, the mRNA was generated from a pEVL construct in order to incorporated a poly(A) tail for stability of the mRNA in the experiment. As shown in **Figure 9**, is the GFP mean fluorescence intensity (MFI) in primary human T cells 24 or 48 hours following mRNA electroporation of Cas9 and Ad5 proteins (wildtype or mutants, as indicated) and transduction of AAV. Cas9 mRNA was generated from a pEVL construct in order to obtain a poly(A) tail for stability of the Cas9 mRNA. Cells were treated with 10% or 1% culture

volume of AAV, 1ug Cas9 mRNA, and 0.03ug of each Ad5 protein. AAV was added 4 hours following electroporation, and cells were incubated at 30°C for 24 hours following electroporation, and left at 37°C thereafter. As such, mRNA generated from the pEVL vector for an increased poly(A) tail can be used for nuclease technologies.

Homopolymeric poly(A) tracts 200 base pair or longer are extremely unstable in circular plasmids.

[0216] BFP followed by poly(A) tract inserts of 70, 172, and 325 base pairs bounded by restriction enzyme sites *DraIII* and *SwaI* were generated via excision with *DraIII* and *SwaI* from the linear plasmid cloning vectors BFP-pEVL-100, BFP-pEVL-200, and BFP-pEVL-300. The inserts were transformed with circular cloning vector pWNY that had been digested with *DraIII* and *SmaI*, and transformed into *E. coli* using standard methods. Transformed bacteria were plated onto agarose under ampicillin selection, and grown at 25 °C. Individual colonies were amplified by PCR using primers flanking the poly(A) tract, and the length of the poly(A) tract was determined based on the resulting band size. Typically, a band was obtained at the expected size, or a smaller size, reflecting shortening of the poly(A) tract during transformation. Colonies were scored for whether the poly(A) tract fragment was approximately of the expected size (open circle, "larger insert"), or was substantially shortened (closed circle) (**Figure 10**).

[0217] To illustrate the difficulties in cloning and propagating extended homopolymeric tracts, we attempted to ligate inserts containing 70, 172, and 325 base pair homopolymeric poly(A) tracts respectively into a standard circular cloning vector plasmid (designated pWNY) (**Figure 10**). To assess the stability of the inserts at 25 °C following transformation and overnight plating onto selective media, PCR across the inserts using DNA isolated from individual bacterial colonies was performed. On analysis by gel electrophoresis, the resulting PCR products resolved primarily into bands of two different sizes (**Figure 10**), those products reflecting expected insert sizes are represented by open circles, and products representing substantially shortened sizes are represented by closed circles. As can be seen, poly(A) tracts longer than approximately 100 base pairs are extremely unstable even over the short term process of ligation/transformation into a standard plasmid, with substantial shortening of the tract occurring at a high frequency.

Homopolymeric poly(A) tracts of at least 500 base pairs can be stably propagated in linear plasmid.

[0218] *DraIII* and *SwaI* were generated via excision with *DraIII* and *SwaI* from the linear plasmid cloning vectors pEVL-100, pEVL-200, and pEVL-300. The inserts were ligated into the circular cloning vector pWNY that had been digested with *DraIII* and *SmaI*, and transformed into *E. coli* using standard electroporation methods, or alternatively were subcloned into pEVL and transformed into electro-competent Big Easy cells (Lucigen, Madison, WI). Transformed bacteria were plated onto agarose under ampicillin (pWNY) or kanamycin (pEVL) selection, and grown at 30 degrees Celsius. Individual colonies were amplified by PCR using primers flanking the poly(A) tract, and the length of the poly(A) tract was determined based on the resulting band size as in **Figure 10**. Typically, a band was obtained at the expected size, or a smaller size, reflecting shortening of the poly(A) tract during transformation. Colonies were scored for whether the poly(A) tract fragment was approximately of the expected size (open circle, "larger insert"), or was substantially shortened (closed circle). D) Stability of the encoded poly(A) tracts following overnight induction of pEVL for template preparation. BFP-pEVL-200 through 500 and BFP-pEVL-300-U, G, UU, and GG were grown overnight with induction at 30C in TB+Kan+arabinose media and then maxi-prepped using the NucleoBond Xtra Maxi kit (Macherey-Nagel), using the manufacturer's instructions for low-copy plasmids (differs from the standard protocol namely in using 8-12mL resuspension, lysis, and neutralization buffers per gram of pellet weight). For each maxi-prepped sample, 2ug of DNA was digested overnight with *BsiWI* and *BsaI* to release the poly(A) tail fragment from the rest of the plasmid. The digest was run on a 3% agarose gel and post-stained with GelStar Nucleic Acid stain (Lonza). GeneRuler 50bp ladder (ThermoScientific) was used to determine poly(A) tail length. E) Stability of encoded poly(A) tracts under extended propagation conditions To test the stability of the poly(A) tail under stringent propagation conditions, pEVL 100 through 500 were grown for 2 weeks at 30°C and 37°C in TB+Kan media. Every 24 hours, 1ul of each sample was seeded into a fresh 1ml aliquot of TB+ Kan media. At days 0, 6, and 13, 1ul of each sample at each temperature was seeded into 1ml of TB+Kan+arabinose media and grown overnight before being mini-prepped. After mini-prep, 2ug of DNA from each sample was digested overnight with *BsiWI* and *BsaI* to release the poly(A) tail fragment from the rest of the plasmid. The digest was run on a 3% agarose gel and post-stained with GelStar Nucleic Acid stain (Lonza). GeneRuler 50bp ladder (Thermo Scientific) was used to determine poly(A) tail length.

[0219] The linear plasmid used was a bacteriophage N15-based linear plasmid system that is reportedly superior in the propagation of genomic DNA from organisms with highly repetitive genomes, but has not been previously reported to support the cloning or propagation of homopolymeric polynucleotide tracts. To determine whether the linear plasmid was capable of cloning or propagating poly(A) tracts, an *in vitro* ligation of chemically synthesized oligonucleotides to generate a population of annealed double stranded poly(A) polynucleotides of varied lengths was performed, they were then gel purified where the polynucleotides had lengths between approximately 100 to 850 base pairs, and it was attempted to ligate this population of oligonucleotide into a derivative of linear plasmid with a custom MCS (**Figure 11A**, top). This ligation reaction led to the isolation of the linear plasmid- derivatives containing c.a. 70 base pair, c.a. 170 base pair, and c.a. 325 base pair poly(A) tracts (**Figure 11A**, bottom; sizes were assessed by gel electrophoresis e.g. as in **Figure 11D**, left panel). To determine the potential for the linear plasmid to stably propagate further extended poly(A) tracts, developed a method was developed for generation of extended poly(A) tracts of arbitrary length (**Figure 11B**). This method required modification of the linear plasmid- to position a unique type IIS restriction enzyme just downstream from an encoded poly(A) tract such that it was able to cleave within the poly(A) tract; to achieve this, all internal BsaI sites from the linear plasmid- were removed to produce linear plasmid-that lacked original BsaI sites (**Figure 11A**, middle panel) Cloning of previously generated 70, 172, and 325 base pair tracts into the linear plasmid generated pEVL-100, pEVL-200 and pEVL-300 respectively, and subsequent cloning of a similarly generated blunt ended poly(A) polynucleotide population into BsaI digested/blunted pEVL-300 resulted in incorporation of additional 100 and 200 base pair tracts of poly(A) at the end of the pEVL-300 poly(A) tract, thus allowing the isolation of pEVL-400 and pEVL-500 (**Figure 11D**, left panel).

[0220] It was found that inserts possessing up to 300 base pair poly(A) sequences are easily subcloned into pEVL (**Figure 11C**, **Figure 11D**). These tracts are also highly stable during extended propagation through a two week course of serial liquid culture (**Figure 11E**, also supported by DNA sequencing in both directions across the pEVL300 poly(A) tail following expansion, freezing to a glycerol stock, and re-expansion - see **Figure 14** with forward and reverse primer electropherograms demonstrating that the pEVL- 300 poly(A) tract extends at least 300 base pairs). In contrast, it is noted that there is a tendency for the pEVL-500 poly(A) tract to shorten during cloning, although it is always possible to identify clones possessing inserts with a poly(A) tract of the original c.a. 500 base pair size. Similarly, extended propagation of pEVL-500 also shows a tendency for the poly(A) tract to shorten (**Figure 11E**), and periodic re-isolation of colonies bearing full length tails is necessary to maintain the poly(A) tract at the desired length.

mRNA incorporating extended polyA tails generated from pEVL templates exhibits superior translation properties.

[0221] The capacity of pEVL for generation of IVT mRNA was validated by cloning blue fluorescent protein (BFP) IVT cassettes upstream from the poly(A) tracts in pEVL-100/200/300/400/500. Using the respective BsaI-cleaved pEVL derivatives as the IVT mRNA templates, IVT mRNA was generated using ARCA capping chemistry from BFP-pEVL-100 through BFP-pEVL-500 (**Figure 12A**, left panel). The resulting mRNA's were of predicted size for each pEVL derivative and highly uniform in comparison to enzymatically polyadenylated RNAs (**Figure 12A**, right panel, shown are examples of typical ARCA capped E. coli poly(A) polymerase (EPAP) enzymatically polyadenylated mRNAs). The potency of the mRNA produced from pEVL was validated by comparing BFP expression following electroporation of mRNAs generated through enzymatic polyadenylation and from pEVL-100 and pEVL-300 into primary human T-cells, followed by analysis by flow cytometry (**Figure 12B**). A striking enhancement of potency was observed, as assessed by peak BFP mean fluorescence intensity at 24 hours post-electroporation, for mRNA generated from pEVL-300 vs. pEVL-100 or enzymatically polyadenylated mRNA. Importantly it was typically observed that pEVL-encoded mRNA produced a cell population with a tighter distribution of BFP fluorescence vs. enzymatically poly-adenylated mRNA (e.g. compare flow plots in **Figure 12B**, bottom panel). Analyses comparing BFP expression driven by mRNA generated from pEVL-100 through pEVL-500 over a period of 7 days (**Figure 12C**) confirmed the above observations -pEVL-100 and pEVL-200 derived mRNA possessing poly(A) tail lengths of 72 base pairs and 165 base pairs respectively exhibited reduced potency vs mRNA's derived from pEVL-300, pEVL-400, or pEVL-500 (poly(A) tails of lengths of approximately 325, 420 and 500 base pairs (lengths are approximate and inferred from gel electrophoresis comparisons with MWV standards in **Figure 11E**). Significant potency differences among pEVL-derived mRNAs possessing encoded poly(A) tracts of 300 was not detected.

[0222] Based on evidence from sequencing of cellular RNAs, which has identified correlations between RNA length and the presence of single or double uridine or guanine residues on mRNA 3' termini 24-26, the pEVL-300 system to generate mRNA's with defined termini consisting of single or double uridine or guanine bases was further used, and their capacity to support fluorescent protein expression following transfection into primary human T-cells was evaluated (**Figure 15**). Consistently, it was observed that mRNA's possessing 3' uridine termini (U or UU) exhibited reduced potency (both reduced total magnitude and duration of expression) relative to a standard poly(A) tail of identical length, while those bearing 3' guanine termini (G or GG) drove BFP expression that was indistinguishable from comparable length standard poly(A) tails.

[0223] Finally, the performance of pEVL-derived IVT mRNA in a gene editing application was evaluated: disruption of the TCR α locus in primary human T-cells (**Figure 13**). For this purpose, ORFs encoding TALENs targeting the TCR α locus were cloned into pEVL-300 and used for IVT mRNA production in parallel with production of TALEN mRNA from a standard circular plasmid. Following electroporation of pEVL-derived TALEN mRNA into primary human T-cells, it was observed that rates of CD3 loss, which occur following disruption of the TCR α gene due to the necessity for TCR α/β holoreceptor/CD3 interaction to support surface expression of CD3, were consistently higher than equal weights of enzymatically polyadenylated mRNA generated in parallel (**Figure 13A**). This was interpreted as being consistent with the general observation that increased expression of gene editing nucleases results in higher rates of target site modification. As a second evaluation of the performance of pEVL-derived mRNA for gene editing applications, the Cas9 nuclease fused through a 2A linker to mCherry was cloned into either a circular plasmid vector or pEVL-200, and used mRNA's derived from these vectors to express Cas9 and mcherry in primary human T-cells, whilst simultaneously expressing a guide RNA targeting the TCR α constant region using an AAV (**Figure 13B**). In the case of expression of Cas9-2A-mCherry using either enzymatically poly-adenylated vs. pEVL-200 derived mRNA, it was again observed that pEVL-derived mRNA produced populations with a tighter distribution of fluorescence, as well as higher average fluorescence, and that this correlated with an enhanced efficiency of CRISPR-mediated gene editing (top panel). Collectively, these data support the concept that level and duration of nuclease expression are important determinants of the cleavage activity observed for a nuclease at a genomic target site in a living cell 27, and that use of mRNAs with >250 base pair poly(A) tails to express gene editing nucleases will support higher gene editing efficiencies vs. use of mRNA with shorter poly(A) tails.

H9 T-cell line growth after transfection with pEVL encoded mRNA.

[0224] H9 T-cells were grown in culture, and 1×10^6 cells were resuspended in neon electroporation buffer. 0.66 ug of BFP mRNA generated by in vitro transcription from the pEVL-300 vector was added to the electroporation cuvette, and cells were electroporated at 1000 volts $\times$ 2 pulses, followed by transfer to and culture at 37°C degrees Celsius in regular culture media. 24 hours following transfection, BFP fluorescence was analyzed by flow cytometry, demonstrating that nearly 60% of the cells expressed BFP, indicative of highly efficient transfection with the pEVL-encoded mRNA. (**Figure 16**).

K562 cells line growth after transfection with pEVL encoded mRNA.

[0225] K562 T-cells were grown in culture, and 1×10^6 cells were resuspended in neon electroporation buffer. 1.0 ug of BFP mRNA generated by in vitro transcription from the pEVL-300 vector was added to the electroporation cuvette, and cells were electroporated at 1000 volts $\times$ 2 pulses, followed by transfer to and culture at 37 degrees Celsius in regular culture media. BFP fluorescence was analyzed by flow cytometry, and demonstrated that 70% of the cells expressed BFP, indicative of highly efficient transfection with the pEVL-encoded mRNA. (**Figure 17**).

Jurkat cells growth after transfection with the pEVL-encoded mRNA.

[0226] 1×10^6 Jurkat cultured T-cells were mock electroplated, or electroporated with 1.5 micrograms of mRNA encoding a Cas9-2A-mCherry protein. Transfected cells were placed in culture for 24 hours, following which the cells

were collected and analyzed for mCherry expression by flow cytometry. As shown in the figure, >75% of cells were demonstrated to be mCherry+, indicative of highly efficient transfection. **(Figure 18).**

HepG2 liver cells growth after transfection with the pEVL-encoded mRNA.

[0227] HepG2 cells were grown in culture, and 3×10^5 cells were resuspended in neon electroporation buffer. 0.25 ug of BFP mRNA generated by in vitro transcription from the pEVL-300 vector was added to the electroporation cuvette, and cells were electroporated at 1295 volts $\times$ 3 pulses, followed by transfer to and culture at 37 degrees Celsius in regular culture media. 24 and 48 hours following transfection, BFP fluorescence was analyzed by flow cytometry, demonstrating that nearly 95% of the cells expressed BFP by 24 hours and remained highly BFP+ at 48 hours, indicative of highly efficient transfection with the pEVL-encoded mRNA. **(Figure 19).**

Human CD34 cells transfected with BFP mRNA.

[0228] Human CD34 cells were cultured in SCGM culture media supplemented with Flt3-L/SCF/TPO/IL-3 and incubated for 48 hours at 37 degrees Celsius. The cells were then resuspended in Neon electroporation buffer. 1.5 ug of BFP mRNA, was generated by in vitro transcription from pEVL-300 vectors which prepared by Trilink was added to 0.2×10^6 /ml cells. Cells were electroporated at 1300 volts, 1 pulse, 20ms, followed by transfer to and culture at 30 degrees for 16 hours, and subsequently at 37 degrees Celsius for additional days. Cells were analyzed by flow cytometry at 24, 48, 72, 96 and 120 hr post-electroporation. The BFP mRNA-transfected cells show >80% BFP signals at 24hr and 48hr post-electroporation. The BFP signals decrease gradually from 80% to 20% at 120 hr post-electroporation. **(Figure 20).**

pELV

[0229] pEVL can act as a template during RNA synthesis. The template can be made with an encoded polyA tail of a predefined length and does not require PCR for its synthesis. pEVL plasmids can have a multiple cloning site (MCS) directly upstream of a polyA tract of a defined length that terminates in a unique Type IIS restriction enzyme, BsaI. BsaI can be oriented such that the cleavage occurs at the end, and within the polyA tract, thus the recognition site for the enzyme is inverted. Multiple pEVL plasmids can exist with polyA lengths ranging from approximately 100 to 500 bp in length; the naming corresponds to the tail length e.g., pEVL100 and pEVL500, respectively. Cloning a T7 RNA polymerase promoter and gene into the MCS, and then cleaving the purified DNA with BsaI, can generate a template for the generation of polyadenylated RNA. This template can be used to generate RNA with a predefined, homogeneous polyA tail length in as little as one enzymatic step.

[0230] The generated pEVL can be kept frozen or at -20°C when not actively in use. pEVL can also be protected by proteins produced by *telN*.

E. coli strain selection.

[0231] *E. coli* can be used with pELV. For example, the strain BigEasy *E. coli* can be used with the pELV, especially when one desires a higher plasmid copy number. pEVL plasmids can also be linear plasmid-OK derivatives, and thus the plasmids can be Kanamycin (Kan) resistant. Kan can be used at 50 µg/mL, 40 µg/mL or 30 µg/mL or any other concentration between any two aforementioned values, preferably, 2,000x (10 mg/mL).

Molecular Cloning.

[0232] During cloning, adapters can be used or other variable recognition sites such as DraIII-BsiWI or DraIII sites.

Digesting the plasmid.

[0233] Digestion of the plasmid can be performed by the appropriate endonuclease and is dependent on the endonuclease recognition site in the plasmid.

Ligations and transformations.

[0234] Ligation of a polyT tract into the plasmid can be performed by standard molecular cloning procedures and standard trouble shooting. The plasmids can be transformed into bacterial cells grown on standard media plates, for example LB agar plates. These procedures are known to those skilled in the art.

[0235] The linear plasmids can also be screened to check for the insert and polyA length maintenance. Without being limiting PCR, colony PCR, sequencing and other methods known to one skilled in the art, can be used to look for polyA shortening.

Growing cells with pEVL.

[0236] Cells harboring the linear plasmids can be grown in a nutritional media such as TB and the appropriate selection antibiotic. Cells such as *E. coli*, BigEasy *E. coli* strains, can be used, for example. Examples of selection antibiotics can include but are not limited to Kanamycin, Ampicillin, Penicillin, and chloramphenicol. If there is an operon or promoter region, a molecule to induce induction can be used. For example, if there is an arabinose operon, arabinose can be used for induction. Cells containing plasmids can be frozen down for later use, such as glycerol stocks, for example.

Growth for DNA preps.

[0237] If the pEVL construct made is a low copy plasmid, the pEVL can require a larger mass of bacteria in order to obtain standard amounts of plasmid from a commercial preparation, (e.g., Macherey Nagel Nucleobond Xtra Maxi). The bacteria can be grown in the presence of a selection antibiotic, depending on the antibiotic resistance.

Plasmid DNA and RNA preps.

[0238] DNA plasmid preps can be made from techniques known to skilled in the art, as well as from commercially available kits, for example (for example, Qiagen Maxi and MiniPrep kits and Invitrogens Maxi kit). Commercially available kits are known to those skilled in the art and the kits can have RNase, lysis buffers, for example. Kits can also be used to retrieve RNA and to avoid RNA contamination and to avoid low yields.

RNA Transcription Using pEVL.

[0239] The pEVL construct can be digested with the proper endonuclease, for example, when the restriction recognition site is BsaI, BsaI can be used. The amount of enzyme required depends on the size of the insert, since larger inserts (and therefore larger plasmids) will have a lower number of sites per μg of DNA, and would therefore requires less enzyme, for example.

[0240] After digestion, purification of the reaction can be performed. Purification can be performed by standard purification techniques known to those skilled in the art, or by a commercially available kit, such as Qiagen PCR

purification columns for example.

Transcription reaction.

[0241] After purification of the template, the template can be used for the transcription reaction.

[0242] Here the development and application of a linear plasmid vector system, (plasmid -Extended Variable Length (pEVL)) is described, which allows for generation of IVT mRNA with poly(A) tracts possessing defined 3' ends of up to at least 500 base pairs. Furthermore, it is shown that IVT mRNA's generated with pEVL have high potency when used in conjunction with electroporation of primary human T-cells, and that the observed potency is highest for transcripts possessing 3' poly(A) tracts greater than approximately 300 base pairs and either adenine or guanine residues at their 3' terminus.

[0243] pEVL using the framework of a linear plasmid was built, in which the linear plasmid was developed for cloning of highly repetitive DNA from bacterial genomes. As schematized in **Figure 11B**, adaptation of the linear plasmid for facile generation of extended poly(A) tracts required its modification such that it possessed a unique target site for the type IIS restriction enzyme BsaI, as the BsaI site 3' to the poly(A) tract is central to the method developed for iterative cloning of blunt end poly (A) oligonucleotides in order to extend the encoded poly(A) tract to a length greater than 300 base pairs. The BsaI site or another restriction site or endonuclease site may also be used for generation of arbitrary alternative terminal residues on the 3' end of the mRNA, that, as it is shown in **Figure 15**, may include one or two terminal guanines following the homo-polymeric poly(A) stretch while still yielding mRNA with potency indistinguishable from a homopolymeric poly(A) tract, at least in primary T-cells.

[0244] One of the most important uses of pEVL will be for large scale generation of mRNA for therapeutic applications. pEVL is easily scaled up for large scale GMP plasmid production using kanamycin selection, and allows for generation of uniform size capped and polyadenylated mRNAs in a single reaction using standard T7 RNA polymerase chemistry. As validating examples for the utility of pEVL at scale, pEVL-200 and pEVL-300 can be used and shown to generate extremely high quality and high potency mRNAs encoding TALENs, BFP, Sleeping Beauty transposase, and Cas9-2A-mCherry at 10 mg scale using standard mRNA IVT methods.

[0245] pEVL also represents a new tool for investigators interested in mRNA biology. By providing a method for generation of transcripts that possess poly(A) tails of arbitrary length and terminal residues, pEVL allows performing of detailed studies of the relationship between mRNA architecture and translational efficiency over a wider range of poly(A) tail lengths than previously possible. pEVL is a significant advance toward simple, efficient, and consistent large scale synthesis of highly potent mRNA's for therapeutic application, as well as a new tool for the study of the architecture and function of mRNAs possessing defined poly(A) tracts in the physiologic (>200 base pair) range.

[0246] With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to plural as is appropriate to the context and/or application. The various singular/plural permutations can be expressly set forth herein for sake of clarity.

[0247] It will be understood by those within the art that, in general, terms used herein, and especially in the appended claims (for example, bodies of the appended claims) are generally intended as "open" terms (for example, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.). It will be further understood by those within the art that if a specific number of an introduced claim recitation is intended, such an intent will be explicitly recited in the claim, and in the absence of such recitation no such intent is present. For example, as an aid to understanding, the following appended claims can contain usage of the introductory phrases "at least one" and "one or more" to introduce claim recitations. However, the use of such phrases should not be construed to imply that the introduction of a claim recitation by the indefinite articles "a" or "an" limits any particular claim containing such introduced claim recitation to alternatives containing only one such recitation, even when the same claim includes the introductory phrases "one or more" or "at least one" and indefinite articles such as "a" or "an" (for example, "a" and/or "an" should be interpreted to mean "at least one" or "one or more"); the same holds true for the use of definite articles used to introduce claim recitations. In addition, even if a specific number of an introduced claim recitation is explicitly

recited, those skilled in the art will recognize that such recitation should be interpreted to mean at least the recited number (for example, the bare recitation of "two recitations," without other modifiers, means at least two recitations, or two or more recitations). Furthermore, in those instances where a convention analogous to "at least one of A, B, and C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (for example, "a system having at least one of A, B, and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). In those instances where a convention analogous to "at least one of A, B, or C, etc." is used, in general such a construction is intended in the sense one having skill in the art would understand the convention (for example, "a system having at least one of A, B, or C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together, etc.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

[0248] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0249] As will be understood by one skilled in the art, for any and all purposes, such as in terms of providing a written description, all ranges disclosed herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a nonlimiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," and the like include the number recited and refer to ranges which can be subsequently broken down into sub-ranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 articles refers to groups having 1, 2, or 3 articles. Similarly, a group having 1-5 articles refers to groups having 1, 2, 3, 4, or 5 articles, and so forth.

[0250] While various aspects and alternatives have been disclosed herein, other aspects and alternatives will be apparent to those skilled in the art. The various aspects and alternatives disclosed herein are for purposes of illustration and are not intended to be limiting, with the true scope and spirit being indicated by the following claims.

[0251] One skilled in the art will appreciate that, for this and other processes and methods disclosed herein, the functions performed in the processes and methods can be implemented in differing order. Furthermore, the outlined steps and operations are only provided as examples, and some of the steps and operations can be optional, combined into fewer steps and operations, or expanded into additional steps and operations without detracting from the essence of the disclosed alternatives.

[0252] One skilled in the art will appreciate that, for this and other processes and methods disclosed herein, the functions performed in the processes and methods can be implemented in differing order. Furthermore, the outlined steps and operations are only provided as examples, and some of the steps and operations can be optional, combined into fewer steps and operations, or expanded into additional steps and operations without detracting from the essence of the disclosed alternatives.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European

patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- [US6206209B \[0001\]](#)
- [US6216110Z \[0001\]](#)
- [WO2007087478A \[0101\]](#)
- [US9029134B \[0103\]](#)

Non-patent literature cited in the description

- **GODISKA et al.** Nucleic Acids Research, 2010, vol. 38, 6 [\[0101\]](#)
- **DOYON Y. et al.** Transient cold-shock enhances zinc-finger nuclease-mediated gene disruption. Nature Methods, 2010, vol. 7, 459-460 [\[0107\]](#)

PATENTKRAV

1. Fremgangsmåde til fremstilling af en nukleinsyre, hvilken fremgangsmåde omfatter:

a) der tilvejebringes en lineær vektor, hvilket omfatter:

5 (i) tilvejebringelse af et lineært plasmid, der omfatter en venstre arm, der omfatter en venstre telomer, en højre arm, der omfatter en højre telomer, og en første nukleinsyresekvens, der omfatter mindst ét endonukleasegenkendelsessted,

(ii) tilvejebringelse af en anden nukleinsyresekvens, der omfatter en flerhed af
10 thyminnukleotider, hvor flerheden af thyminnukleotider omfatter mindst, mere end, lig med eller et hvilket som helst antal mellem 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 eller 550 kovalent bundne konsekutive thyminnukleotider, og

(iii) insertion af den anden nukleinsyre i det lineære plasmid, hvorved den
15 første nukleinsyresekvens forbindes med den anden nukleinsyresekvens, således at den anden nukleinsyre omfatter et endonukleasespaltningssted, der ligger inden for flerheden af thyminnukleotider eller inden for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 eller 40 nukleotider fra den 5'-terminale thymin i flerheden af
20 thyminnukleotider, og

hvor den første nukleinsyre er kovalent bundet til den anden nukleinsyre i den ene ende af den anden nukleinsyre, og hvor endonukleasegenkendelsesstedet ligger inden for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33,
25 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54 eller 55 nukleotider fra endonukleasespaltningsstedet, og

(b) den lineære vektor bringes i kontakt med en RNA-polymerase i nærværelse af nukleotider, hvorved nukleinsyren genereres.

2. Fremgangsmåde ifølge krav 1, hvor endonukleasespaltningsstedet ligger inden for flerheden af thyminnukleotider.
3. Fremgangsmåde ifølge krav 1 eller 2, hvor det lineære plasmid omfatter en venstre arm, der omfatter en venstre telomer; en højre arm, der omfatter en
5 højre telomer; og hvor fremgangsmåden omfatter en kloningsregion, der er lokaliseret mellem den venstre arm og den højre arm.
4. Fremgangsmåde ifølge et hvilket som helst af kravene 1-3, hvor det lineære plasmid yderligere omfatter en tredje nukleinsyre, der omfatter et gen, som koder for en nuklease, der er en endonuklease.
- 10 5. Fremgangsmåde ifølge et hvilket som helst af kravene 1-4, hvor endonukleasen omfatter aminosyresekvensen, der er angivet i SEQ ID NO. 6 eller kodes for af nukleotidsekvensen, der er angivet i en hvilken som helst af SEQ ID NO: 7-14.
6. Fremgangsmåde ifølge et hvilket som helst af kravene 1-5, hvor det
15 mindst ene endonukleasegenkendelsessted er et genkendelsessted for type II-restriktionsendonukleaser, såsom et genkendelsessted for type IIS-restriktionsendonukleaser.
7. Fremgangsmåde ifølge et hvilket som helst af kravene 1-6, hvor det mindst ene endonukleasegenkendelsessted er et genkendelsessted for Bsal-
20 restriktionsendonukleaser eller et genkendelsessted for Stul-restriktionsendonukleaser.
8. Fremgangsmåde ifølge et hvilket som helst af kravene 1-7, hvor den genererede nukleinsyre er RNA, såsom et RNA, der omfatter en flerhed af adeninnukleotider, hvor flerheden af adeninnukleotider omfatter mere end, lig med
25 eller et hvilket som helst antal mellem 130, 140, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475 eller 500 kovalent bundne konsekutive adeniner.
9. Fremgangsmåde til stabilisering af et RNA under *in vitro*-translation, hvilken fremgangsmåde omfatter:

nukleinsyren, der fremstilles ved hjælp af fremgangsmåden ifølge et hvilket som helst af kravene 1-8, tilvejebringes, og nukleinsyren bringes i kontakt med et ribosom i nærværelse af aminosyrer og tRNA'er, hvorved der genereres et RNA, hvor RNA'et er stabilt ved en temperatur i et område fra 30 °C til 37 °C og i
5 et tidsrum på mere end eller lig med 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 eller 100 timer eller i et tidsrum inden for et område, der er defineret af hvilke som helst to af ovennævnte tider.

10. Fremgangsmåde ifølge krav 9, hvor RNA'et yderligere omfatter et gen, som koder for et protein til en terapi, eller en nuklease, såsom en endonuklease.

10 11. Fremgangsmåde til stabilisering af et RNA under *in vivo*-translation i en celle, såsom en human celle, såsom en celle, der er valgt fra gruppen bestående af en primær celle, en human primær T-celle, en CD4+-celle og en CD8+-celle, hvilken fremgangsmåde omfatter:

transficering, i en celle, af nukleinsyren, der fremstilles ved hjælp af
15 fremgangsmåden ifølge et hvilket som helst af kravene 1-8;

placering af cellen i en dyrkningsbeholder med medier;

forsyning af cellen med næringsstoffer og aminosyrer til translationen; og

inkubering af cellen i et tidsrum på mere end eller lig med 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 eller 100 timer eller i et tidsrum inden for et område, der er
20 defineret af hvilke som helst to af ovennævnte tider, og ved en temperatur i et område fra 30 °C til 37 °C.

12. Fremgangsmåde til forøgelse og/eller stabilisering af en ekspression af et protein, hvilken fremgangsmåde omfatter generering af en nukleinsyre ved udførelse af fremgangsmåden ifølge et hvilket som helst af kravene 1-8; og
25 translatering af nukleinsyren i et peptid.

DRAWINGS

Drawing



FIG. 1A

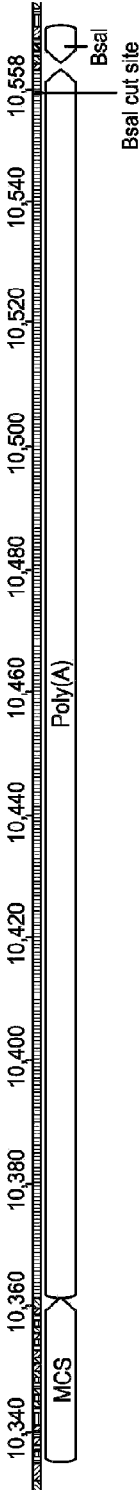


FIG. 1B

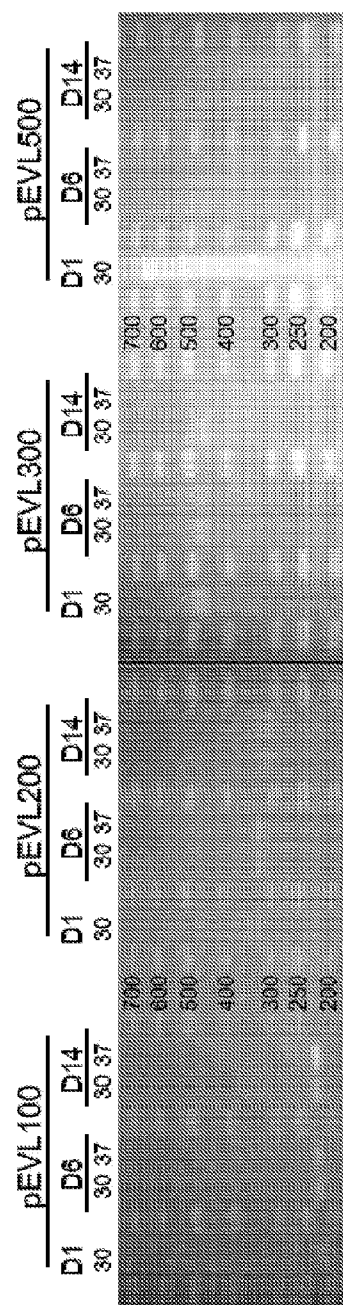


FIG. 2

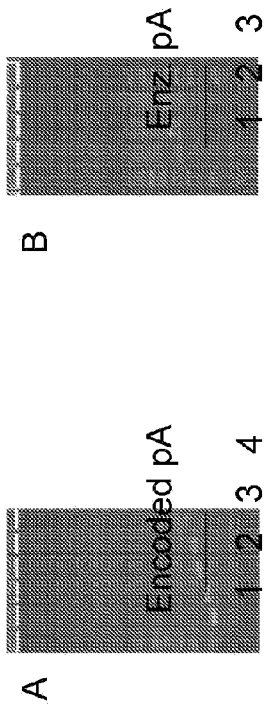


FIG. 3

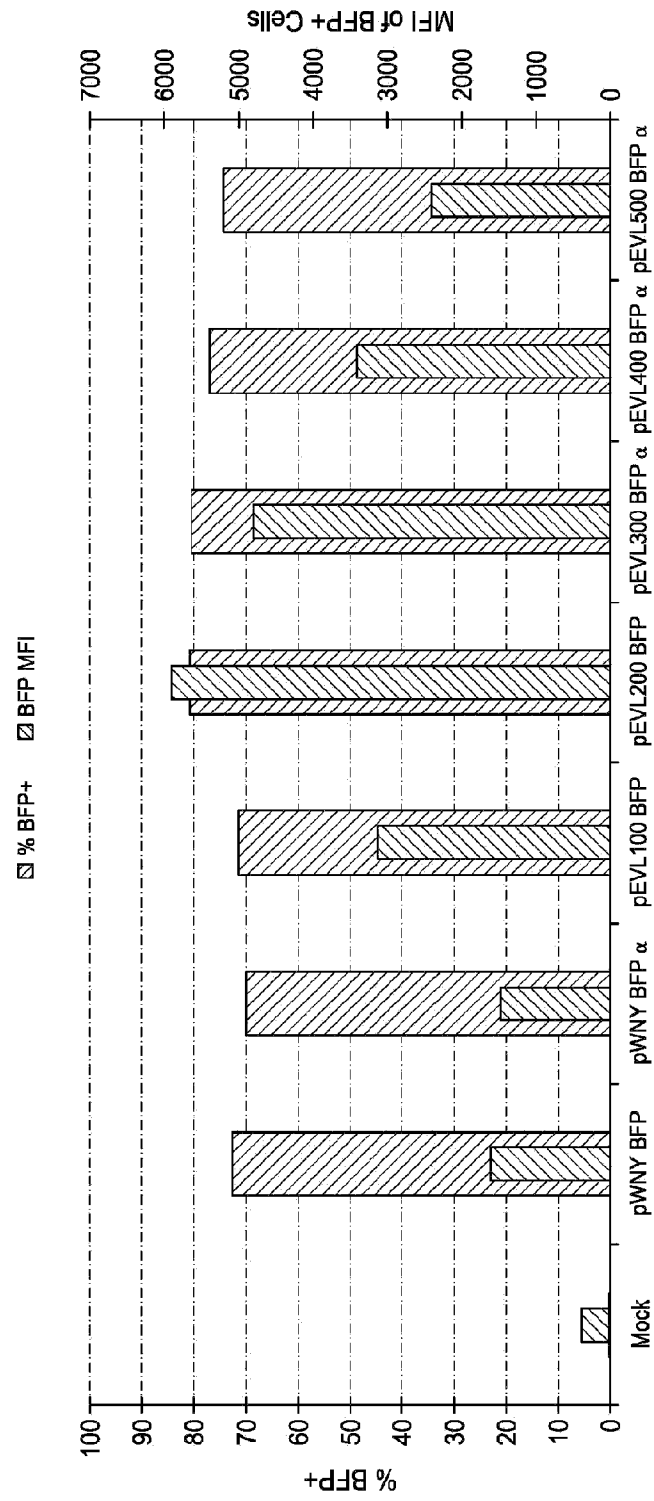


FIG. 4

Enzymatic & Encoded pA BFP vs Time

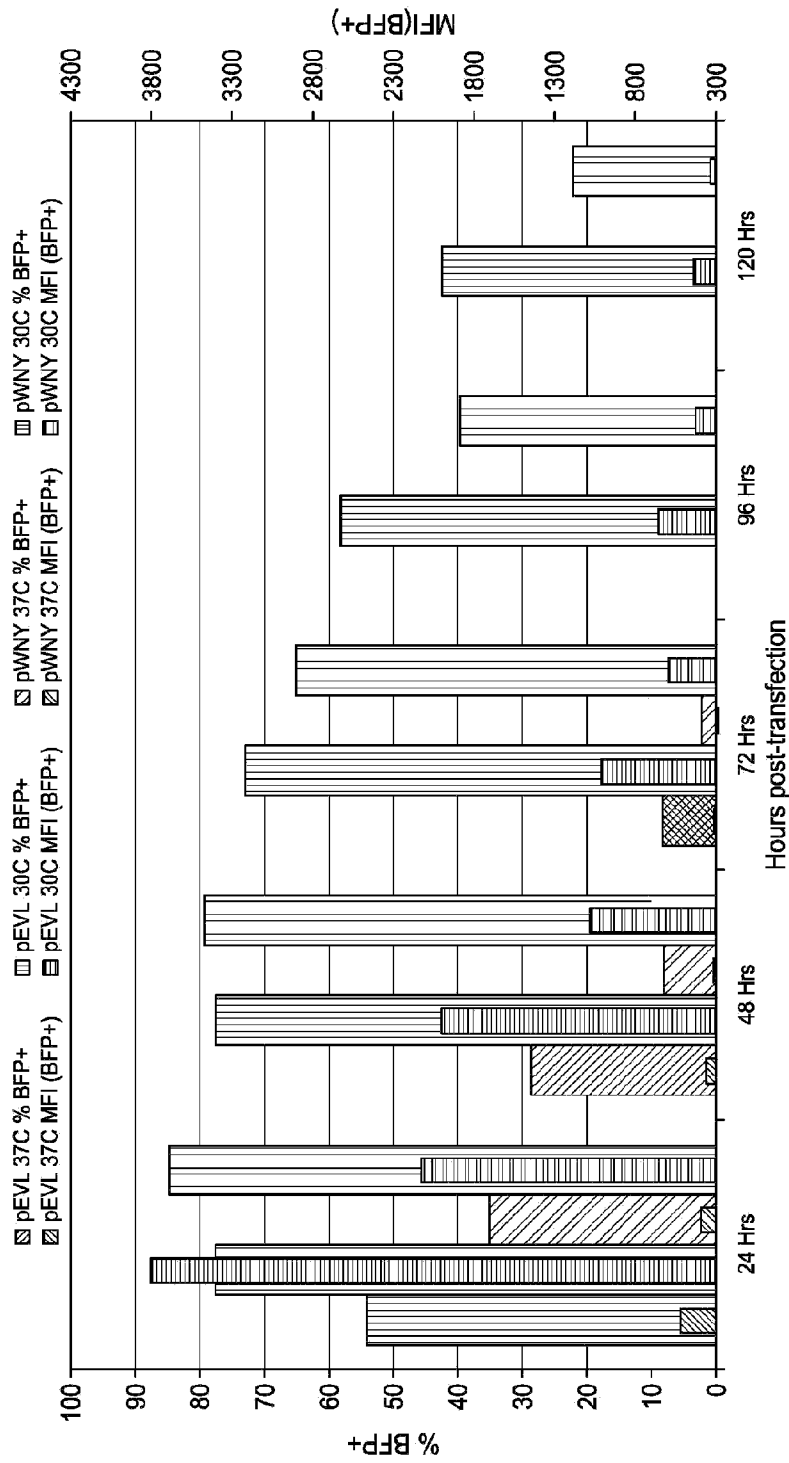
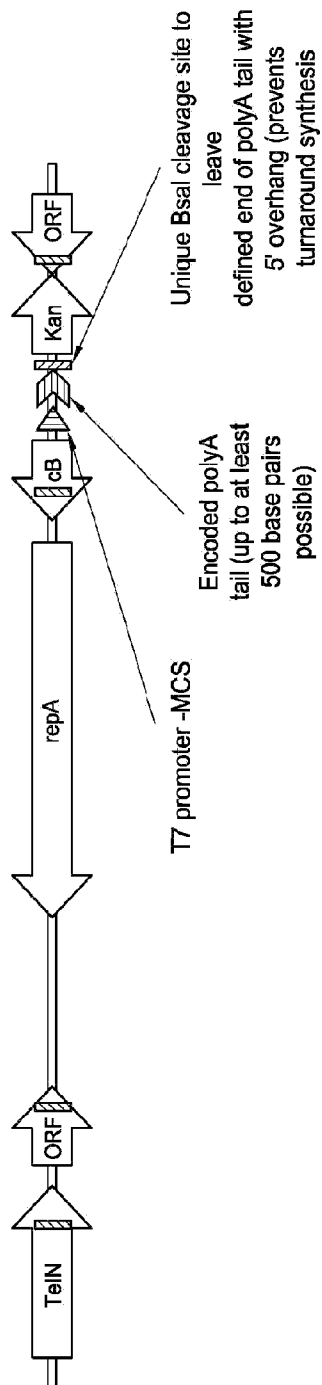


FIG. 5

*FIG. 6A*

TALEN TCR α Knockout

▨ 30 C ▨ 37 C

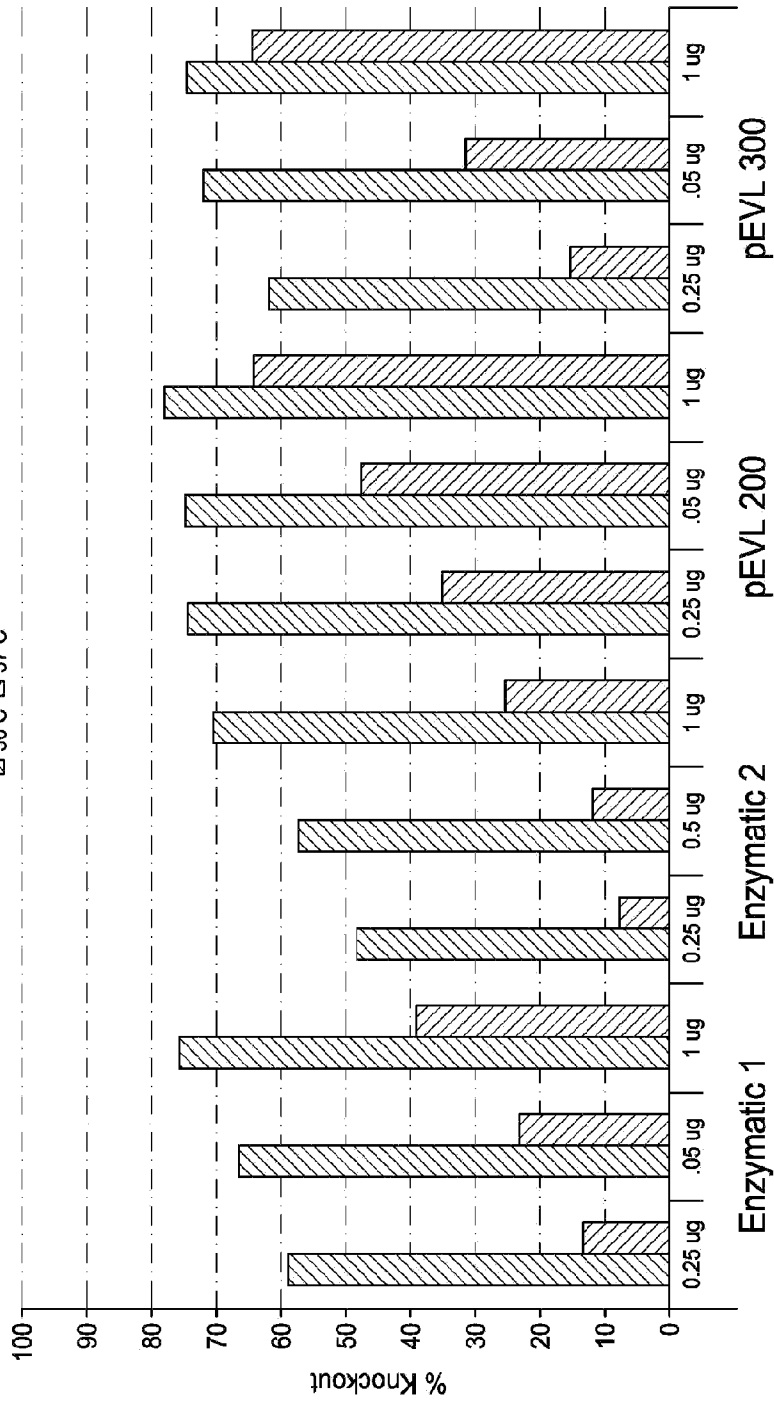


FIG. 6B

TCRa CRISPR guide#4 generates the highest TCRa-KO (knockout)

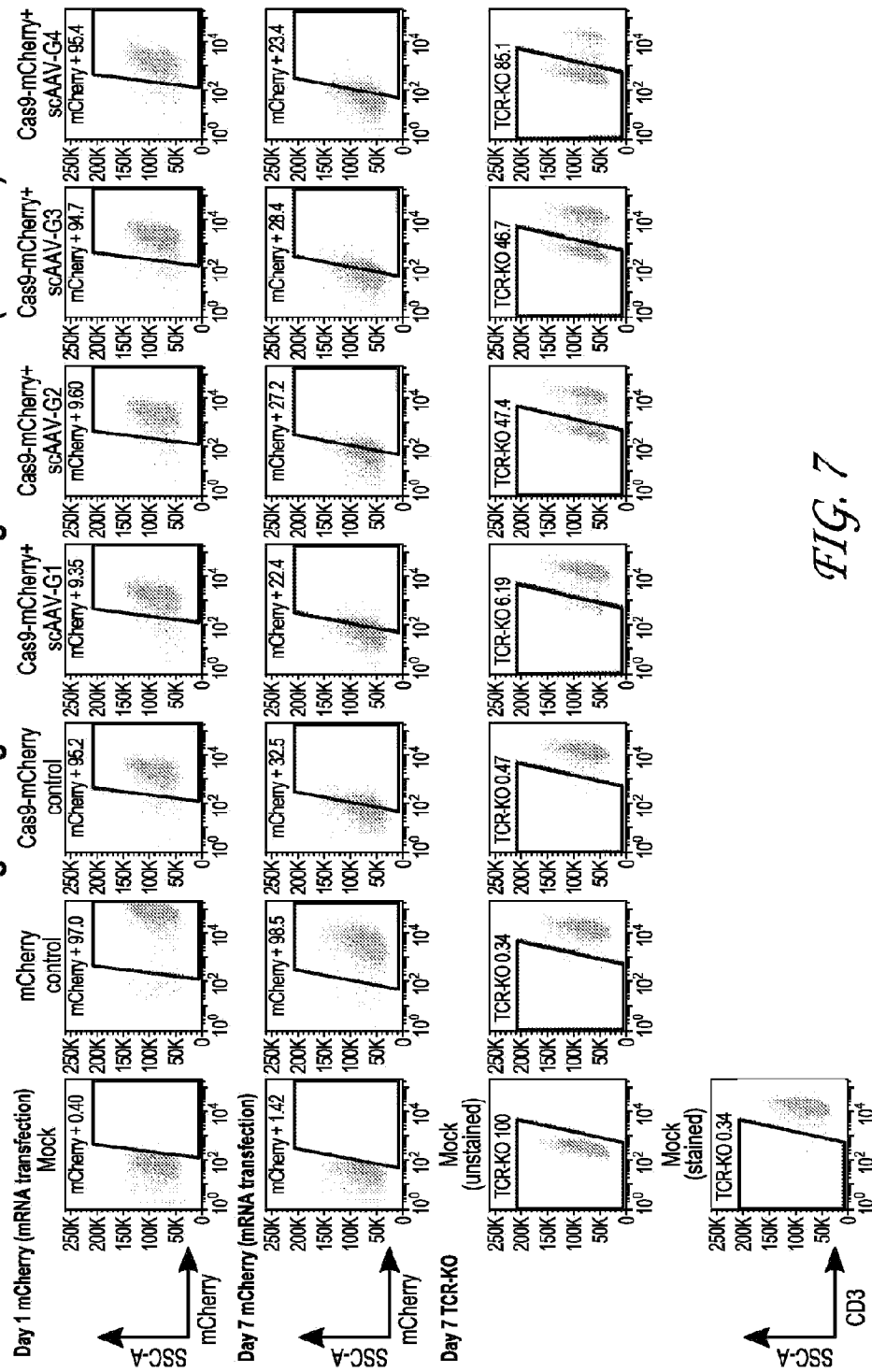


FIG. 7

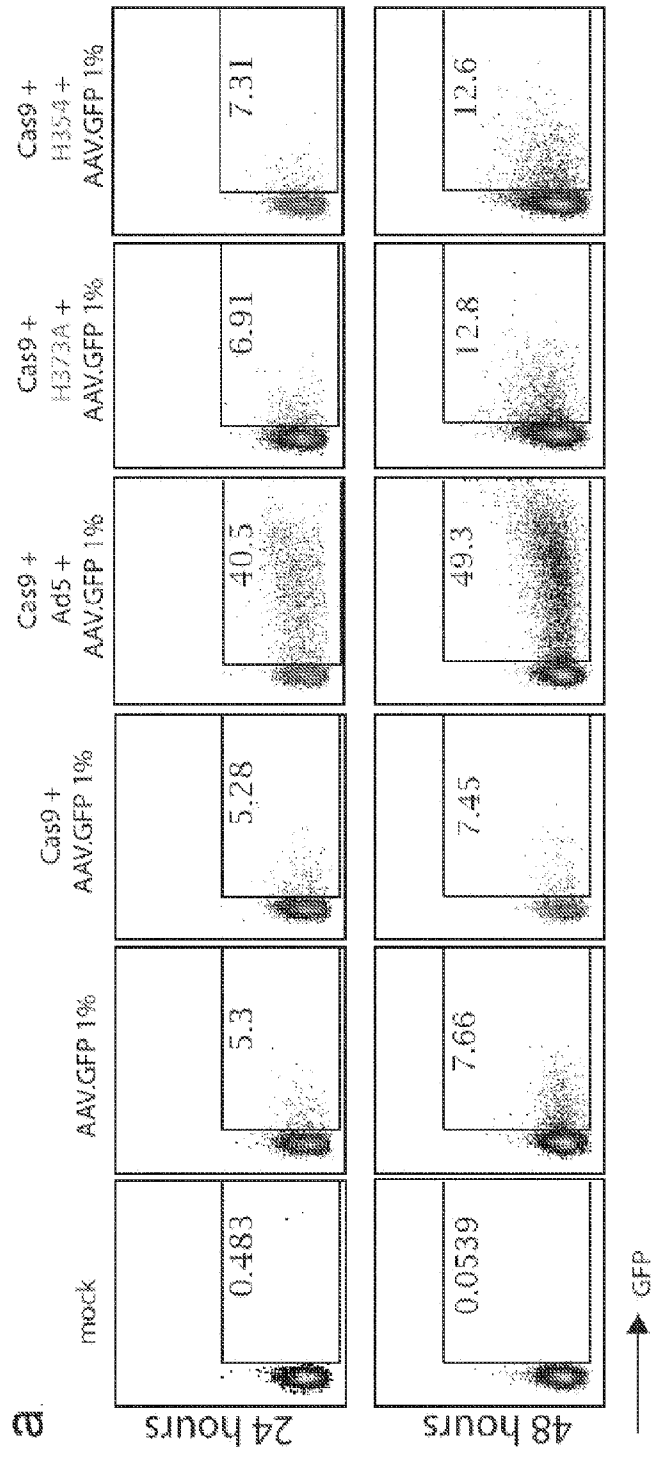


FIG. 8

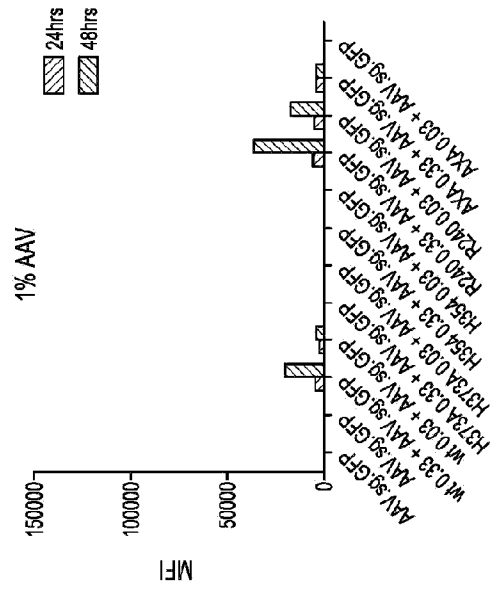
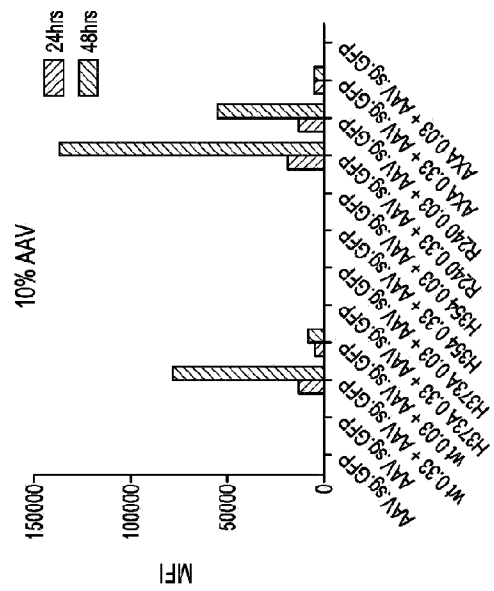


FIG. 9



PolyA shortening at 25 °C following
transformation into a circular plasmid

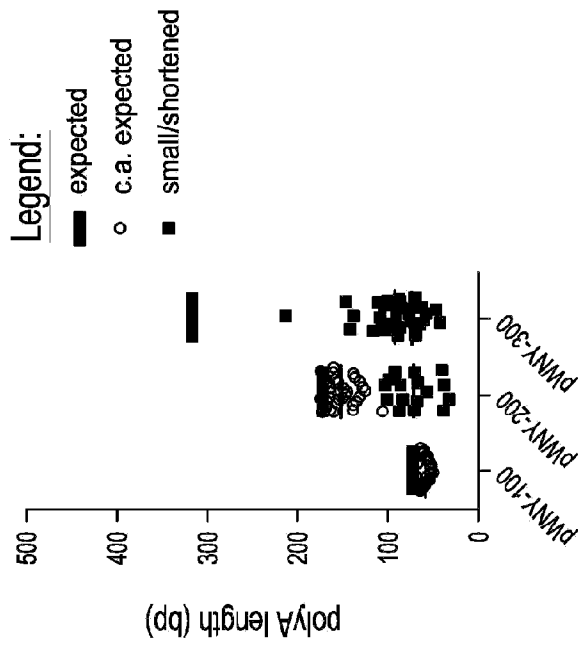
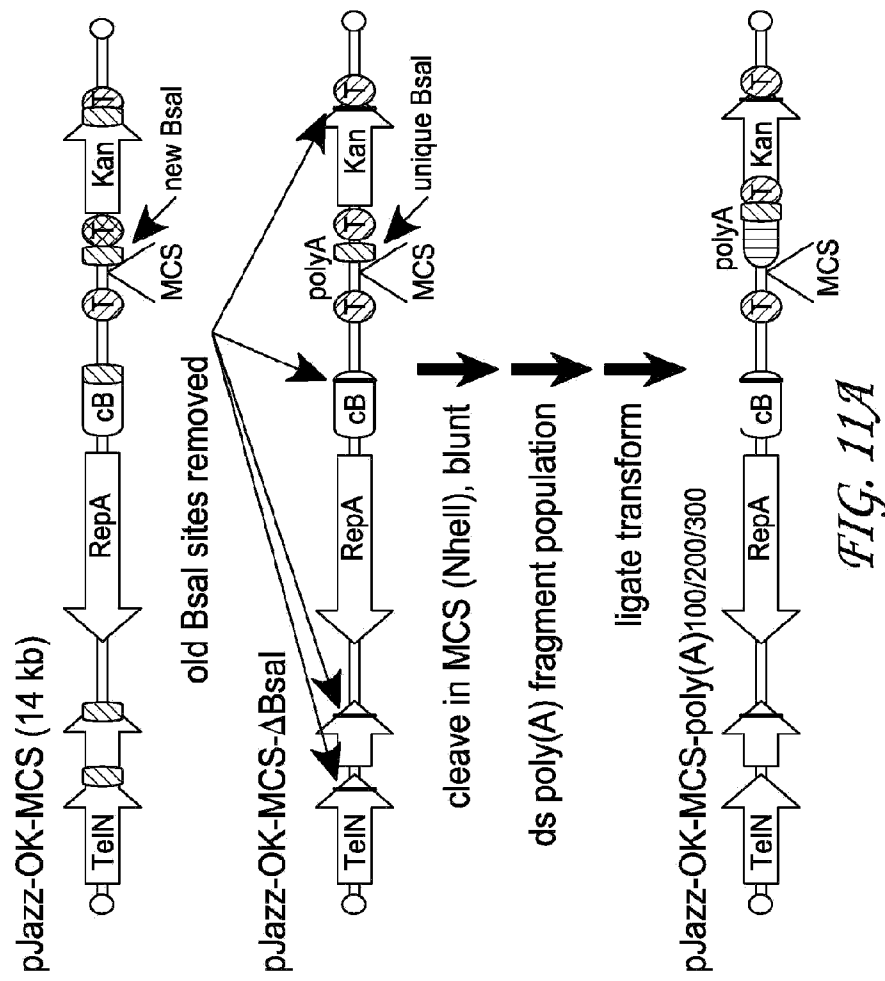
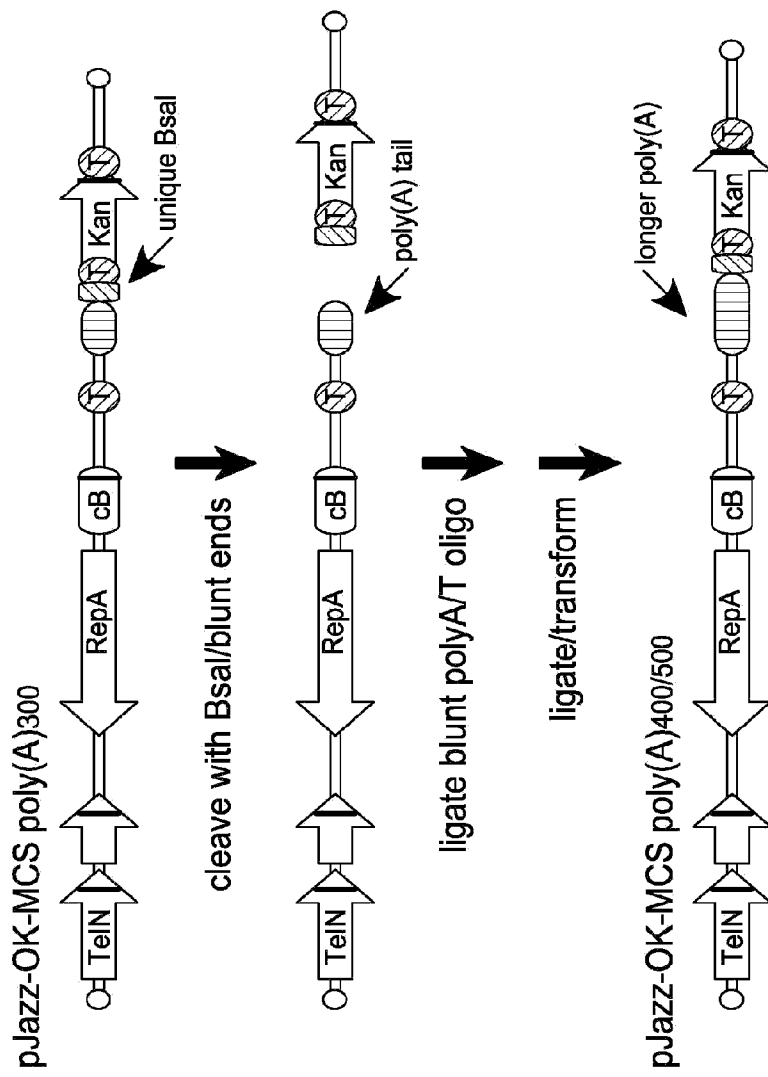


FIG. 10



*FIG. 11B*

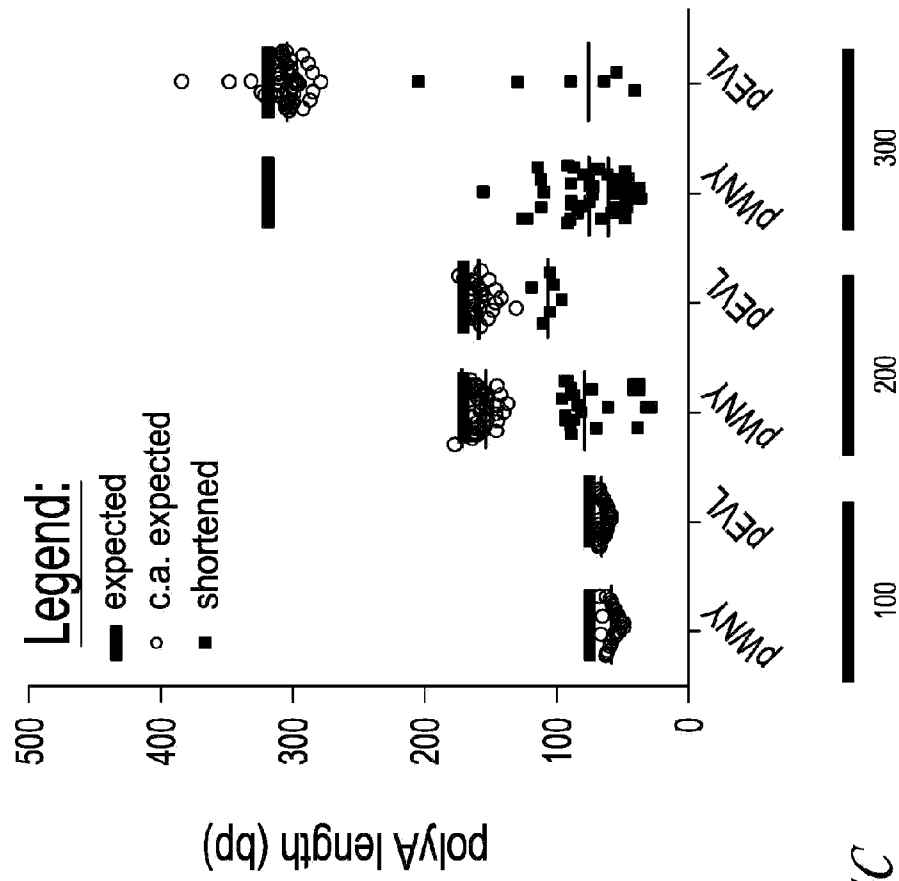


FIG. 11C

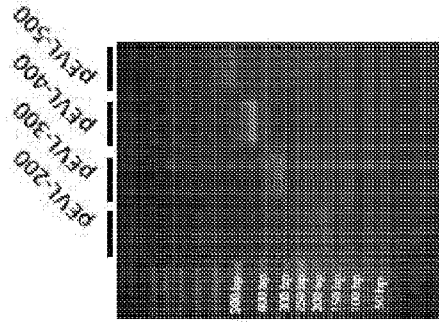


FIG. 11D

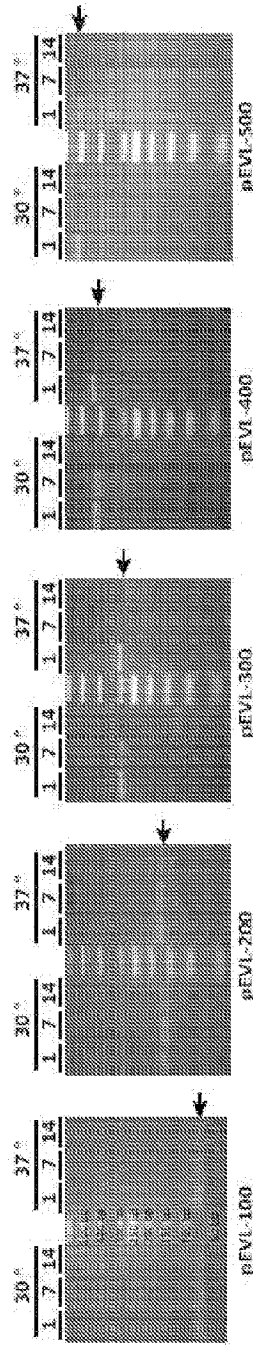
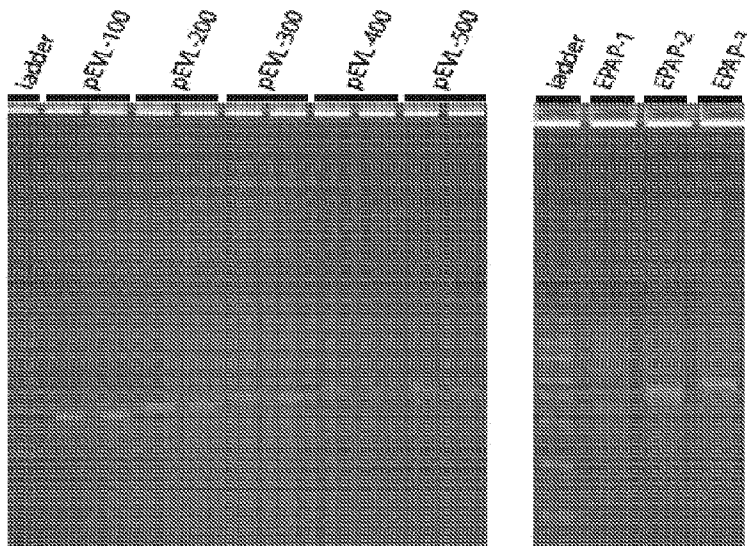
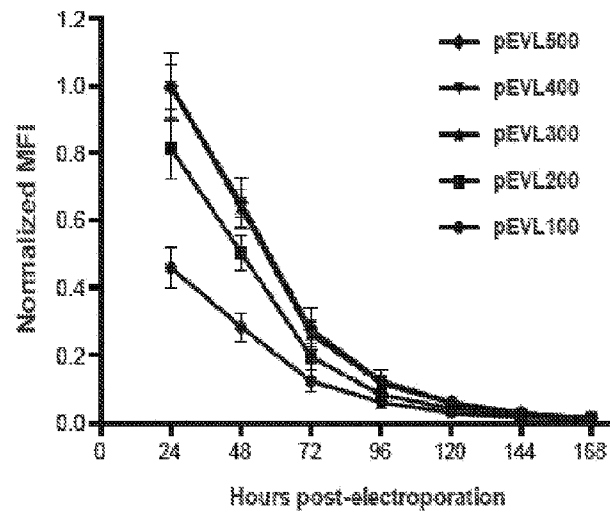


FIG. 11E

*FIG. 12A**FIG. 12C*

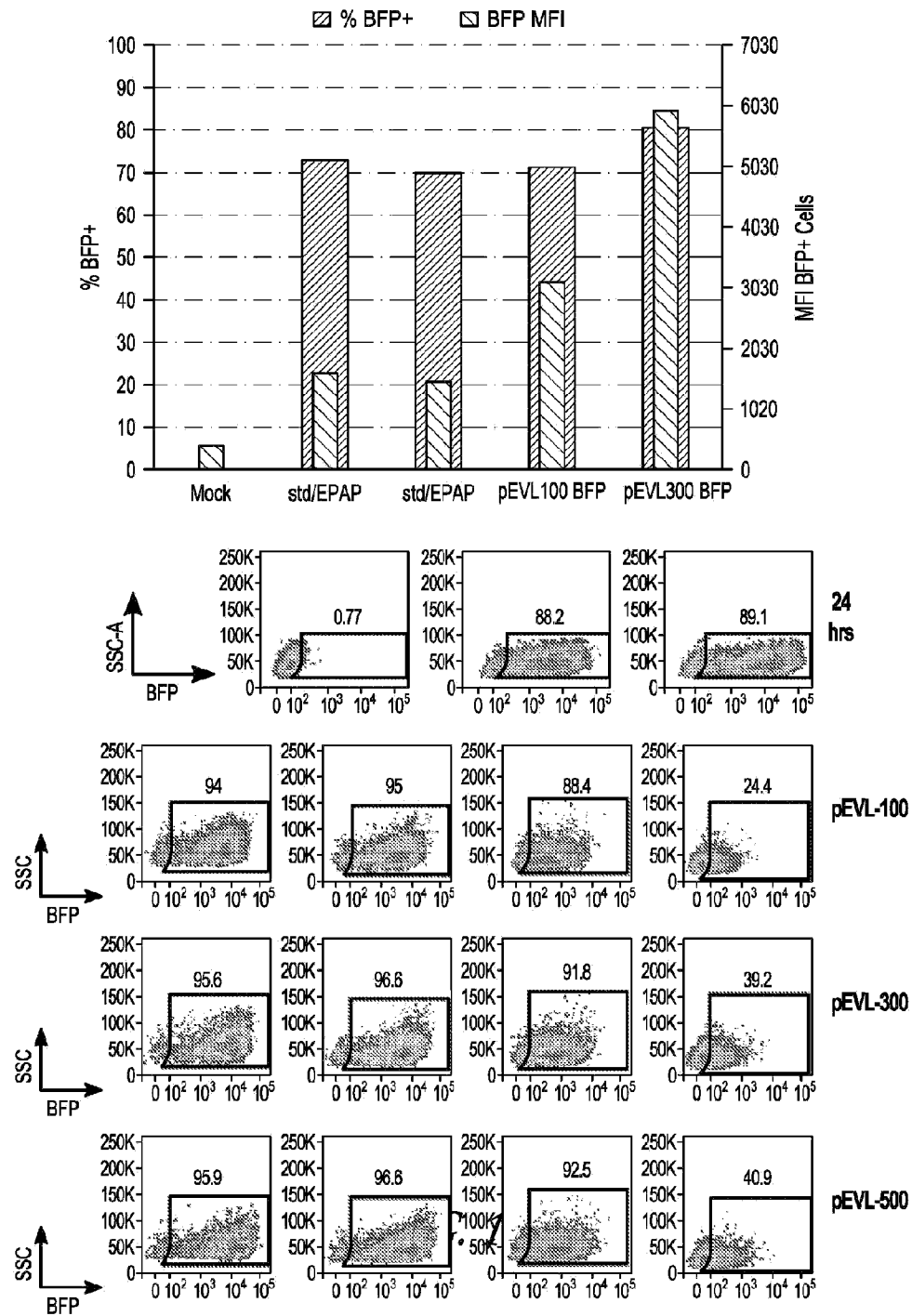


FIG. 12B

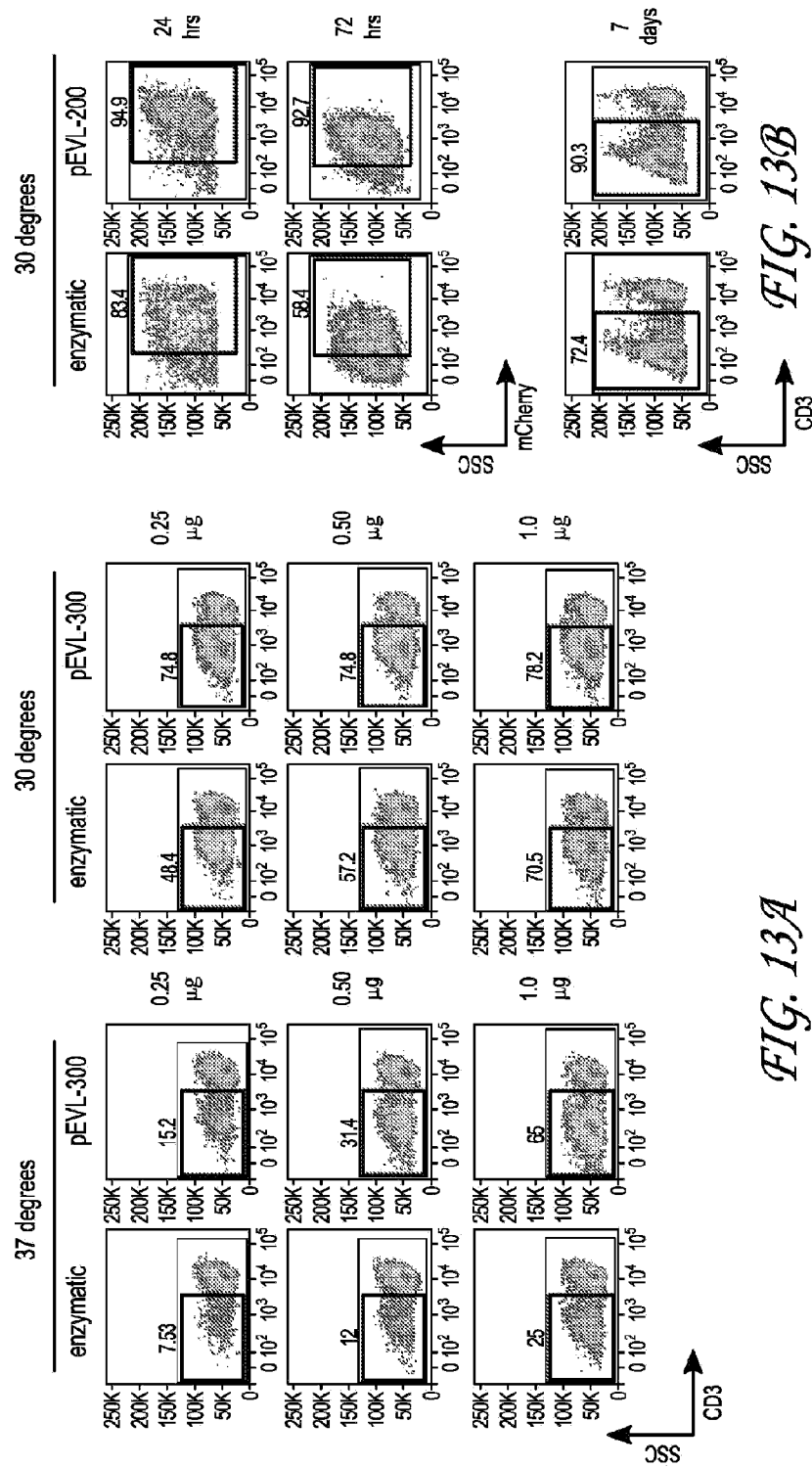
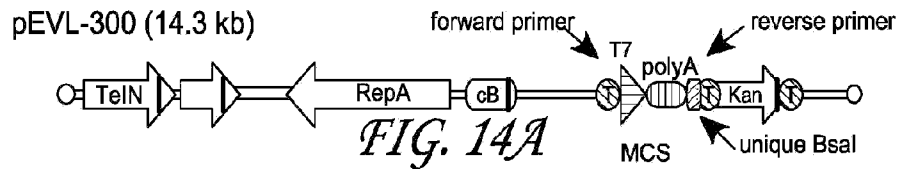
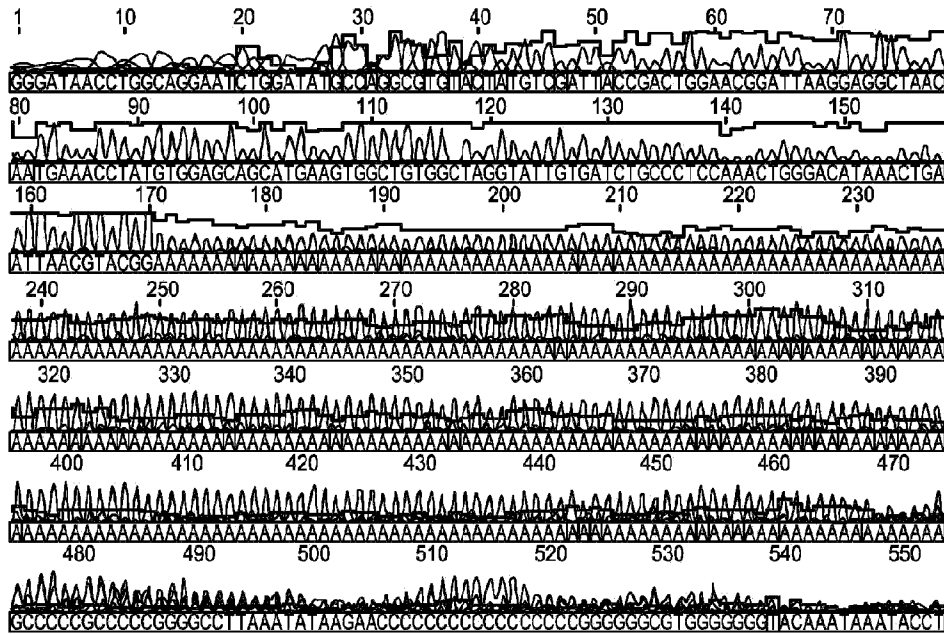


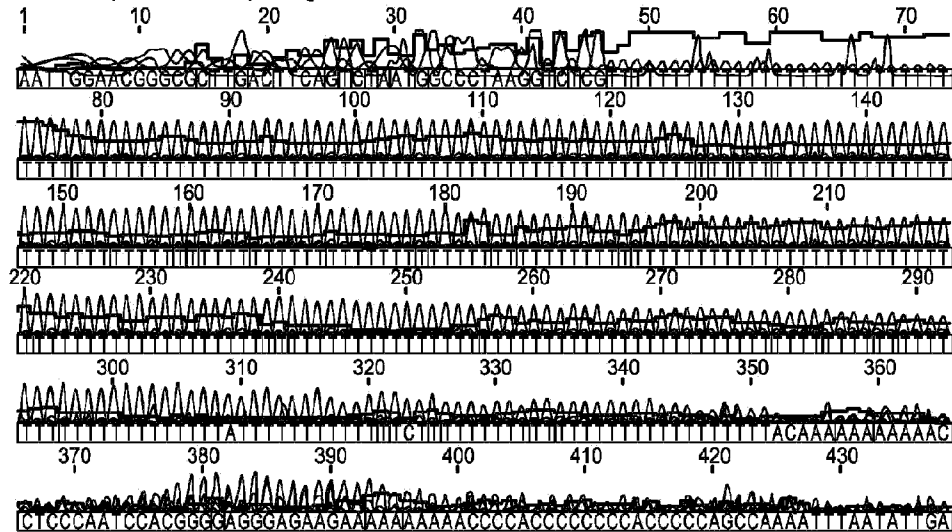
FIG. 13B

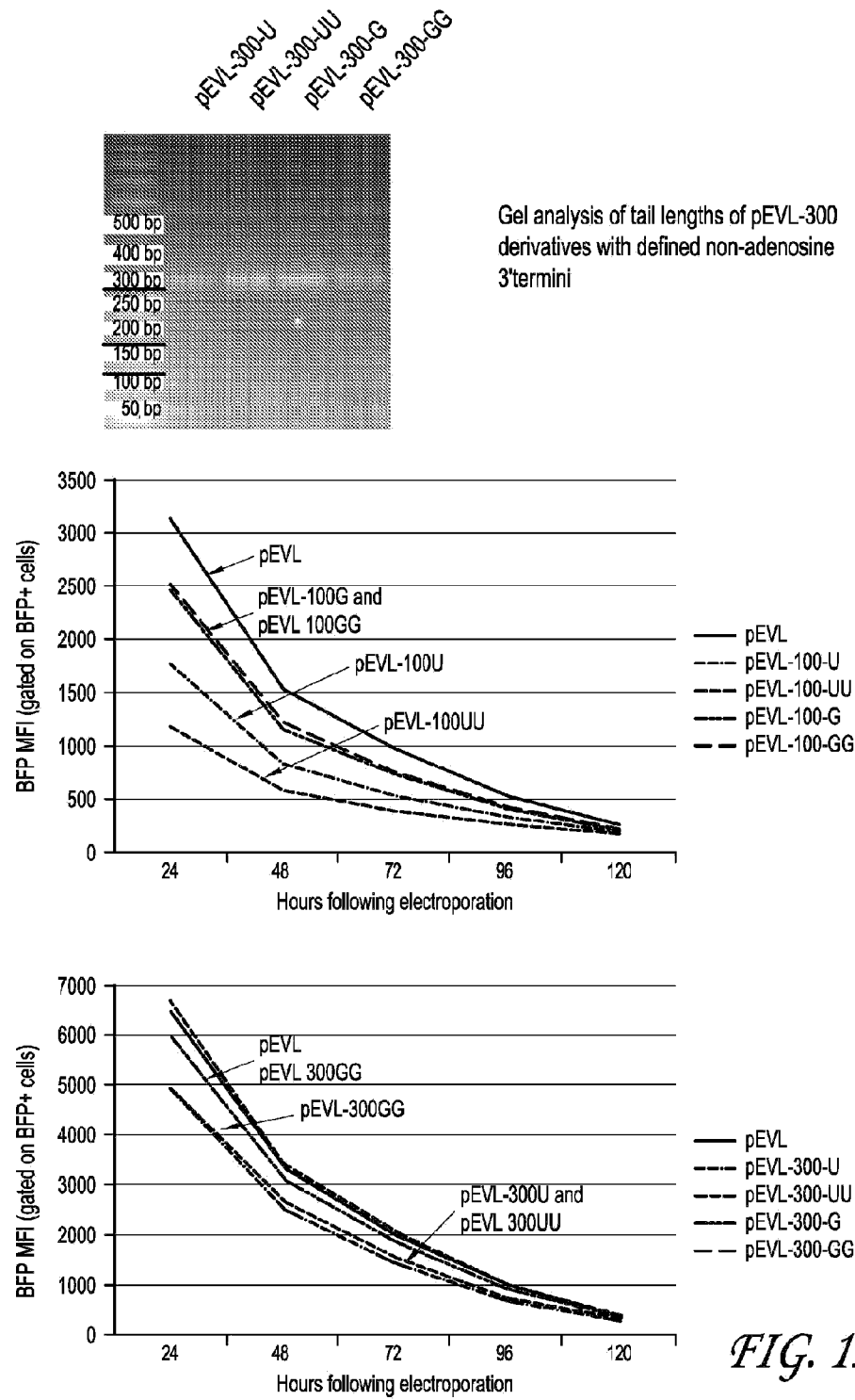


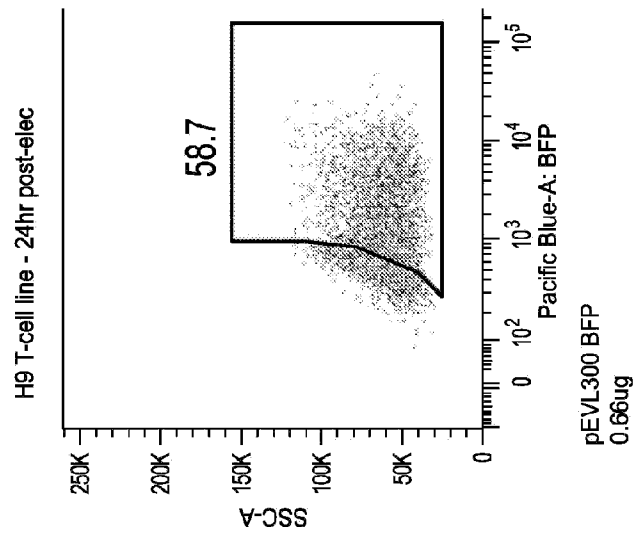
Forward primer electropherogram

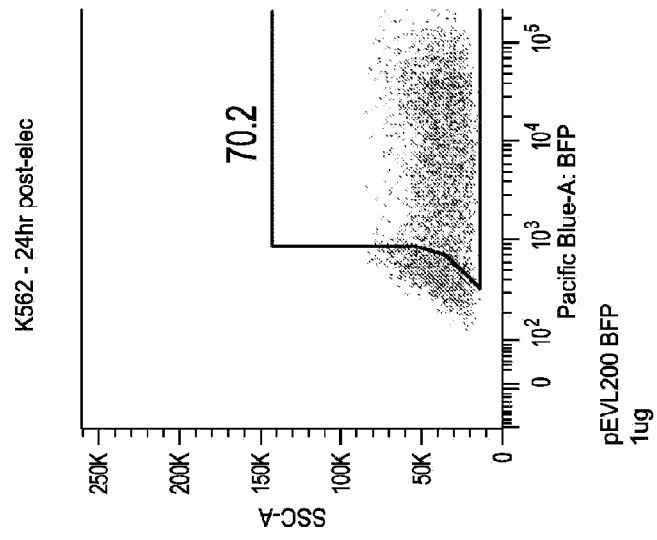
*FIG. 14B*

Reverse primer electropherogram

*FIG. 14C*



*FIG. 16*

*FIG. 17*

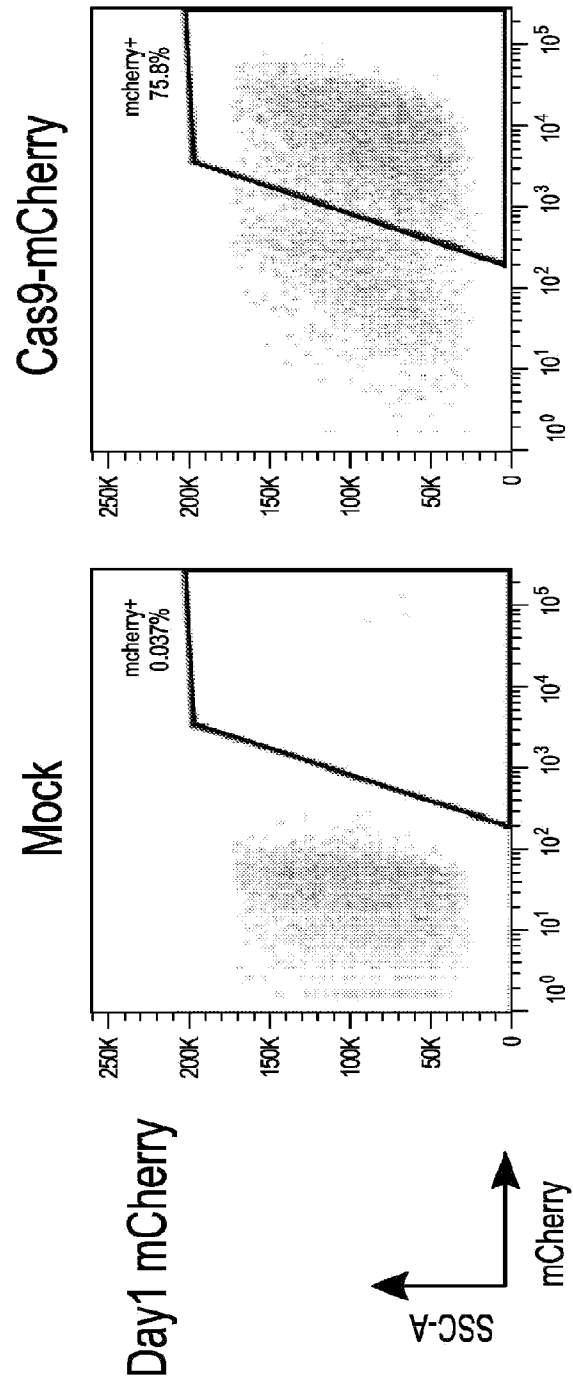
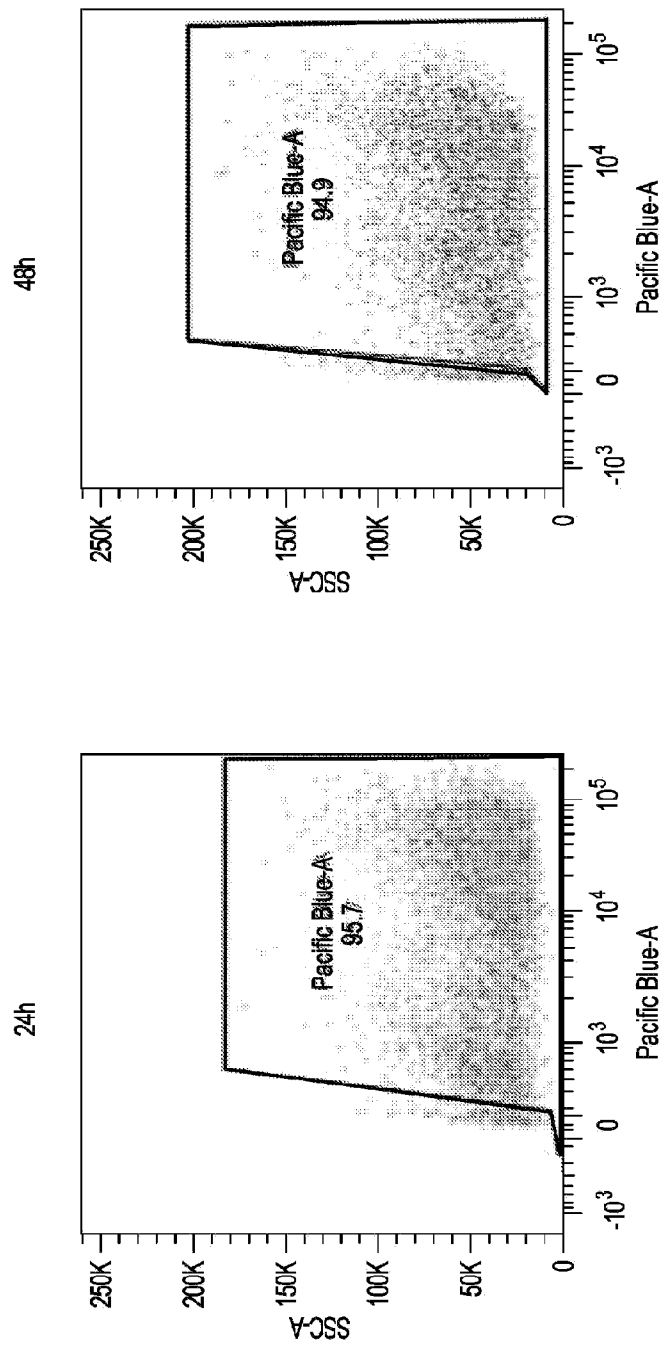


FIG. 18

pEVL300 BFP mRNA in HepG2 cells

*FIG. 19*

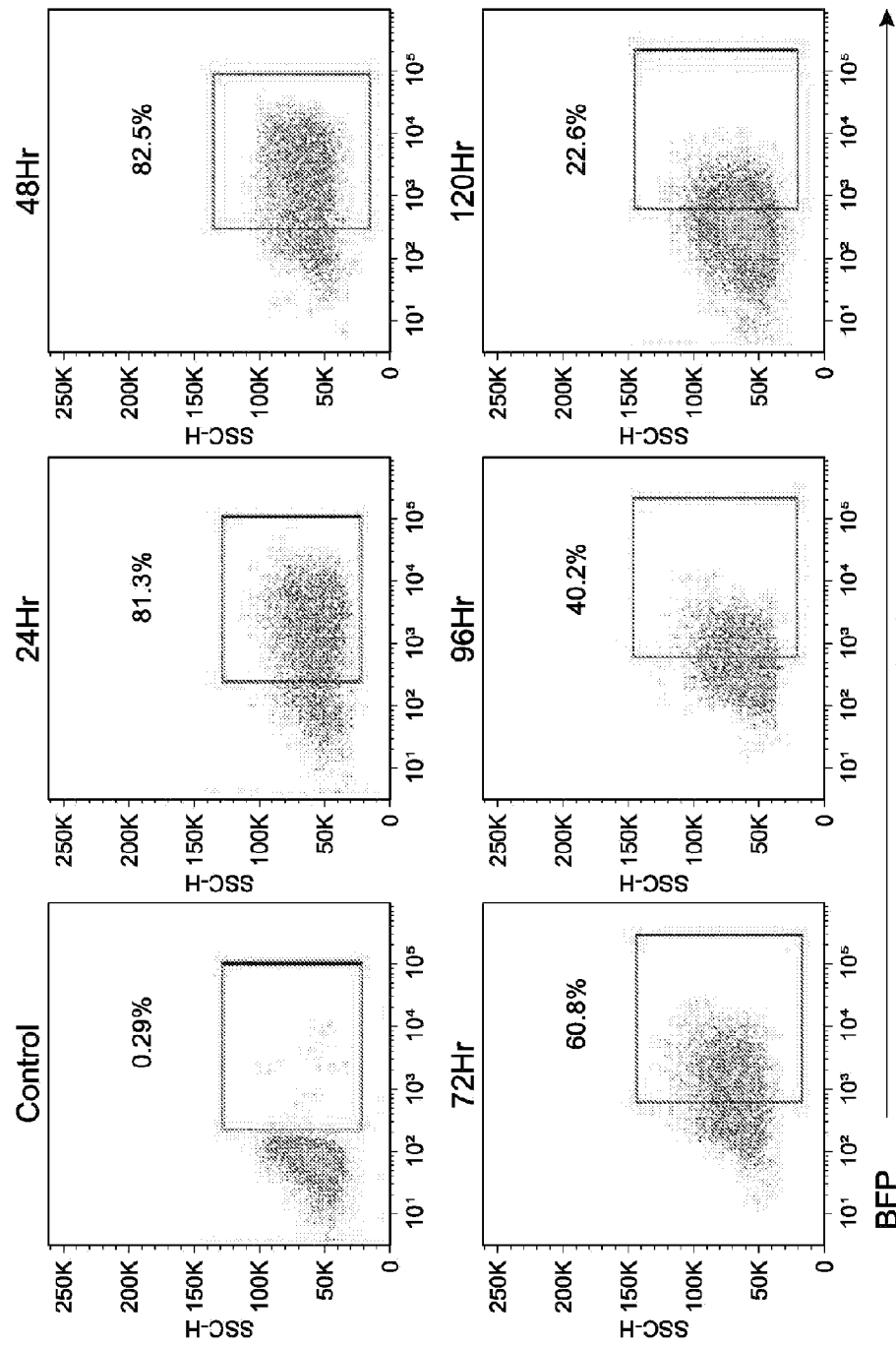


FIG. 20

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.

