

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2015364282 B2**

(54) Title
CRISPR-based compositions and methods of use

(51) International Patent Classification(s)
C12N 15/11 (2006.01)

(21) Application No: **2015364282** (22) Date of Filing: **2015.12.18**

(87) WIPO No: **WO16/100951**

(30) Priority Data

(31) Number	(32) Date	(33) Country
62/093,588	2014.12.18	US
62/239,546	2015.10.09	US

(43) Publication Date: **2016.06.23**

(44) Accepted Journal Date: **2021.04.15**

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(56) Related Art
M. JINEK ET AL, "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity", SCIENCE, US, (2012-08-17), vol. 337, no. 6096, doi:10.1126/science.1225829, ISSN 0036-8075, pages 816 - 821
US 20140273232 A1
WO 2014144592 A2
WO 2014065596 A1
WO 2014124226 A1

CRISPR-Based Compositions and Methods of Use

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority under 35 U.S.C. 119 to U.S. provisional patent applications bearing serial numbers 62/093,588 and 62/239,546, filed December 18, 2014 and October 9, 2015, and entitled “CRISPR-BASED COMPOSITIONS AND METHODS OF USE,” the contents of which are herein incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing that has been submitted in ASCII format and is hereby incorporated by reference in its entirety. The ASCII copy, created on March 03, 2021, is named IDT01-008-PCT-AU_ST25.txt, and is 280,066 bytes in size.

FIELD OF THE INVENTION

[0003] This invention pertains to modified compositions for use in CRISPR systems, and their methods of use.

BACKGROUND OF THE INVENTION

[0004] The use of clustered regularly interspaced short palindromic repeats (CRISPR) and associated Cas proteins (CRISPR-Cas system) for site-specific DNA cleavage has shown great potential for a number of biological applications. CRISPR is used for genome editing; the genome-scale-specific targeting of transcriptional repressors (CRISPRi) and activators (CRISPRa) to endogenous genes; and other applications of RNA-directed DNA targeting with Cas enzymes.

CRISPR-Cas systems are native to bacteria and Archaea to provide adaptive immunity against viruses and plasmids. There are three classes of CRISPR-Cas systems that could potentially be adapted for research and therapeutic reagents, but Type-II CRISPR systems have a desirable characteristic in utilizing a single CRISPR associated (Cas) nuclease (specifically Cas9) in a complex with the appropriate guide RNAs –

either a 2-part RNA system similar to the natural complex in bacteria comprising a CRISPR-activating RNA:trans-activating crRNA (crRNA:tracrRNA) pair or an artificial chimeric single-guide-RNA (sgRNA) – to mediate double-stranded cleavage of target DNA. In mammalian systems, these RNAs have been introduced by transfection of DNA cassettes containing RNA Pol III promoters (such as U6 or H1) driving RNA transcription, viral vectors, and single-stranded RNA following *in vitro* transcription (see Xu, T., et al., Appl Environ Microbiol, 2014. 80(5): p. 1544-52).

[0006] In the CRISPR-Cas9 system, using, for example, the system present in *Streptococcus pyogenes* as an example (*S.py.* or *Spy*), native crRNAs are about 42 bp long, containing a 5'-region of about 20 bases complementary to a target sequence (also referred to as a protospacer sequence) and a 3' region typically about 22 bases long that corresponds to a complementary region of the tracrRNA sequence. The native tracrRNAs are about 85-90 bases long, having a 5'-region containing the region complementary to the crRNA as well as about a 10-base region 5'-upstream. The remaining 3' region of the tracrRNA includes secondary structures (herein referred to as the "tracrRNA 3'-tail").

[0007] Jinek et al. extensively investigated the portions of the crRNA and tracrRNA that are required for proper functioning of the CRISPR-Cas9 system (Science, 2012. 337(6096): p. 816-21). They devised a truncated crRNA:tracrRNA fragment that could still function in CRISPR-Cas9 wherein the crRNA was the wild type 42 nucleotides and the tracrRNA was truncated to 75 nucleotides. They also developed an embodiment wherein the crRNA and tracrRNA are attached with a linker loop, forming a single guide RNA (sgRNA), which varies between 99-123 nucleotides in different embodiments. The configuration of the native 2-part crRNA:tracrRNA complex is shown in FIG. 1 and the 99 nucleotide embodiment of the artificial sgRNA single guide is shown in FIG. 2.

[0008] At least two groups have elucidated the crystal structure of *Streptococcus pyogenes* Cas9 (*SpyCas9*). In Jinek, M., et al., the structure did not show the nuclease in complex with either a guide RNA or target DNA. They carried out molecular modeling experiments to reveal predictive interactions between the protein in complex with RNA and DNA (Science, 2014. 343, p. 1215, DOI: 10.1126/science/1247997).

[0009] In Nishimasu, H., et al., the crystal structure of SpyCas9 is shown in complex with sgRNA and its target DNA at 2.5 angstrom resolution (Cell, 2014. 156(5): p. 935-49, incorporated herein in its entirety). The crystal structure identified two lobes to the Cas9 enzyme: a recognition lobe (REC) and a nuclease lobe (NUC). The sgRNA:target DNA heteroduplex (negatively charged) sits in the positively charged groove between the two lobes. The REC lobe, which shows no structural similarity with known proteins and therefore likely a Cas9-specific functional domain, interacts with the portions of the crRNA and tracrRNA that are complementary to each other.

[0010] Another group, Briner et al. (Mol Cell, 2014. 56(2): p. 333-9, incorporated herein in its entirety), identified and characterized the six conserved modules within native crRNA:tracrRNA duplexes and sgRNA.

[0011] The CRISPR-Cas9 system is utilized in genomic engineering as follows: a portion of the crRNA hybridizes to a target sequence, a portion of the tracrRNA hybridizes to a portion of the crRNA, and the Cas9 nuclease binds to the entire construct and directs cleavage. The Cas9 contains two domains homologous to endonucleases HNH and RuvC, wherein the HNH domain cleaves the DNA strand complementary to the crRNA and the RuvC-like domain cleaves the noncomplementary strand. This results in a double-stranded break in the genomic DNA. When repaired by non-homologous end joining (NHEJ) the break is typically shifted by 1 or more bases, leading to disruption of the natural DNA sequence and in many cases leading to a frameshift mutation if the event occurs in the coding exon of a protein-encoding gene. The break by also be repaired by homology dependent recombination (HDR), which permits insertion of new genetic material via experimental manipulation into the cut site created by Cas9 cleavage.

[0012] Some of the current methods for guide RNA delivery into mammalian cells include transfection of double-stranded DNA (dsDNA) containing RNA Pol III promoters for endogenous transcription, viral delivery, transfection of RNAs as *in vitro* transcription (IVT) products, or microinjection of IVT products. There are disadvantages to each of these methods. Unmodified exogenous RNA introduced into mammalian cells is known to initiate the innate immune response via recognition by Toll-like Receptors (TLRs), RIG-I, OAS1 and others receptors that recognize pathogen-associated molecular

patterns (PAMPs). However, in most published studies, RNA which has been *in vitro* transcribed (IVT) by a T7 RNA polymerase is delivered to the cells. This type of RNA payload has been shown to be a trigger for the innate immune response. The alternative delivery methods described above each have their own disadvantages as well. For example, dsDNA cassettes can lead to integration, guide RNA transcription driven endogenously by a RNA Pol II promoter can persist constitutively, and the amount of RNA transcribed is uncontrollable.

[0013] RNA is quickly degraded by nucleases present in serum and in cells. Unmodified CRISPR RNA triggers (crRNAs, tracrRNAs, and sgRNAs) made by IVT methods or chemical synthesis are quickly degraded during delivery or after delivery to mammalian cells. Greater activity would be realized if the RNA was chemically modified to gain nuclease resistance. The most potent degradative activity present in serum and in cells is a 3'-exonuclease (Eder et al., *Antisense Research and Development* 1:141-151, 1991). Thus "end blocking" a synthetic oligonucleotide often improves nuclease stability. Chemical modification of single-stranded antisense oligonucleotides (ASOs) and double-stranded small interfering RNAs (siRNAs) has been well studied and successful approaches are in practice today (for reviews, see: Kurreck, *Eur. J. Biochem.*, 270:1628-1644, 2003; Behlke, *Oligonucleotides*, 18:305-320, 2008; Lennox et al., *Gene Therapy*, 18:1111-1120, 2011). It is therefore desirable to devise chemical modification strategies for use with the RNA components of CRISPR/Cas. While the basic toolbox of chemical modifications available is well known to those with skill in the art, the effects that site-specific modification have on the interaction of a RNA species and an effector protein are not easily predicted and effective modification patterns usually must be empirically determined. In some cases, sequence of the RNA may influence the effectiveness of a modification pattern, requiring adjustment of the modification pattern employed for different sequence contexts, making practical application of such methods more challenging.

[0014] There is therefore a need to modify the guide RNA to reduce its toxicity to cells and to extend lifespan and functionality in mammalian cells while still performing their intended purpose in the CRISPR-Cas system. The methods and compositions of the invention described herein provide RNA and modified RNA oligonucleotides for use in a CRISPR-Cas system. These and other advantages of the invention, as well as additional

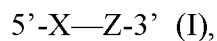
inventive features, will be apparent from the description of the invention provided herein.

BRIEF SUMMARY OF THE INVENTION

[0015] This invention pertains to modified compositions for use in CRISPR systems, and their methods of use. The compositions include modified internucleotide linkages and 2'-O-alkyl and 2'-O-fluoro modified RNA oligonucleotides to serve as the guides strands (crRNA:tracrRNA or sgRNA) for the CRISPR-Cas system. Compositions also include end-modifications such as an inverted-dT base or other non-nucleotide modifiers that impeded exonuclease attack (such as the propanediol group (C3 spacer), naphthyl-azo modifier, or others as are well known in the art).

[0016] In a first aspect, isolated tracrRNA including a length-modified form of SEQ ID NO.:18 is provided. The isolated tracrRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.

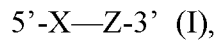
[0017] In a second aspect, an isolated crRNA including a length-modified form of formula (I) is provided:



wherein X represents sequences comprising a target-specific protospacer domain comprising about 20 target-specific nucleotides, and Z represents sequences comprising a universal tracrRNA-binding domain comprising about 20 nucleotides. The isolated crRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.

[0018] In a third aspect, an isolated tracrRNA including a chemically-modified form of one of SEQ ID NOs.:2, 18, 30-33 and 36-39 is provided. The isolated tracrRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.

[0019] In a fourth aspect, isolated crRNA including a chemically-modified form of formula (I) is provided:



wherein X represents sequences comprising a target-specific protospacer domain comprising from about 17 nucleotides to about 20 nucleotides, and Z represents sequences comprising a universal tracrRNA-binding domain comprising about 12 nucleotides to about 19 nucleotides. The isolated crRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIG. 1 is an illustration of a wild-type (WT) natural 2-part crRNA:tracrRNA complex with a 42 base unmodified crRNA (SEQ ID No. 46) and an 89 base unmodified tracrRNA (SEQ ID No. 18). Lowercase letters represent RNA.

[0021] FIG. 2 is an illustration of a 99 base artificial single-guide RNA (SEQ ID NO: 428) (sgRNA) that fuses the crRNA and tracrRNA elements into a single sequence through the addition of a new hairpin loop. Lowercase letters represent RNA.

[0022] FIG. 3 shows an alignment of the full-length and truncated tracrRNA species studied in Example 2. Sequences are RNA and are shown 5'-3'. Alignment is based upon the 89 base WT tracrRNA sequence at the top (SEQ ID No. 18). Internal gaps represent sites of internal truncation/deletion. Uppercase letters represent RNA.

[0023] FIG. 4 shows an alignment of the full-length and truncated crRNA and tracrRNA species studied in Example 3. Alignment is based upon the 42 base WT crRNA (SEQ ID No. 46) and 89 base WT tracrRNA (SEQ ID No. 18) sequences at the top of their respective groupings. The 20 base 5'-domain in the crRNAs is sequence-specific and targets human *HPRT1*. The 3'-domain in underlined and binds to a region towards the 5'-end of the tracrRNA. The 5'-domain in the tracrRNA is underlined that binds the 3'-end of the crRNA. Uppercase letters represent RNA.

[0024] FIG. 5 is an illustration of a truncated 2-part crRNA:tracrRNA complex with a 36 base crRNA (SEQ ID No. 48) and a 67 base tracrRNA (SEQ ID No. 2). Lowercase letters represent RNA.

[0025] FIG. 6 is a schematic showing structure of one embodiment of an optimized truncated and chemically-modified tracrRNA (SEQ ID No. 134). Length is 67 bases. RNA is lower case and 2'OMe RNA is uppercase. Phosphorothioate (PS) internucleotide linkages are indicated by “*”. Residues which lead to substantial loss of function when converted from RNA to 2'OMe RNA are identified by large arrows and residues which lead to a moderate loss of function when converted from RNA to 2'OMe RNA are identified by small arrows.

[0026] FIG. 7 is a schematic showing structure of one embodiment of an optimized truncated and chemically-modified crRNA (SEQ ID No. 239). Length is 36 bases. RNA is lower case and 2'OMe RNA is uppercase. Phosphorothioate (PS) internucleotide linkages are indicated by “*”. Residues which lead to substantial loss of function when converted from RNA to 2'OMe RNA are identified by large arrows and residues which lead to a moderate loss of function when converted from RNA to 2'OMe RNA are identified by small arrows. The 5'-end 20 base protospacer target-specific guide domain is indicated, which in this case is sequence specific to the human *HPRT1* gene. The 3'-end 16 base tracrRNA binding domain is indicated.

[0027] FIG. 8 is a schematic showing structure of one embodiment of the optimized truncated/modified crRNA:tracrRNA complex as employed in Example 8. The crRNA is positioned at the top with the 5'-protospacer domain 20 base underlined, which in this case is specific for target human *HPRT1* site 38285; the 3'-end is the 16 base tracrRNA binding domain. The tracrRNA is aligned below. RNA is lower case, 2'OMe RNA is uppercase, and “*” indicates a phosphorothioate internucleotide linkage modification. This figure shows the complex formed by crRNA SEQ ID No. 178 and tracrRNA SEQ ID No. 100.

[0028] FIG. 9 is a schematic showing structure of one embodiment of the optimized truncated/modified crRNA:tracrRNA complex that is highly modified. The crRNA is positioned at the top with the 5'-protospacer domain 20 base underlined, which in this case is specific for target human *HPRT1* site 38285; the 3'-end is the 16 base tracrRNA binding domain. The tracrRNA is aligned below. RNA is lower case, 2'OMe RNA is uppercase, and “*” indicates a phosphorothioate internucleotide linkage modification.

This figure shows the complex formed by crRNA SEQ ID No. 446 and tracrRNA SEQ ID No. 134.

[0029] FIG. 10 is a schematic showing the crRNA modification patterns employed in Example 10. Oligonucleotide sequences (SEQ ID NOS 429-439, respectively, in order of appearance) are shown 5'-3'. Lowercase = RNA; Underlined = 2'-O-methyl RNA; C3 = C3 spacer (propanediol modifier); * = phosphorothioate internucleotide linkage; ZEN = naphthyl-azo modifier. The 5'-target specific protospacer domain is indicated. Bases are indicated by "N" in this domain as sequence is different for each target site, although the modification pattern employed remains constant. The 3'-universal tracrRNA binding domain is indicated. Modification patterns are numbered for reference between Table 10 and FIG. 10.

[0030] FIG. 11 is a plot of the data in Table 10 showing the functional gene editing observed using the T7E1 assay in mammalian cells using crRNAs made with 11 different modification patterns tested at 12 different sites in the human *HPRT1* gene. All crRNA variants were paired with an optimized, modified tracrRNA (SEQ ID No. 100).

[0031] FIG. 12 is a schematic showing structure of one embodiment of the optimized truncated/modified crRNA:tracrRNA complex that is highly modified using crRNA Mod Pattern 6 that is universal and can be applied in any sequence context. The crRNA (SEQ ID NO: 440) is positioned at the top with the 5'-protospacer domain 20 base underlined (N-bases); the 3'-end is the 16 base tracrRNA binding domain. The tracrRNA is aligned below (SEQ ID No. 134). RNA is lower case, 2'OMe RNA is uppercase, and "*" indicates a phosphorothioate internucleotide linkage modification.

[0032] FIG. 13 shows a plot of RT-qPCR data from HEK-Cas9 cells transfected with different CRISPR gRNAs showing relative expression levels of IFIT1 and IFITM1, 2 genes involved in interferon signaling pathways.

DETAILED DESCRIPTION OF THE INVENTION

[0033] Aspects of this invention relate to modified compositions for use in CRISPR systems, and their methods of use.

[0034] The term “oligonucleotide,” as used herein, refer to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base (a single nucleotide is also referred to as a “base” or “residue”). There is no intended distinction in length between the terms “nucleic acid”, “oligonucleotide” and “polynucleotide”, and these terms can be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. For use in the present invention, an oligonucleotide also can comprise nucleotide analogs in which the base, sugar or phosphate backbone is modified as well as non-purine or non-pyrimidine nucleotide analogs. An oligonucleotide may comprise ribonucleotides, deoxyribonucleotides, modified nucleotides (e.g., nucleotides with 2' modifications, synthetic base analogs, etc.) or combinations thereof.

[0035] Compositions of the present invention include any modification that potentially reduces activation of the innate immune system. Modifications can be placed or substituted at a conventional phosphodiester linkage, at the ribose sugar, or at the nucleobase of RNA. Such compositions could include, for example, a modified nucleotide such as 2'-O-methyl-modified RNAs.

[0036] More broadly, the term “modified nucleotide” refers to a nucleotide that has one or more modifications to the nucleoside, the nucleobase, pentose ring, or phosphate group. For example, modified nucleotides exclude ribonucleotides containing adenosine monophosphate, guanosine monophosphate, uridine monophosphate, and cytidine monophosphate and deoxyribonucleotides containing deoxyadenosine monophosphate, deoxyguanosine monophosphate, deoxythymidine monophosphate, and deoxycytidine monophosphate. Modifications include those naturally occurring that result from modification by enzymes that modify nucleotides, such as methyltransferases. Modified nucleotides also include synthetic or non-naturally occurring nucleotides. Modifications also include base analogs and universal bases. Synthetic or non-naturally occurring modifications in nucleotides include those with 2' modifications, e.g., 2'-O-alkyl (including 2'-O-methyl), 2'-fluoro, 2'-methoxyethoxy, 2'-allyl, 2'-O-[2-(methylamino)-2-oxoethyl], 4'-thio, bicyclic nucleic acids, 4'-CH₂—O-2'-bridge, 4'-(CH₂)₂—O-2'-bridge, 2'-LNA, and 2'-O-(N-methylcarbamate) or those comprising base analogs. Such

modified groups are described, e.g., in Eckstein et al., U.S. Pat. No. 5,672,695 and Matulic-Adamic et al., U.S. Pat. No. 6,248,878.

[0037] The use of 2'-O-methyl has been documented in siRNA literature (See Behlke, M.A., *Oligonucleotides*, 2008. **18**(4): p. 305-19) as well as in mRNA delivery (see Sahin, U. et al., *Nat Rev Drug Discov*, 2014. **13**(10): p. 759-80). Sahin et al., describes modifications of mRNA therapeutics that extend beyond 2'-OMe modification and "non-immunogenic" mRNA.

[0038] The term "ribonucleotide" encompasses natural and synthetic, unmodified and modified ribonucleotides. Modifications include changes to the sugar moiety, to the base moiety and/or to the linkages between ribonucleotides in the oligonucleotide.

[0039] The term "Cas9 protein" encompasses wild-type and mutant forms of Cas9 having biochemical and biological activity when combined with a suitable guide RNA (for example sgRNA or dual crRNA:tracrRNA compositions) to form an active CRISPR-Cas endonuclease system. This includes orthologs and Cas9 variants having different amino acid sequences from the *Streptococcus pyogenese* Cas9 employed as example in the present invention.

[0040] The term "length-modified," as that term modifies RNA, refers to a shortened or truncated form of a reference RNA lacking nucleotide sequences or an elongated form of a reference RNA including additional nucleotide sequences.

[0041] The term "chemically-modified," as that term modifies RNA, refers to a form of a reference RNA containing a chemically-modified nucleotide or a non-nucleotide chemical group covalently linked to the RNA. Chemically-modified RNA, as described herein, generally refers to synthetic RNA prepared using oligonucleotide synthesis procedures wherein modified nucleotides are incorporated during synthesis of an RNA oligonucleotide. However, chemically-modified RNA also includes synthetic RNA oligonucleotides modified with suitable modifying agents post-synthesis.

[0042] Applicants have discovered novel crRNA and tracrRNA oligonucleotide compositions that display robust activity in the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system. The oligonucleotide compositions include length-modified forms of crRNA and

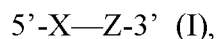
tracrRNA, as well as chemically-modified forms of crRNA and tracrRNA. The length-modified forms of crRNA and tracrRNA enable one to prepare active forms of these RNAs with cost-effective and efficient oligonucleotide synthesis protocols routinely available. The chemically-modified forms of crRNA and tracrRNA provide one with active agents tunable with certain specific properties, such as improved stability in cellular and *in vivo* contexts. The length-modified forms of crRNA and tracrRNA can also include modifications, thereby enabling access to a broad range of compositions having activity in CRISPR-Cas endonuclease system contexts. These oligonucleotide compositions and their properties in the CRISPR-Cas endonuclease system are described below.

[0043] Length-modified forms of crRNA and tracrRNA

[0044] FIG.1 depicts a representation of the wild-type *S. pyogenes* crRNA:tracrRNA complex, wherein an exemplary isolated crRNA (SEQ ID No. 46) is paired with an isolated tracrRNA (SEQ ID No. 18). In a first aspect, an isolated tracrRNA including a length-modified form of SEQ ID NO.:18 is provided. The isolated tracrRNA displays activity in the CRISPR-Cas endonuclease system. In one respect, the isolated tracrRNA includes a length-modified form of SEQ ID NO.:18 nucleotide having deleted sequence information. In some embodiments, the length-modified form of SEQ ID NO.:18 includes shortened or truncated forms of SEQ ID NO.:18, wherein SEQ ID NO.:18 can be shortened by 1 to 20 nucleotides at the 5'-end and by 1-10 nucleotides at the 3'-end. Such shortened or truncated forms of SEQ ID NO.:18 retain activity when paired with a functionally competent crRNA in the CRISPR-Cas endonuclease system. Where shortening of the 5'-end of the tracrRNA is performed and extends into sequence that pairs with the 3'-end of the crRNA, improved activity may be obtained using chemical modification that enhance binding affinity in these domains. Where shortening of the 3'-end of the crRNA is performed and extends into sequence that pairs with the 5'-end of the tracrRNA, improved activity may be obtained using chemical modification that enhance binding affinity in these domains. Preferred examples of a length-modified form of SEQ ID NO.:18 having a shortened or truncated form include SEQ ID NOs:2, 30-33 and 36-39. A highly preferred example of a length-modified form of SEQ ID NO.:18 having a shortened or truncated form includes SEQ ID NO:2. For each of the foregoing exemplary length-modified forms of SEQ ID NO.:18 having a shortened or truncated

form, SEQ ID NOs.:2, 30-33 and 36-69 can consist of chemically non-modified nucleotides.

[0045] In a second aspect, an isolated crRNA comprising a length-modified form of formula (I) is provided:



wherein X represents sequences including a target-specific protospacer domain, and Z represents sequences including a tracrRNA-binding domain.

[0046] The target-specific protospacer domain (X domain of formula (I)) typically includes about twenty nucleotides having complementarity to a region of DNA targeted by the CRISPR-Cas endonuclease system. The tracrRNA-binding domain (the Z domain of formula (I)) typically includes about 20 nucleotides in most CRISPR endonuclease systems (in the native *S.py.* version, this domain is 22 nucleotides). The isolated crRNA displays activity in the CRISPR-Cas endonuclease system.

[0047] In one respect, the isolated crRNA includes a length-modified form of formula (I) having deleted sequence information. In some embodiments, the length-modified form of formula (I) includes shortened or truncated forms of formula (I), wherein formula (I) can be shortened by 1-8 nucleotides at the 3'-end of the Z domain. The length-modified form of formula (I) can be shortened at the 5-end of the X-domain to accommodate a target-specific protospacer domain having 17, 18, 19 or 20 nucleotides. Highly preferred examples of such length-modified form of formula (I) include target-specific protospacer domain having 19 or 20 nucleotides. The exemplary length-modified forms of formula (I) having a shortened or truncated form with a target-specific protospacer (X-domain) of 17-20 nucleotides in length and/or lacking 1-8 nucleotides at the 3'-end of the Z-domain can consist of chemically non-modified nucleotides.

[0048] Such shortened or truncated forms of formula (I) retain activity when paired with a competent tracrRNA in the CRISPR-Cas endonuclease system. Preferred embodiments of isolated crRNA of formula (I) having a length-modified form of formula (I) can include chemically non-modified nucleotides and chemically modified nucleotides.

[0049] Chemically-modified forms of crRNA and tracrRNA

[0050] In a third aspect, an isolated tracrRNA including a chemically-modified nucleotide or a non-nucleotide chemical modifier is provided. The isolated tracrRNA displays activity in the CRISPR-Cas endonuclease system. In one respect, the isolated tracrRNA includes a chemically-modified nucleotide having a modification selected from a group consisting of a ribose modification, an end-modifying group, and internucleotide modifying linkages. Exemplary ribose modifications include 2'O-alkyl (e.g., 2'OMe), 2'F, bicyclic nucleic acid, and locked nucleic acid (LNA). Exemplary end-modifying groups include a propanediol (C3) spacer and naphthyl-azo modifier (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, or "ZEN"), and an inverted-dT residue. Exemplary internucleotide modifying linkages include phosphorothioate modification. In one respect, the isolated tracrRNA having a chemically-modified form include SEQ ID NO.:46 and length-modified forms thereof, such as shortened or truncated forms of SEQ ID NO.:46. Preferred shortened or truncated forms of SEQ ID NO.:46 having a chemically-modified nucleotide include SEQ ID NOs:2, 30-33 and 36-39 having a chemically-modified nucleotide. Yet other examples of isolated tracrRNA having a chemically-modified nucleotide with robust activity in the CRISPR-Cas endonuclease system are presented in the Examples.

[0051] In a fourth aspect, an isolated crRNA including a chemically-modified nucleotide is provided. The isolated crRNA displays activity in the CRISPR-Cas endonuclease system. In one respect, the isolated crRNA includes a chemically-modified nucleotide having a modification selected from a group consisting of a ribose modification, an end-modifying group, and internucleotide modifying linkage. Exemplary ribose modifications include 2'O-alkyl (e.g., 2'OMe), 2'F, bicyclic nucleic acid, and locked nucleic acid (LNA). Exemplary end-modifying groups include a propanediol (C3) spacer and naphthyl-azo modifier (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, or "ZEN"), and an inverted-dT residue. Exemplary internucleotide modifying linkages include phosphorothioate modification. In one respect, the isolated crRNA having a chemically-modified form include crRNA of formula (I) and length-modified forms thereof. Preferred shortened or truncated forms of crRNA of formula (I) having a chemically-modified nucleotide include SEQ ID NOs.:429-439. Highly preferred examples of an isolated crRNA having a chemically-modified nucleotide

include SEQ ID NOs.:434 and 435. These particular isolated crRNA species represent “universal” crRNAs having a chemically-modified nucleotide showing high activity when combined with a competent tracrRNA in the CRISPR-Cas endonuclease system. Yet other examples of isolated crRNA having a chemically-modified nucleotide with robust activity in the CRISPR-Cas endonuclease system are presented in the Examples.

[0052] The foregoing isolated, length-modified and chemically-modified of crRNA and tracrRNA preferably include chemical modifications at the 2'-OH groups (for example, 2'OMe, 2'F, bicyclic nucleic acid, locked nucleic acid, among others) and end-blocking modifications (for example, ZEN, C3 spacer, inverted-dT). Use of both types of general modifications provides isolated, length-modified and chemically-modified of crRNA and tracrRNA with biochemical stability and immunologic tolerance for isolated, length-modified and chemically-modified of crRNA and tracrRNA in biological contexts.

[0053] The foregoing isolated, length-modified and chemically-modified of crRNA and tracrRNA can be mixed in different combinations to form active crRNA:tracrRNA as the guide RNA for Cas9. For example, an isolated, length-modified tracrRNA can be combined with an isolated chemically-modified crRNA to form an active crRNA:tracrRNA as the guide RNA for Cas9. The Examples provide illustrations of different combinations of isolated, length-modified and chemically-modified of crRNA and tracrRNA resulting in active crRNA:tracrRNA as the guide RNA for Cas9.

[0054] The extent to which one needs particular chemically-modified nucleotides included in one (or both) of the isolated, length-modified and chemically-modified crRNA and tracrRNA depends upon the application for which the resultant active crRNA:tracrRNA serves as the guide RNA for Cas9. In certain biochemical assays of the CRISPR-Cas endonuclease system, particularly where nucleases can be minimized or absent, one may not need extensively chemically-modified crRNA and tracrRNA to effect robust activity of the resultant guide RNA for Cas9 of the CRISPR-Cas endonuclease system. This is attributed to the fact that chemically-modified nucleotides that confer resistance to nucleases are not necessary when nucleases are minimal or absent. In certain biological (*in vivo*) contexts, wherein a mixture including crRNA and tracrRNA is delivered to cells inside carrier vehicles, such as liposome nanoparticles, the

isolated length-modified and chemically-modified crRNA and tracrRNA may require less extensive chemically-modified nucleotides than mixtures of crRNA and tracrRNA delivered directly into the blood stream or injected into organ systems as isolated, “naked,” RNA mixtures. The extent of chemical modification present in chemically-modified crRNA and tracrRNA can dictate the half-life of the relevant RNA molecules *in vivo* (that is, in the relevant biological context, such as, for example, in the blood stream or inside cells). Accordingly, the modification profile of chemically-modified crRNA and tracrRNA can be used to fine tune the biochemical and biological activity of the resultant crRNA:tracrRNA duplexes as a guide RNA for Cas9 in the CRISPR-Cas endonuclease system.

[0055] Although the prior art focuses on the structure of Cas9 as it interacts with a sgRNA, the disclosed design patterns described herein contemplate the aforementioned crRNA:tracrRNA dual RNA systems. A single strand guide RNA offers several benefits, such as simplicity of a therapeutic design. However, standard solid phase phosphoramidite RNA synthesis shows diminishing yields for oligonucleotides as length increases and this problem becomes more apparent as length exceeds 60-70 bases. This precludes robust, cost-effective synthesis of some tracrRNAs as well as the chimeric sgRNA, especially at larger scales needed for some commercial or therapeutic applications. For this reason, the invention contemplates embodiments of not only sgRNA, but also alternate dual crRNA:tracrRNA as the guide RNA for Cas9. However, an isolated guide RNA having robust activity when combined with Cas9 in the CRISPR-Cas endonuclease system can be engineered by linkage or synthesis of appropriate crRNA and tracrRNA as an artificial, unimolecular sgRNA based upon the isolated, length-modified and chemically-modified forms of crRNA and tracrRNA provided herein. Long single guides of this type may be obtained by direct synthesis or by post-synthetic chemical conjugation of shorter strands.

[0056] The design of length-modified and chemically-modified tracrRNA compositions addresses the potential synthetic issues associated with tracrRNA oligonucleotides that are >80 nucleotides in length. The coupling efficiency of 2'-OMe-modified RNA monomers (effectively containing a protecting group on the 2'-OH) is greater than RNA monomer coupling. Incorporating 2'-OMe modified RNAs provides some advantages. First, it allows for longer oligonucleotides to be synthesized as either

full 2'-OMe or RNA/2'-OMe mixed oligonucleotides. Secondly, the methods and compositions of the invention lead to synthesis and transfection of crRNA:tracrRNA that can evade detection by the immune system. It is well known that exogenous, unmodified RNAs trigger an innate immune response in mammalian cells as well as whole animals. Using 2'-OMe-modified oligonucleotides can confer RNA stability to nucleases (a third advantage) as well as reduce cell death and toxicity associated with immunogenic triggers. These advantages are not unique to 2'-OMe modification, per se, as the other disclosed modified nucleotides having different chemical moieties (for example, 2'-F, other 2'-O-alkyls, LNA, and other bicyclic nucleotides) can offer similar benefits and advantages in terms of conferring resistance to nucleases.

[0057] In another embodiment, the tracrRNA portion complementary to the crRNA contains at least one modified nucleotide, and in a further embodiment the tracrRNA portion complementary to the crRNA is comprised of more than 10% modified residues, and in a further embodiment the tracrRNA portion not complementary to the crRNA is comprised of more than 50% modified residues, and a further embodiment the tracrRNA portion not complementary to the crRNA is comprised of more than 90% modified residues.

[0058] In another embodiment, the crRNA portion is unmodified and the tracrRNA portion is comprised of at least one modified nucleotide. In a further embodiment the crRNA portion is unmodified and the tracrRNA portion is comprised of more than 10% modified bases.

[0059] In another embodiment, an isolated crRNA of formula (I) is designed with modifications that are empirically determined. As depicted in FIGs. 7 and 10, the 12 nucleotides at the 3'-end of the Z domain (the tracrRNA-binding domain) and the 10-12 nucleotides at the 5'-end of the X domain (within the protospacer domain) represent universal nucleotides amenable to substitution with chemically-modified nucleotides, wherein the resultant RNAs retain robust activity in the CRISPR-Cas endonuclease system. Yet other nucleotides within the 5'-end of the Z domain (the tracrRNA-binding domain) are intolerant to substitution with chemically-modified nucleotides (FIG. 7). Yet the ability of other sites within an isolated crRNA of formula (I) to accept chemically-modified nucleotides and retain activity in the CRISPR-Cas endonuclease system is

largely determined empirically. The tracrRNA binding domain (Z domain) of the crRNA is constant (i.e., sequence does not change as target site varies), so the modifications patterns described herein are universal to all crRNAs regardless of target site and can be broadly applied. The protospacer (X domain) of the crRNA varies with target, and the tolerance of some of the base positions within this domain to chemical modification vary with sequence context and, if maximal chemical modification of a site is desired, may benefit from empiric optimization. However, some of the residues within the target-specific protospacer (X) domain can be modified without consideration to sequence context. The 10-12 residues at the 5'-end of this domain can be substituted with 2'-modified residues with the expectation that full activity of the modified crRNA will be maintained. The remaining 8-10 bases towards the 3'-end of the protospacer (X) domain may tolerate modification or may not, depending on sequence context. One sequence context where 17 out of the 20 bases of the protospacer (X) domain can be modified while maintaining full activity are shown in FIG. 7. Sites where modification compromised activity are indicated.

[0060] The applications of Cas9-based tools are many and varied. They include, but are not limited to: plant gene editing, yeast gene editing, rapid generation of knockout/knockin animal lines, generating an animal model of disease state, correcting a disease state, inserting a reporter gene, and whole genome functional screening.

[0061] The utility of the present invention is further expanded by including mutant versions of Cas enzymes, such as a D10A and H840a double mutant of Cas9 as a fusion protein with transcriptional activators (CRISPRa) and repressors (CRISPRi) (see Xu, T., et al., Appl Environ Microbiol, 2014, **80**(5): p. 1544-52). The Cas9-sgRNA complex also can be used to target single-stranded mRNA as well (see O'Connell, M.R., et al., Nature, 516:263, 2014). In the same way as targeting dsDNA, crRNA:tracrRNA can be used with a PAMmer DNA oligonucleotide to direct Cas9 cleavage to the target mRNA or use it in the mRNA capture assay described by O'Connell.

[0062] By utilizing an approach to deliver synthetic RNA oligonucleotides for CRISPR/Cas9 applications, it is possible to 1) use mass spectroscopy to confirm discrete RNA sequences, 2) selectively insert 2'-OMe modified RNAs in well-tolerated locations to confer stability and avoid immunogenicity yet retain functional efficacy, 3)

specifically control the amount of RNA that is introduced into cells for a controlled transient effect, and 4) eliminate concern over introducing dsDNA that would be endogenously transcribed to RNA but could also become substrate in either homology-directed repair pathway or in non-homologous end joining resulting in an integration event. These integration events can lead to long term undesired expression of crRNA or tracrRNA elements. Further, integration can disrupt other genes in a random and unpredictable fashion, changing the genetic material of the cell in undesired and potentially deleterious ways. The present invention is therefore desirable as a means to introduce transient expression of elements of the CRISPR pathway in cells in a way which is transient and leaves no lasting evidence or change in the genome outside of whatever alteration is intended as directed by the crRNA guide.

[0063] CRISPR-Cas endonuclease systems

[0064] A competent CRISPR-Cas endonuclease system includes a ribonucleoprotein (RNP) complex formed with isolated Cas9 protein and isolated guide RNA selected from one of a dual crRNA:tracrRNA combination and a chimeric sgRNA. In some embodiments, isolated length-modified and/or chemically-modified forms of crRNA and tracrRNA are combined with purified Cas9 protein (for example, SEQ ID NOs.:407-410), an isolated mRNA encoding Cas9 protein (for example, SEQ ID NO.:413), or a gene encoding Cas9 protein (for example, SEQ ID NOs.: 411 and 412) in an expression vector. In certain assays, isolated length-modified and/or chemically-modified forms of crRNA and tracrRNA can be introduced into cell lines that stably express Cas9 protein from an endogenous expression cassette encoding the Cas9 gene. In other assays, a mixture of length-modified and/or chemically-modified forms of crRNA and tracrRNA in combination with either Cas9 mRNA or Cas9 protein can be introduced into cells.

EXAMPLE 1

[0065] This example illustrates functioning of chemically modified and truncated guide RNAs in an *in vitro* Cas9 DNA cleavage assay.

[0066] CrRNA and tracrRNA oligonucleotides were synthesized having various chemical modifications and truncations relative to the WT sequences as indicated (Table 1).

Table 1: In vitro biochemical studies of cleavage of HPRT1 target DNA by Cas9 endonuclease with various crRNA and tracrRNA pairs.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage
		tracrRNA Sequence		
1A	1	uuauauccaacacacuucgugguuuuagagcuaugcu	35	+++
	2	agcauagcaaguuaaaauaaggcuaguccguaucaacuuga aaaaguggcaccgagucggugcuuu	67	
1B	1	uuauauccaacacacuucgugguuuuagagcuaugcu	35	-
	3	<u>guuggaacc<u>auu</u>caaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> aaaaaguggcaccgagucggugcuu uuuuu	89	
1C	1	uuauauccaacacacuucgugguuuuagagcuaugcu	35	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
2A	5	uuauauccaacacacuucgugguuuuagagcuaugcuguuuug	41	+++
	2	agcauagcaaguuaaaauaaggcuaguccguaucaacuuga aaaaguggcaccgagucggugcuuu	67	
2B	5	uuauauccaacacacuucgugguuuuagagcuaugcuguuuug	41	-
	6	<u>guuggaacc<u>auu</u>caaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> aaaaaguggcaccgagucggugcuu uuuuu	89	
2C	5	uuauauccaacacacuucgugguuuuagagcuaugcuguuuug	41	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
3A	7	<u>uuauauccaacacacuucgugguuuuagagcuaugcuguuuug</u>	41	-
	2	agcauagcaaguuaaaauaaggcuaguccguaucaacuuga aaaaguggcaccgagucggugcuuu	67	
3B	7	<u>uuauauccaacacacuucgugguuuuagagcuaugcuguuuug</u>	41	-
	6	<u>guuggaacc<u>auu</u>caaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> aaaaaguggcaccgagucggugcuu uuuuu	89	
3C	7	<u>uuauauccaacacacuucgugguuuuagagcuaugcuguuuug</u>	41	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
4A	8	uuauauccaacacacuucgugguuuuagagcuaugcuguuuug	41	-

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage
		tracrRNA Sequence		
	2	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
4B	8	<u>uuauauccaacacuucgugguuuuagagcuaugcuguuuug</u>	41	-
	6	<u>guuggaaccbauucaaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
4C	8	<u>uuauauccaacacuucgugguuuuagagcuaugcuguuuug</u>	41	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
5A	9	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	2	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
5B	9	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	6	<u>guuggaaccbauucaaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
5C	9	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
6A	10	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	2	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
6B	10	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	6	<u>guuggaaccbauucaaaaacagcauagcaaguuaaaauaaggcu</u> <u>aguccguaucaacuuga</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
6C	10	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	-
	4	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
1G	1	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	+++
	11	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
1K	1	<u>uuauauccaacacuucgugguuuuagagcuaugcu</u>	35	++
	12	<u>agcauagcaaguuaaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	

cr/tracr RNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage
		tracrRNA Sequence		
1L	1	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	13	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
14A	14	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	2	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
14G	14	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	11	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
14K	14	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	12	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
14L	14	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	13	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
15A	15	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	2	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
15G	15	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	11	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
15K	15	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	12	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
15L	15	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	13	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
16A	16	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++
	2	agcauagcaaguu <u>aaaauaaggcuaguccguaucaacuuga</u> aaaaguggcaccgagucggugcuuu	67	
16G	16	uuauauccaacac <u>uucgugguuuuagagcu</u> augcu	35	+++

cr/tracr RNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage
		tracrRNA Sequence		
	11	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
16K	16	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	+++
	12	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
16L	16	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	+++
	13	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
1H	1	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	+++
	17	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
2D	5	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcuuuug</u>	41	+++
	18	<u>guuggaacc</u> <u>auu</u> <u>caaaacagcauagcaaguu</u> <u>aaaauaaggcu</u> <u>aguccguaucaacuug</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
2E	5	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcuuuug</u>	41	+++
	19	<u>guuggaacc</u> <u>auu</u> <u>caaaacagcauagcaaguu</u> <u>aaaauaaggcu</u> <u>aguccguaucaacuug</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
2F	5	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcuuuug</u>	41	+++
	20	<u>guuggaacc</u> <u>auu</u> <u>caaaacagcauagcaaguu</u> <u>aaaauaaggcu</u> <u>aguccguaucaacuug</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
2I	5	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcuuuug</u>	41	++
	21	<u>guuggaacc</u> <u>auu</u> <u>caaaacagcauagcaaguu</u> <u>aaaauaaggcu</u> <u>aguccguaucaacuug</u> <u>aaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
7A	22	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	-
	2	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
9A	23	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	+++
	2	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	
10A	24	<u>uuauauccaacacuucgugguuuuagagcuau</u> <u>gcu</u>	35	+++
	2	<u>agcauagcaaguu</u> <u>aaaauaaggcuaguccguaucaacuuga</u> <u>aaaaguggcaccgagucggugcuuu</u>	67	

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage
		tracrRNA Sequence		
3D	7	<u>uuauauccaacac<u>uucgugguuuu</u>agagcuaugcuguuuug</u>	41	-
	18	<u>guuggaaccau<u>ucaaaa</u>cagcauagcaagu<u>uaaa</u>uaaggcu</u> <u>aguccguuaucaacuugaaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
4D	8	<u>uuauauccaacac<u>uucgugguuuu</u>agagcuaugcuguuuug</u>	41	-
	18	<u>guuggaaccau<u>ucaaaa</u>cagcauagcaagu<u>uaaa</u>uaaggcu</u> <u>aguccguuaucaacuugaaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
8D	25	<u>uuauauccaacac<u>uucgugguuuu</u>agagcuaugcuguuuug</u>	41	-
	18	<u>guuggaaccau<u>ucaaaa</u>cagcauagcaagu<u>uaaa</u>uaaggcu</u> <u>aguccguuaucaacuugaaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
13D	26	<u>uuauauccaacac<u>uucgugguuuu</u>agagcuaugcuguuuug</u>	41	+++
	18	<u>guuggaaccau<u>ucaaaa</u>cagcauagcaagu<u>uaaa</u>uaaggcu</u> <u>aguccguuaucaacuugaaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	
13I	26	<u>uuauauccaacac<u>uucgugguuuu</u>agagcuaugcuguuuug</u>	41	+++
	21	<u>guuggaaccau<u>ucaaaa</u>cagcauagcaagu<u>uaaa</u>uaaggcu</u> <u>aguccguuaucaacuugaaaaaguggcaccgagucggugcuu</u> <u>uuuuu</u>	89	

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA, Underlined = 2'-O-methyl RNA, Italics = 2'-fluoro RNA. Lengths of the RNA oligonucleotides are indicated (bases). The relative efficiency of cleavage of the DNA target by recombinant Cas9 with each of the crRNA:tracrRNA pairs as visualized by agarose gel electrophoresis is indicated with “+++” indicating complete cleavage, “++” and “+” indicating intermediate levels of cleavage, and “-” indicating no cleavage.

[0067] The crRNAs contained a 19 base protospacer guide sequence matching a site in the human *HPRT1* gene adjacent to a suitable ‘NGG’ PAM site. A 938 base pair region from the human *HPRT1* gene was cloned into the pCR-Blunt vector (Life Technologies). The plasmid was linearized by digestion with the restriction endonuclease XmaI (New England BioLabs) prior to use in the Cas9 cleavage assay. Sequence of the *HPRT1* target fragment is shown below. The target PAM site is indicated in bold font and the protospacer guide sequence binding site is underlined.

HPRT1 target sequence. SEQ ID No. 27.

```
GAATGTTGTGATAAAAGGTGATGCTCACCTCTCCACACCCTTTTATAGTTTAGGGATTGTATTTCCAAGG
TTTCTAGACTGAGAGCCCTTTTCATCTTTGCTCATTGACACTCTGTACCCATTAATCCTCCTTATTAGCTC
CCCTTCAATGGACACATGGGTAGTCAGGGTGCAGGTCTCAGAACTGTCCTTCAGGTTCCAGGTGATCAACC
AAGTGCCTTGTCTGTAGTGTCAACTCATTGCTGCCCCCTTCCTAGTAATCCCCATAATTTAGCTCTCCATTT
CATAGTCTTTCTTGGGTGTGTTAAAAAGTGACCATGGTACACTCAGCACGGATGAAATGAAACAGTGTTTA
GAAACGTCAGTCTTCTCTTTTGTAATGCCCTGTAGTCTCTCTGTATGTTATATGTCACATTTTGTAATTAA
CAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAGCCAGACTGTAAGTGAATTACTTTTTTTTG
TCAATCATTTAACCATCTTTAACCTAAAAGAGTTTTATGTGAAATGGCTTATAATTGCTTAGAGAATATTT
GTAGAGAGGCACATTTGCCAGTATTAGATTTAAAAAGTGATGTTTTCTTTATCTAAATGATGAATTATGATT
CTTTTTAGTTGTTGGATTTGAAATTCCAGACAAGTTTGTGTAGGATATGCCCTTGACTATAATGAATACT
TCAGGGATTTGAATGTAAGTAATTGCTTCTTTTTCTCACTCATTTTTTCAAAACACGCATAAAAAATTTAGGA
AAGAGAATTGTTTTCTCCTTCCAGCACCTCATAATTTGAACAGACTGATGGTTCCCATTAGTCACATAAAG
CTGTAGTCTAGTACAGACGTCCTTAGAACTGGAACCTGGCCAGGCTAGGGTGACACTTCTTGTGGCTGAA
ATAGTTGAACAGCTT
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[0068] The crRNA and tracrRNA pairs were tested for the ability to direct degradation of a linearized plasmid DNA containing a cloned fragment of the human *HPRT1* gene by recombinant Spy Cas9 (New England BioLabs). The crRNA:tracrRNA were annealed in Duplex Buffer (30 mM HEPES pH 7.5, 100 mM potassium acetate) at 150 nM concentration. Spy Cas9 (15 nM) was preincubated with the crRNA:tracrRNA for 10 min at 37°C at a 1:1 molar ratio. Subsequently, 3 nM of the linearized target plasmid was added and incubated at 37°C for 1 hour. The reaction products were separated on a 1% agarose gel at 125 V for 1 hour. Bands were visualized by GelRed (Biotium) post-staining according to the manufacturer's protocol. The gel was imaged on a UV-transilluminator and results are summarized in Table 1 above.

[0069] Native wild-type (WT) CRISPR RNAs have a 19-20 base protospacer domain (guide, which binds to a target nucleic acid) at the 5'-end and a 22 base domain at the 3'-end that binds to the tracrRNA. Thus WT crRNAs are 41-42 bases long. The WT tracrRNA is 89 bases long. We observed that a WT type crRNA:tracrRNA pair supported full cleavage of the target DNA (cr/tracrRNA pair 2D). We additionally observed that a truncated version of the reagents with a 35 base crRNA (19 base protospacer and 16 base tracrRNA binding domain) paired with a 67 base tracrRNA supported full cleavage of the target RNA (cr/tracrRNA pair 1A). The crRNA tracrRNA binding region was truncated 6 bases at the 3'-end (SEQ ID No. 1). The tracrRNA was truncated at both ends (SEQ ID No. 2). Pairwise combinations of the short crRNA with the long tracrRNA showed cleavage as well as the long crRNA with the short tracrRNA (pair 2A). These findings are significant as it permits use of shorter RNA components to

direct Cas9 target recognition and cleavage. Shorter RNA oligonucleotides are less expensive and less difficult for chemical synthesis, requiring less purification and giving higher yields than longer RNA oligonucleotides.

[0070] Some elements of the native crRNA and tracrRNA (Fig. 1) were deleted to make a functional sgRNA (Fig. 2). However, the amount of duplex nucleic acid binding the crRNA to the tracrRNA in the sgRNA is limited to 11 base pairs, which is typically too short for duplex formation in biological salt conditions. The complex is stable in sgRNA format due to the unimolecular hairpin structure, however the same sequences split into 2 RNAs would be unstable. It was therefore unclear what length of duplex domain was needed to make a minimal yet functional 2-molecule (2-part) CRISPR complex, or if this complex would function to direct target cleavage by Cas9. The present example demonstrates that having as little of 15 bases base paired permits a function 2-part crRNA:tracrRNA complex that is competent to direct Cas9 nuclease activity against a target complementary to the crRNA protospacer domain (SEQ ID Nos. 1 and 2).

[0071] Complete chemical modification of the crRNA with 2'OMe RNA was not tolerated (pair 3A and pair 5A). Further, complete 2'OMe modification of the 22 base tracrRNA binding domain of the crRNA did not support target cleavage (pair 4A, pair 6A) and complete 2'OMe modification of the protospacer guide domain did not support cleavage (pair 7A). Complete chemical modification of the tracrRNA with 2'OMe RNA was also not tolerated (pair 1B, 1C and pair 2B, 2C).

[0072] Importantly, some highly 2'OMe-modified versions of both CRISPR RNA species did support cleavage. Pair 1K shows high cleavage activity with a tracrRNA having 29 2'OMe residues at the 3'-end (SEQ ID No. 11). Pair 1L shows high cleavage activity with 9 2'OMe residues at the 5'-end and 29 2'OMe residues at the 3'-end (SEQ ID No. 13). Thus 38 out of 67 RNA residues in the short version of the tracrRNA can be converted to 2'OMe RNA (57%) with no loss of activity in an *in vitro* cleavage assay.

[0073] Pair 14A demonstrates that 11 bases at the 3'-end of the crRNA (50% of the 22 base tracrRNA binding domain) can be modified with 2'OMe RNA and support target cleavage (SEQ ID No. 14). The modified crRNA retains full activity when paired with the modified tracrRNA (pair 14L, Seq ID Nos. 13 and 14). Modification of 11

bases towards the 5'-end of the crRNA (in the guide, protospacer domain, bases 2-12) supports target cleavage (pair 15A) and this modification is also functional when paired with the modified tracrRNA (pair 15L, SEQ ID Nos. 13 and 15). The 2'OMe modifications towards the 5'-end and 3'-end of the crRNA can be combined (SEQ ID No.16) such that 22 out of 35 residues are modified (63%) and still support cleavage (pair 16A), even when paired with the modified tracrRNA (pair 16L, SEQ ID Nos. 13 and 16).

[0074] The crRNA:tracrRNA pairs mentioned above all employed 2'OMe RNA as the modifier. Additional studies showed that 2'F modification was also tolerated by Cas9 and enabled cleavage of a target DNA. Pair 9A employs a crRNA with 2'F modification at all pyrimidine bases (SEQ ID No. 23) and this design supported complete target cleavage. Likewise complete 2'F modification of the crRNA supported complete target cleavage (pair 10A, SEQ ID No. 24). Combined use of 2'OMe and 2'F modifications may permit complete modification of both the crRNA and tracrRNA. The studies in the present example utilized *in vitro* biochemical analyses. Performance may vary in the context of mammalian gene editing where the sequences have to function in the milieu of a cell nucleus.

EXAMPLE 2

[0075] This example demonstrates functioning of truncated tracrRNAs to direct genome editing by the Spy Cas9 nuclease in mammalian cells.

[0076] Both functional Cas9 nuclease and the RNA triggers (a single sgRNA or dual crRNA:tracrRNA pair) must be present in the nucleus of mammalian cells for CRISPR genome editing to take place. Transfection of large plasmid vectors expressing Cas9 is inefficient and adds variability to experimental results. In order to accurately assess the impact that changes in length and chemical composition of the crRNA and tracrRNA have in mammalian cells in the absence of other variables, a cell line that stably expresses Spy Cas9 was constructed.

[0077] A HEK293 cell line having constitutive expression of SpyCas9 (human codon-optimized) with stable vector integration and selection under G418 was developed as described below. Human optimized Spy Cas9 was ligated into a pcDNA3.1

expression vector (Life Technologies) and transfected into HEK293 cells using Lipofectamine2000 (Life Technologies). The transfected cells were allowed to grow for 2 days before being placed under selective pressure using Neomycin. After 7 days, cells were plated to single colonies using limiting dilution techniques. Monoclonal colonies were screened for Cas9 activity and the clone having highest level of expression was used for future studies. A single copy integration event for Cas9 was determined using droplet digital PCR (ddPCR). Western blot using an anti-Cas9 antibody showed low but constant expression of Cas9 protein. This cell line is henceforth referred to as “HEK-Cas9”.

[0078] The HEK-Cas9 cell line was employed in subsequent studies. In a reverse transfection format, anti-*HPRT1* crRNA:tracrRNA complexes were mixed with Lipofectamine RNAiMAX (Life Technologies) and transfected into the HEK-Cas9 cells. Transfections were done with 40,000 cells per well in 96 well plate format. RNAs were introduced at a final concentration of 30 nM in 0.75 μ l of the lipid reagent. Cells were incubated at 37°C for 48 hours. Genomic DNA was isolated using QuickExtract solution (Epicentre). Genomic DNA was amplified with KAPA HiFi DNA Polymerase (Roche) and primers targeting the HPRT region of interest (HPRT forward primer: AAGAATGTTGTGATAAAAGGTGATGCT (SEQ ID No. 28); HPRT reverse primer: ACACATCCATGGGACTTCTGCCTC (SEQ ID No. 29)). PCR products were melted and re-annealed in NEB buffer 2 (New England BioLabs) to allow for heteroduplex formation followed by digestion with 2 units of T7 endonuclease 1 (T7EI; New England BioLabs) for 1 hour at 37°C. The digested products were visualized on a Fragment Analyzer (Advanced Analytics). Percent cleavage of targeted DNA was calculated as the average molar concentration of the cut products / (average molar concentration of the cut products + molar concentration of the uncut band) $\times$ 100.

[0079] TracrRNAs (Table 2) were synthesized having deletions at the 5'-end, 3'-end, internal or combinations thereof. The tracrRNAs were complexed with unmodified truncated anti-*HPRT1* crRNA SEQ ID No. 1 (Table 1) which has a 19 base protospacer domain targeting *HPRT1* at the 5'-end and a 16 base tracrRNA binding domain at the 3'-end. The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described above. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch

endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer. A representation of the wild-type *S. pyogenes* crRNA:tracrRNA complex is shown in FIG. 1 (which pairs crRNA SEQ ID No. 46 with tracrRNA SEQ ID No. 18). The relative location of deletions in the tracrRNA tested in this example are shown in sequence alignment format in FIG. 3.

Table 2: Effect of length truncations in the tracrRNA on efficiency of gene editing in mammalian cells by Cas9 endonuclease.

SEQ ID No.	tracrRNA Sequence 5'-3'	Cleavage (%)	Length	Truncation positions
18	guuggaaccgauucaaacagcauagcaaguua aaauaaggcuaguccguuaucaacuugaaaaa guggcaccgagucggugcuuuuuuu	38	89	-
30	caaaacagcauagcaaguuaaaauaaggcuag uccguuaucaacuugaaaaaguggcaccgagu cggugcuuuu	26	74	5' - 12 bases 3' - 3 bases
31	aacagcauagcaaguuaaaauaaggcuagucc guuaucaacuugaaaaaguggcaccgagucgg ugcuuu	32	70	5' - 15 bases 3' - 4 bases
2	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucggugc uuu	57	67	5' - 18 bases 3' - 4 bases
32	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucggugc u	47	65	5' - 18 bases 3' - 6 bases
33	cauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcu	27	63	5' - 20 bases 3' - 6 bases
34	agcauagcaaguuaaaauaaguuaucacuuga aaaaguggcaccgagucggugcu	0	55	5' - 18 bases Int - 10 bases 3' - 6 bases
35	agcauagcaaguuaaaauaacuugaaaaagu ggcaccgagucggugcu	0	49	5' - 18 bases Int - 16 bases 3' - 6 bases
36	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucggugc	53	64	5' - 18 bases 3' - 7 bases
37	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucggug	56	63	5' - 18 bases 3' - 8 bases
38	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucgggu	56	62	5' - 18 bases 3' - 9 bases
39	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaaguggcaccgagucggg	53	61	5' - 18 bases 3' - 10 bases
40	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaagugccgagucggg	5	58	5' - 18 bases Int - 3 bases

SEQ ID No.	tracrRNA Sequence 5'-3'	Cleavage (%)	Length	Truncation positions
				3' - 10 bases
41	agcauagcaaguuaaaauaaggcuaguccaac uugaaaaaguggcaccgagucggugcu	0	59	5' - 18 bases Int - 6 bases 3' - 6 bases
42	agcauagcaaguuaaaauaaggcuaguccaac uugaaaaaguggcaccgagucgg	0	55	5' - 18 bases Int - 6 bases 3' - 10 bases
43	agcauagcaaguuaaaauaaggcuaguccaac uugaaaaaguggcaccgagucgg	0	52	5' - 18 bases Int - 9 bases 3' - 10 bases
44	agcauagcaaguuaaaauaaggcuaguccguu aucaacuugaaaaagug	0	49	5' - 18 bases 3' - 22 bases
45	agcauagcaaguuaaaauaaggcuaguccguu aucagcaccgagucggugcu	0	52	5' - 18 bases Int - 13 bases 3' - 6 bases
427	agcauagcaaguuaaaauaaggcuaguccguc aacuugaaaaaguggcaccgagucggugcuuu	4	64	5' - 18 bases Int - 3 bases 3' - 4 bases

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA. Lengths of the RNA oligonucleotides are indicated (bases). The number of RNA residues removed in truncation studies at the 5'-end, 3'-end, and internal (int) are indicated. The relative functional activity of each species is indicated by the % cleavage in a T7EI heteroduplex assay.

[0080] This example demonstrates that for purposes of gene editing in mammalian cells that the tracrRNA can tolerate significant deletions from both the 5'-end and 3'-end and retain full functionality. Deletion of 18 bases from the 5'-end was well tolerated. Deletion of 20 bases from the 5'-end led to reduced activity, possibly due to lower affinity of binding of the crRNA. It is possible that this reduced length or even shorter might be functional if Tm-enhancing modifications were employed to stabilize the short duplex forming region. Deletion of up to 10 bases from the 3'-end was well tolerated. Additional deletions resulted in loss of activity. Internal deletions that disrupted hairpin elements or spacing between hairpin elements were not functional.

[0081] In summary, this example demonstrates that truncation of the tracrRNA from the 89 base length of the wild-type (WT, SEQ ID No. 18) to a 67 base length (SEQ ID No. 2) or to a 62 base length (SEQ ID No. 38), or to a 61 base length (SEQ ID No. 39)

retained high functional activity. Use of shortened tracrRNAs of this kind will be less costly and easier to manufacture by chemical methods than the WT 89 base RNA. Some of the truncated species (SEQ ID No. 2, SEQ ID No. 38, and SEQ ID No. 39) showed increased functional activity over the 89 base WT tracrRNA. Therefore in addition to being less costly and easier to manufacture by chemical methods, the shortened tracrRNAs of the present invention showed improved activity.

EXAMPLE 3

[0082] Examples 1 and 2 demonstrated that crRNA:tracrRNA complexes shorter than the WT lengths of 42 and 89 bases, respectively, can show higher functional activity in mammalian gene editing. The present example shows further optimization of the lengths of these RNA species.

[0083] A series of crRNAs and tracrRNAs (Table 3) were synthesized having different lengths as indicated. Truncations were made at the 3'-end of the crRNA, the 5'-end of the tracrRNA, and/or the 3'-end of the tracrRNA. The crRNAs and tracrRNA were paired as indicated in Table 3. The crRNAs all employed a 20 base protospacer domain targeting *HPRT1* at the 5'-end and variable length 3'-ends (tracrRNA binding domains). An alignment of the crRNA and tracrRNA sequences studied in this example is shown in FIG. 4 to make clear the positions of truncations relative to each functional domain.

[0084] The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described in Example 2. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer. Results are shown in Table 3. The relative location of deletions are shown in sequence alignment format in FIG. 4.

Table 3: Effect of length truncations in both the crRNA and tracrRNA on efficiency of gene editing in mammalian cells by Cas9 endonuclease.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
42/89	46	cuuauauccaacacucgugguuuuagagcuaugcu guuuug	42	25

cr/tracr RNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
	18	guuggaaccauucaaaacagcauagcaaguuaaaau aaggcuaguccguuaucacuugaaaaaguggcacc gagucggugcuuuuuuu	89	
39/89	47	cuuauauccaacacacuucgugguuuuagagcuaugcu guu	39	31
	18	guuggaaccauucaaaacagcauagcaaguuaaaau aaggcuaguccguuaucacuugaaaaaguggcacc gagucggugcuuuuuuu	89	
36/89	48	cuuauauccaacacacuucgugguuuuagagcuaugcu	36	38
	18	guuggaaccauucaaaacagcauagcaaguuaaaau aaggcuaguccguuaucacuugaaaaaguggcacc gagucggugcuuuuuuu	89	
34/89	49	cuuauauccaacacacuucgugguuuuagagcuaug	34	21
	18	guuggaaccauucaaaacagcauagcaaguuaaaau aaggcuaguccguuaucacuugaaaaaguggcacc gagucggugcuuuuuuu	89	
42/74	46	cuuauauccaacacacuucgugguuuuagagcuaugcu guuuug	42	35
	50	caaaacagcauagcaaguuaaaaauaaggcuaguccg uuaucaacuugaaaaaguggcaccgagucggugcuu uu	74	
39/74	47	cuuauauccaacacacuucgugguuuuagagcuaugcu guu	39	34
	50	caaaacagcauagcaaguuaaaaauaaggcuaguccg uuaucaacuugaaaaaguggcaccgagucggugcuu uu	74	
36/74	48	cuuauauccaacacacuucgugguuuuagagcuaugcu	36	26
	50	caaaacagcauagcaaguuaaaaauaaggcuaguccg uuaucaacuugaaaaaguggcaccgagucggugcuu uu	74	
34/74	49	cuuauauccaacacacuucgugguuuuagagcuaug	34	20
	50	caaaacagcauagcaaguuaaaaauaaggcuaguccg uuaucaacuugaaaaaguggcaccgagucggugcuu uu	74	
42/70	46	cuuauauccaacacacuucgugguuuuagagcuaugcu guuuug	42	55
	51	aacagcauagcaaguuaaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuuu	70	
39/70	47	cuuauauccaacacacuucgugguuuuagagcuaugcu guu	39	48
	51	aacagcauagcaaguuaaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuuu	70	
36/70	48	cuuauauccaacacacuucgugguuuuagagcuaugcu	36	32

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
	51	aacagcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuuu	70	
34/70	49	cuuauauccaacacucugugguuuuagagcuauag	34	9
	51	aacagcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuuu	70	
42/67	46	cuuauauccaacacucugugguuuuagagcuauagcu guuuug	42	36
	2	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcuuu	67	
39/67	47	cuuauauccaacacucugugguuuuagagcuauagcu guu	39	41
	2	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcuuu	67	
36/67	48	cuuauauccaacacucugugguuuuagagcuauagcu	36	57
	2	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcuuu	67	
34/67	49	cuuauauccaacacucugugguuuuagagcuauag	34	44
	2	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcuuu	67	
42/65	46	cuuauauccaacacucugugguuuuagagcuauagcu guuuug	42	50
	52	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcu	65	
39/65	47	cuuauauccaacacucugugguuuuagagcuauagcu guu	39	46
	52	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcu	65	
36/65	48	cuuauauccaacacucugugguuuuagagcuauagcu	36	47
	52	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcu	65	
34/65	49	cuuauauccaacacucugugguuuuagagcuauag	34	16
	52	agcauagcaaguuaaaauaaggcuaguccguuauc acuugaaaaaguggcaccgagucggugcu	65	
42/63	46	cuuauauccaacacucugugguuuuagagcuauagcu guuuug	42	6
	53	cauagcaaguuaaaauaaggcuaguccguuaucac uugaaaaaguggcaccgagucggugcu	63	

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
39/63	47	cuuauauccaacacucgugguuuuagagcuaugcuguu	39	13
	53	cauagcaaguuaaaauaaggcuaguccguaucaacuugaaaaaguggcaccgagucggugcu	63	
36/63	48	cuuauauccaacacucgugguuuuagagcuaugcu	36	28
	53	cauagcaaguuaaaauaaggcuaguccguaucaacuugaaaaaguggcaccgagucggugcu	63	
34/63	49	cuuauauccaacacucgugguuuuagagcuaug	34	33
	53	cauagcaaguuaaaauaaggcuaguccguaucaacuugaaaaaguggcaccgagucggugcu	63	

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA. Lengths of the RNA oligonucleotides are indicated (bases). The relative functional activity of each crRNA:tracrRNA pair is indicated by the % cleavage in a T7EI heteroduplex assay.

[0085] All of the compounds studied directed CRISPR/Cas editing at the HPRT1 locus in HEK-Cas9 cells. Efficiency varied widely from 6% to 57%. The most effective crRNA+tracrRNA combination was the 36 base crRNA (SEQ ID No. 48) with the 67mer tracrRNA (SEQ ID No. 2). A schematic representation of the truncated, optimized crRNA:tracrRNA complex is shown in FIG. 5. In this case the tracrRNA binding domain of the crRNA was truncated to 16 bases from the WT 22 base sequence (3'-end). This hybridizes to the crRNA binding domain at the 5'-end of the tracrRNA. The tracrRNA was truncated 18 bases at the 5'-end and 4 bases at the 3'-end to product the active 67 base product. For this pair, a blunt end is formed upon hybridization of the 3'-end of the crRNA with the 5'-end of the tracrRNA. Other versions also showed high activity, including the 42 base (WT) crRNA (SEQ ID No. 46) paired with the 70 base tracrRNA (SEQ ID No. 51).

[0086] The shortest crRNA tested was 34 bases in length (SEQ ID No. 49) and, in general, showed lower activity than the longer variants. The shorter duplex domain formed between this variant and the tracrRNA has reduced binding affinity (T_m) compared to the 36 base crRNA variant and that 34 base complex was less stable at 37°C. Use of chemical modification that increase binding affinity (T_m), such as 2'OMe RNA, 2'F RNA, or LNA residues, will increase stability of this short duplex domain and

will lead to improved activity, permitting use of very short crRNAs of this design. Extensive use of Tm-enhancing modifications will permit use of even shorter tracrRNA binding domains in the crRNA, such as 13 base, or 12 base, or 11 base, or 10 base, or 9 base, or 8 base or shorter, depending on the kind and number of modified residues employed.

EXAMPLE 4

[0087] Examples 1, 2, and 3 demonstrated that crRNA:tracrRNA complexes shorter than the WT lengths of 42 and 89 bases, respectively, can show higher functional activity in mammalian gene editing. In those examples, all truncations were made in the universal domains of the RNAs. The present example tests the effects that truncations have on the target-specific protospacer domain of the guide crRNA.

[0088] A series of crRNAs (Table 4) were synthesized having protospacer domain lengths of 20, 19, 18, or 17 bases as indicated. Truncations were made at the 5'-end of the crRNA, using a 16mer universal tracrRNA binding sequence at the 3'-end. The crRNAs were paired with an unmodified 67mer tracrRNA (SEQ ID No. 2). The crRNAs targeted 4 different sites in the same exon of the human *HPRT1* gene.

[0089] The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described in Example 2. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer. Results are shown in Table 4.

Table 4: Effect of length truncations in the 5'-protospacer domain of the crRNA on efficiency of gene editing in mammalian cells by Cas9 endonuclease.

SEQ ID No.	crRNA Sequence 5'-3'	Length Protospacer domain	Cleavage (%)	<i>HPRT1</i> site
48	<u>cuuauauccaacac</u> uucgugguuuuagagc uagcu	20	64	38285
1	<u>uuauauccaacac</u> uucgugguuuuagagcu augcu	19	62	
54	<u>uauauccaacac</u> uucgugguuuuagagcua	18	57	

SEQ ID No.	crRNA Sequence 5'-3'	Length Protospacer domain	Cleavage (%)	HPRT1 site
	ugcu			38087
55	<u>auauccaacac</u> uucgugguuuuagagcuau gcu	17	42	
56	<u>aaauaugggg</u> auuacuaggaguuuuagagc uauugcu	20	78	
57	<u>auuauaugggg</u> auuacuaggaguuuuagagcu augcu	19	81	38087
58	<u>uuauaugggg</u> auuacuaggaguuuuagagcua ugcu	18	82	
59	<u>uaugggg</u> auuacuaggaguuuuagagcuau gcu	17	82	
60	<u>auuucacauaaa</u> acucuuuuuguuuuagagc uauugcu	20	52	38358
61	<u>uuucacauaaa</u> acucuuuuuguuuuagagcu augcu	19	30	
62	<u>uucacauaaa</u> acucuuuuuguuuuagagcua ugcu	18	12	
63	<u>ucacauaaa</u> acucuuuuuguuuuagagcuau gcu	17	0	
64	<u>uccauuucau</u> agucuuuccuguuuuagagc uauugcu	20	70	38094
65	<u>ccauuucau</u> agucuuuccuguuuuagagcu augcu	19	71	
66	<u>cauuucau</u> agucuuuccuguuuuagagcua ugcu	18	52	
67	<u>auuucau</u> agucuuuccuguuuuagagcuau gcu	17	0	

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA. The target-specific protospacer domain is underlined and length is indicated (bases). The relative functional activity of each species is indicated by the % cleavage in a T7EI heteroduplex assay.

[0090] Of the 4 sites studied, one (site 38087) showed high activity for all 4 crRNAs with no changes seen as the protospacer domain was shortened. Site 38285 similar

efficacy for the 20 and 19 base protospacer crRNAs (SEQ ID Nos. 48 and 1), a slight decrease for the 18 base version (SEQ ID No. 54), and a large decrease for the 17 base version (SEQ ID No. 55). Site 38094 showed similar efficacy for the 20 and 19 base protospacer crRNAs (SEQ ID Nos. 64 and 65), a moderate decrease for the 18 base version (SEQ ID No. 66), and no activity for the 17 base version (SEQ ID No. 67). Site 38358 showed good activity for the 20 base version (SEQ ID No. 60), lower activity for the 19 base version (SEQ ID No. 61), even lower activity for the 18 base version (SEQ ID No. 62) and no activity for the 17 base version (SEQ ID No. 63).

[0091] The use of shortened 17 base protospacer guide domains can lower the occurrence of undesired off-target events compared to the wild-type 20 base domain (Fu et al., Nature Biotechnol., 32:279, 2014). We observe that on-target efficacy varies in a sequence-context specific fashion and that 20 base and 19 base protospacer guide domains are generally effective but that activity begins to decrease when 18 base protospacer domains are used and activity significantly decreases when 17 base protospacer domains are used. Therefore, to maintain desired on-target efficiency, use of 20 and 19 base target-specific protospacer guide domains are employed herein. Significant truncation of the protospacer guide domain in many cases lowers on-target cleavage of a DNA target by the Cas9 endonuclease. Use of chemical modifications that enhance T_m (increase binding affinity of the protospacer target-specific domain of the crRNA to the target DNA sequence) may permit use of shorter sequences such that a 17 base protospacer guide may show similar on-target efficacy as an unmodified 20 base protospacer guide domain.

EXAMPLE 5

[0092] This example demonstrates that truncated crRNA:tracrRNA complex show improved gene editing activity at multiple sites. The prior examples studied efficacy of the truncated RNAs as triggers of CRISPR gene editing in mammalian cells at a single site in the human *HRPT1* gene. Site/sequence specific effects may exist. The present example demonstrates improved performance of the truncated species of the present invention at 12 sites in an exon of the human *HPRT1* gene.

[0093] A series of crRNAs (Table 5) were synthesized having a protospacer domain lengths of 20 bases specific to 12 sites in the human HPRT1 gene with a 16mer universal

tracrRNA binding sequence at the 3'-end. The crRNAs were paired with an unmodified 67mer tracrRNA (SEQ ID No. 2). The same 12 sites were studied using WT length crRNA:tracrRNA complexes wherein the crRNA comprised a 20 base protospacer guide paired with a 22mer universal tracrRNA binding sequence at the 3'-end complexed with the WT 89mer tracrRNA (SEQ ID No. 18).

[0094] The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described in Example 2. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer. Results are shown in Table 5.

Table 5: Effect of length truncations in both the crRNA and tracrRNA on efficiency of gene editing in mammalian cells by Cas9 endonuclease.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
38094 short	64	uccauuucauagucuuuccuguuuuagagcuau gcu	36	55
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38094 long	68	uccauuucauagucuuuccuguuuuagagcuau gcuguuuug	42	31
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38231 short	69	uuuuguaauuaacagcuugcguuuuagagcuau gcu	36	7
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38231 long	70	uuuuguaauuaacagcuugcguuuuagagcuau gcuguuuug	42	0
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38371 short	71	cuuagagaauauuuguagagguuuuagagcuau gcu	36	57
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38371 long	72	cuuagagaauauuuguagagguuuuagagcuau gcuguuuug	42	27

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
	18	guuggaaccbauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38509 short	73	uugacuauaaugaauacuucguuuuagagcuau gcu	36	56
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38509 long	74	uugacuauaaugaauacuucguuuuagagcuau gcuguuuug	42	7
	18	guuggaaccbauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38574 short	75	caaaacacgcuaaaaaauuuguuuuagagcuau gcu	36	58
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38574 long	76	caaaacacgcuaaaaaauuuguuuuagagcuau gcuguuuug	42	22
	18	guuggaaccbauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38087 short	56	aauiuaggggauiuacuaggaguuuuagagcuau gcu	36	60
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38087 long	77	aauiuaggggauiuacuaggaguuuuagagcuau gcuguuuug	42	53
	18	guuggaaccbauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38133 short	78	ggucacuuuuuacacacccaguuuuagagcuau gcu	36	53
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38133 long	79	ggucacuuuuuacacacccaguuuuagagcuau gcuguuuug	42	37
	18	guuggaaccbauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38285 short	48	cuuauauccaacacuucgugguuuuagagcuau gcu	36	38
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38285 long	46	cuuauauccaacacuucgugguuuuagagcuau gcuguuuug	42	8

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Length	Cleavage %
		tracrRNA Sequence		
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38287 short	80	ggcuuauauccaacacucgguuuuagagcuau gcu	36	48
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38287 long	81	ggcuuauauccaacacucgguuuuagagcuau gcuguuuug	42	6
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38358 short	60	auuucacauaaaacucuuuuuguuuagagcuau gcu	36	42
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38358 long	82	auuucacauaaaacucuuuuuguuuagagcuau gcuguuuug	42	8
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38636 short	83	ucaaauuauagaggugcuggaguuuuagagcuau gcu	36	26
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38636 long	84	ucaaauuauagaggugcuggaguuuuagagcuau gcuguuuug	42	16
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	
38673 short	85	uacagcuuuauugugacuaauuuuagagcuau gcu	36	45
	2	agcauagcaaguuaaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu u	67	
38673 long	86	uacagcuuuauugugacuaauuuuagagcuau gcuguuuug	42	32
	18	guuggaaccauucaaaacagcauagcaaguuaa aauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcuuuuuuu	89	

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA. Lengths of the RNA oligonucleotides are indicated (bases). The relative functional activity of each crRNA:tracrRNA pair is indicated by the % cleavage in a T7EI heteroduplex assay.

[0095] The relative efficiency of CRISPR mediated gene editing in the HEK-Cas9 cells varied with sequence context. However, in all cases the shorter optimized RNA guides (36mer crRNA and 67mer tracrRNA) showed higher efficiency than the WT RNAs (42mer crRNA and 89mer tracrRNA). Use of the shortened, optimized guide RNAs of the present invention improve Cas9 cleavage of targeted DNAs relative to the WT RNAs, improving the gene editing rates.

EXAMPLE 6

[0096] Example 1 described chemical modification patterns that functioned with Cas9 in an *in vitro* biochemical target DNA cleavage assay. This example demonstrates functioning of chemically modified tracrRNAs to direct genome editing by the Spy Cas9 nuclease in mammalian cells. Optimal modification patterns differ between *in vitro* and *in vivo* use.

[0097] A series of tracrRNAs (Table 6) were synthesized having a variety of chemical modifications, including: the ribose modifications 2'OMe RNA and LNA; the end-modifying groups propanediol spacer and naphthyl-azo modifier (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, or "ZEN"); and select internucleotide linkages with phosphorothioate modifications. See: Lennox et al., Molecular Therapy Nucleic Acids 2:e117 2013 for structure of the naphthyl-azo modified and use of the naphthyl-azo modifier and propanediol modifier for use as end-groups to block exonuclease attack. The tracrRNAs listed in Table 6 were complexed with unmodified truncated anti-*HPRT1* crRNA SEQ ID No. 1 (Table 1) which has a 19 base protospacer domain targeting *HPRT1* at the 5'-end and a 16 base tracrRNA binding domain at the 3'-end. The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described above. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer.

Table 6: Optimization of tracrRNA oligonucleotide modification patterns in mammalian cells.

SEQ ID No.	tracrRNA Sequence (5'-3')	Cleavage (%)
2	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	65
4	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	0
11	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	56
17	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaguggca ccgagucggugcuuu	12
12	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	20
13	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	64
87	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	61
88	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	60
89	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcuuu	64
90	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	60
91	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	61
92	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	59
93	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	57
94	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	57
95	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	62
96	agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaaguggca ccgagucggugcu	62
97	C3-agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaagugg gcaccgagucggugcuuu-C3	53
98	a*g*cauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaagugg caccgagucggugcu*u*u	58
99	C3-agcauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaagugg gcaccgagucggugcuuu-C3	20
100	a*g*cauagcaaguuuuuuuagggcuaguccguuaucaacuugaaaaagugg caccgagucggugcu*u*u	63

SEQ ID No.	tracrRNA Sequence (5'-3')	Cleavage (%)
101	a*g*c*auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc <u>*u*u*u</u>	55
102	agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc <u>uuu</u>	39
103	a*g*c*auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc <u>*u*u*u</u>	54
104	agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc <u>uuu</u> - ZEN	55
105	ZEN-agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc <u>uuu</u> - ZEN	23
106	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucg</u> *g*u	58
107	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	8
108	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	0
109	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	11
110	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	61
111	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	61
112	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	62
113	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	62
114	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	61
115	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	14
116	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	60
117	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	60
118	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	15
119	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	0
120	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	7
121	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	14
122	a*g*c <u>auagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccgagucggugc</u> *u*u	11

SEQ ID No.	tracrRNA Sequence (5'-3')	Cleavage (%)
123	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
124	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
125	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
126	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	64
127	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
128	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
129	+a*+g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagu ggcaccgagucggugcu*+t*+t	57
130	C3- <u>agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagug</u> <u>gcaccgagucggugcuuu-InvT</u>	
131	C3- <u>agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagug</u> <u>gcaccgagucgggu-C3</u>	58
132	C3- <u>agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagug</u> <u>gcaccgagucgggu-InvT</u>	59
133	<u>agcauagca*a*g*u*u*a*a*a*a*u*a*a*g*g*c*u*a*g*u*c*c*g*</u> <u>u*u*a*u*c*a*a*a*cuugaaaaaguggcaccgagucggugcuuu</u>	0
134	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	58
135	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	19
136	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucg*g*u</u>	54
137	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucg*g*u</u>	13
138	<u>a*g*cauagcaagTAAAAATAAGGCTAGTCCGTTaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
139	<u>a*g*cauagcaagTAAAAATAAGGcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
140	<u>a*g*cauagcaaguuaaaauaaggGCTAGTCCGTTaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
141	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
142	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	4
143	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	0
144	<u>a*g*cauagcaaguuaaaauaaggcuaguccguuaucaacuugaaaaagugg</u> <u>caccgagucggugcu*u*u</u>	52

SEQ ID No.	tracrRNA Sequence (5'-3')	Cleavage (%)
145	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	63
146	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	0
147	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	62
148	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	57
149	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	47
150	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	61
151	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	61
152	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	61
153	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	61
154	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	50
155	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	46
156	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	59
157	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	2
158	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	18
159	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	50
160	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	58
161	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	14
162	<u>a</u> * <u>g</u> *cauagcaaguu ⁺ aaaauaaggcuaguccguu <u>aucaacuugaaaaagugg</u> <u>caccgagucggugcu</u> * <u>u</u> * <u>u</u>	8

Oligonucleotide sequences are shown 5'-3'. Uppercase = DNA; Lowercase = RNA; Underlined = 2'-O-methyl RNA; Italics = 2'-fluoro RNA; +a, +c, +t, +g = LNA; C3 = C3 spacer (propanediol modifier); * = phosphorothioate internucleotide linkage; ZEN – naphthyl-azo modifier; Inv-dT = inverted-dT. The relative functional activity of each species is indicated by the % cleavage in a T7EI heteroduplex assay.

[0098] Modification is usually necessary for synthetic nucleic acids to function well in an intracellular environment due to the presence of exonucleases and endonucleases that degrade unmodified oligonucleotides. A wide range of modifications have been described that confer nuclease resistance to oligonucleotides. The precise combination and order of modifications employed that works well for a given application can vary with sequence context and the nature of the protein interactions required for biological function. Extensive prior work has been done relating to chemical modification of antisense oligonucleotides (which interact with RNase H1) and siRNAs (which interact with DICER, AGO2, and other proteins). It is expected that chemical modification will improve function of the CRISPR crRNA:tracrRNA complex. However, it is not possible to predict what modifications and/or pattern of modifications will be compatible with functional complexation of the synthetic RNAs with Cas9. The present invention defines minimal, moderate, and extensive chemical modification patterns for the tracrRNA that retain high levels of function to direct Cas9 mediated gene editing in mammalian cells.

[0099] The results in Table 6 demonstrate that extensive modification is tolerated throughout the 5' and 3' end domains of the tracrRNA. Modification of the internal domains of the tracrRNA showed reduced activity, likely due to altered structure of the folded RNA and/or blocking of protein contact points with the 2'-OH of key RNA residues by the 2'OMe modification. For example, compound SEQ ID No. 100 has 39/67 residues modified with 2'OMe RNA (58%) and retains full activity compared with the unmodified sequence. SEQ ID No. 134 has 46/67 residues modified with 2'OMe RNA (69%) and retains near full activity compared with the unmodified sequence (FIG. 6). SEQ ID No. 134 is a truncated 67mer variant of the tracrRNA. Using SEQ ID No. 134 as a model, modification of 11 sequential residues in the 5'-domain with 2'OMe RNA was tolerated with no loss of activity. Modification of 35 sequential residues in the 3'-domain with 2'OMe RNA was tolerated with not loss of activity. Of note, the two hairpin structures present in the 3'-domain are necessary for function as deletion of either of these features results in loss of activity (Example 2, FIG. 3), yet both of these domains can be completely modified with 2'OMe RNA without compromising function. Note that both SEQ ID Nos. 134 and 100 also have phosphorothioate (PS) modified

internucleotide linkages at the 5'- and 3'-ends, which provides additional protection against exonuclease attack.

[00100] Specific residues were identified that led to large reductions or complete loss of activity when modified. Using the 67 base tracrRNA (for example, SEQ ID No. 134) as reference, starting from the 5'-end of the sequence substitution of 2'OMe RNA for the natural RNA at residues U12, A15, G26, U27, G30, U31, and U32 led to substantial loss of activity (FIG. 6). Specific residues were also identified that led to smaller yet significant reductions in activity when modified. Using the 67 base tracrRNA (for example, SEQ ID No. 134) as reference, starting from the 5'-end of the sequence substitution of 2'OMe RNA for the natural RNA at residues U13, U18, C23, U24, and C28 led to reduced activity (FIG. 6). This study was performed using 2'OMe RNA. Use of other modifications, such as 2'F, LNA, DNA, etc. at these positions may be better tolerated. The central 21 residue domain of unmodified RNA in SEQ ID No. 134 was modified with 2'-F RNA either completely (SEQ ID No. 141) or partially (SEQ ID Nos. 142 and 143). These variants were not functional. The central 21 residue domain of unmodified RNA in SEQ ID No. 134 was modified with DNA either completely (SEQ ID No. 138) or partially (SEQ ID Nos. 139 and 140). These variants were not functional. Modification of isolated residues in this domain may work, however large continuous blocks of modification in this domain reduce activity of the tracrRNA.

[00101] To further investigate which individual residues can be modified using 2'OMe RNA within the central domain of the tracrRNA, a single base modification 2'OMe RNA 'walk' was done (SEQ ID Nos. 144-162). Within this series, modification as residues A14, A19, A20, G21, G22, A25, and C29 showed no loss of activity and are candidates for modification.

[00102] Antisense oligonucleotides are often made using complete PS modification, where every internucleotide linkage is phosphorothioate modified. This extensive level of modification is possible because the protein effector molecule RNase H1 (which mediates ASO-directed mRNA degradation) tolerates the PS modification in the ASO when forming a functional substrate/enzyme complex. On the other hand, siRNAs do not tolerate full PS modification; extensive PS modification disrupts productive interaction with the effector protein AGO2 (which mediates siRNA-directed mRNA

degradation). Extensive PS modification of the tracrRNA in the internal RNA loops disrupts functional interaction with Cas9 (Seq ID No. 133; 29 PS modifications). Limited PS end-modification can be done with no loss of activity (SEQ ID Nos. 98 and 101; 2-3 PP linkages on each end). Less extensive PS modification may be tolerated in the central domain. In particular, RNase cleavage mapping (where incubation of the tracrRNA in a series of serum or cell extract dilutions are used to find the sites that are most sensitive to RNase attack) may be used to identify critical sites where PS modification of only one or a few linkages may stabilize the RNA without disrupting function.

[00103] There are applications where the PS modification contributes to chemical toxicity. In this case use of other methods to block exonuclease attack are desirable. Options include end-modifiers such as inverted-dT or abasic groups such as dSpacer, C3 spacer (propanediol), ZEN (naphthyl-azo modifier), and others. Placement of such end-modifying groups can eliminate the need for terminal PS internucleotide linkages.

EXAMPLE 7

[00104] Example 1 described chemical modification patterns that functioned with Cas9 in an *in vitro* biochemical target DNA cleavage assay. This example demonstrates functioning of chemically modified crRNAs to direct genome editing by the Spy Cas9 nuclease in mammalian cells. Optimal modification patterns differ between *in vitro* and *in vivo* use.

[00105] A series of crRNAs (Table 7) were synthesized having a variety of chemical modifications, including: the ribose modifications 2'OMe RNA, 2'F, and LNA; the end-modifying groups propanediol spacer and naphthyl-azo modifier (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, or "ZEN"), and an inverted-dT residue; and select internucleotide linkages with phosphorothioate modifications. See: Lennox et al., Molecular Therapy Nucleic Acids 2:e117 2013 for structure of the naphthyl-azo modified and use of the naphthyl-azo modifier and propanediol modifier for use as end-groups to block exonuclease attack. The crRNAs had either a 19 base protospacer domain targeting *HPRT1* at the 5'-end (SEQ ID Nos. 1, 9 10, 14-16, 22-24, 163-173) or a 20 base protospacer domain targeting the same site (SEQ ID Nos. 48, 174-237) with a 16 base tracrRNA binding domain at the 3'-end. The crRNAs listed in Table 7 were

complexed with unmodified truncated (67 base) tracrRNA SEQ ID No. 2 (Table 1) or chemically-modified truncated (67 base) tracrRNA SEQ ID No. 100 (Table 6). The use of two tracrRNAs enables determination if function of chemical modified crRNAs varies if paired with a modified tracrRNA. The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described previously.

Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay, with quantitative measurement of products done using the Fragment Analyzer.

Table 7: Optimization of crRNA oligonucleotide modification patterns in mammalian cells.

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
1	uuauauccaacac <u>uucgugguuuuagagcuaugcu</u>	63	61
9	<u>uuauauccaacacac</u> uucgugguuuuagagcuaugcu	1	0
10	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	0	1
22	<u>uuauauccaacacac</u> uucgugguuuuagagcuaugcu	1	1
23	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	5	ND
24	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	3	5
14	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	63	26
15	<u>uuauauccaacacac</u> uucgugguuuuagagcuaugcu	5	3
16	<u>uuauauccaacacac</u> uucgugguuuuagagcuaugcu	5	5
163	C3-uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u> -C3	65	49
164	u*u*a*uauc <u>caacacac</u> uucgugguuuuagagcuaugcu *g*c*u	65	65
165	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	0	3
166	<u>uuauauccaacacac</u> uucgugguuuuagagcuaugcu	54	42
167	uuauauccaacacac <u>uucgugguuuuagagcuaugcu</u>	49	58

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
168	uuauauccaacac <u>u</u> ucgugguuuuagag <u>c</u> ua <u>u</u> gc <u>u</u>	64	60
169	uuauauccaacac <u>u</u> ucgugguuuuagag <u>c</u> ua <u>u</u> gc <u>u</u>	16	16
170	uuauauccaacac <u>u</u> ucgugguuuuagagcuaugcu	3	3
171	uuauauccaacac <u>u</u> ucgugguuuuagagcuaugcu	42	62
172	uuauau <u>c</u> caacac <u>u</u> ucgugguuuuagagcuaugcu	4	13
173	uuauauccaacac <u>u</u> ucgugguuuuagagcuaugcu	1	1
48	cuuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u	61	60
174	<u>c</u> uuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u	60	59
175	cuuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u	62	60
176	<u>c</u> uuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u	61	59
177	c*u*u*a <u>u</u> auccaacac <u>u</u> ucgugguuuuagagcua u*g*c <u>u</u>	60	59
178	c*u*u*a <u>u</u> auccaacac <u>u</u> ucgugguuuuagagcua u*g*c <u>u</u>	61	59
179	C3-cuuauauccaacac <u>u</u> ucgugguuuuagagcua ugcu	61	58
180	cuuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u-C3	57	59
181	C3-cuuauauccaacac <u>u</u> ucgugguuuuagagcua ugcu-C3	62	59
182	ZEN-cuuauauccaacac <u>u</u> ucgugguuuuagagcua augcu	64	62
183	cuuauauccaacac <u>u</u> ucgugguuuuagagcuaugc u-ZEN	62	60
184	ZEN-cuuauauccaacac <u>u</u> ucgugguuuuagagcua augcu-ZEN	64	64
185	ZEN-cuuauauccaacac <u>u</u> ucgugguuuuagagcua augcu-ZEN	60	63
186	u*u*a <u>u</u> auccaacac <u>u</u> ucgugguuuuagagcua u*g*c <u>u</u>	64	62
187	c*u*u*a <u>u</u> auccaacac <u>u</u> ucgugguuuuagagcua u*g*c <u>u</u>	65	65

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
188	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
189	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	63	64
190	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	64	62
191	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	64	63
192	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	64	64
193	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	64	65
194	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	60	63
195	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	63	62
196	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	62	63
197	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	61	64
198	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	61	64
199	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	63	68
200	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	59	67
201	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	63	67
202	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	60	69
203	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	53	67
204	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	54	67
205	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	59	62
206	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	58	61
207	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	50	60
208	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	7

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
209	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
210	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
211	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
212	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	56	68
213	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	41	64
214	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	53	67
215	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	2
216	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
217	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
218	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
219	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
220	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
221	+c*+t*uauauccaacacuucguggguuuuagagcu aug*+c*+t	58	61
222	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	31	54
223	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	6	60
224	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	27	57
225	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	2
226	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	2	25
227	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	3	31
228	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	4	35
229	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
230	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
231	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	1
232	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
233	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	33	67
234	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	24	66
235	C3- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> u <u>g</u> <u>c</u> <u>u</u> -C3	56	65
236	C3- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> u <u>g</u> <u>c</u> <u>u</u> -C3	11	55
237	C3- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> u <u>g</u> <u>c</u> <u>u</u> -InvT	62	65
238	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	17	67
239	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	39	66
240	C3- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> u-C3	27	63
241	C3- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> u-C3	14	46
242	ZEN- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> u-ZEN	41	67
243	ZEN- <u>c</u> <u>u</u> <u>a</u> <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> u-ZEN	23	24
244	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	60
245	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	65
246	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	64
247	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	64
248	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	63
249	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	ND	53
250	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>a</u> <u>a</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>U</u> <u>U</u> <u>C</u> <u>G</u> <u>U</u> <u>G</u> <u>G</u> <u>U</u> <u>U</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	2

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
251	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>U</u> <u>U</u> <u>C</u> <u>G</u> <u>U</u> <u>G</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
252	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>G</u> <u>U</u> <u>U</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	18
253	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	3
254	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	5	0
255	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
256	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	27	53
257	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	10	50
258	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	29	47
259	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>*u</u> * <u>g</u> * <u>c</u>	7	45
260	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>*</u> <u>a</u> * <u>u</u> * <u>g</u>	0	4
261	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>*u</u> <u>*a</u> * <u>u</u>	0	0
262	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>*c</u> <u>u</u> * <u>a</u>	0	0
263	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>*g</u> * <u>c</u> <u>*u</u>	0	0
264	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	50	62
265	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>*u</u> * <u>u</u> * <u>u</u> * <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>*g</u> * <u>c</u> * <u>u</u>	45	59
266	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>*a</u> * <u>c</u> * <u>a</u> * <u>c</u> * <u>u</u> * <u>u</u> * <u>c</u> * <u>g</u> * <u>u</u> * <u>g</u> * <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>*g</u> * <u>c</u> * <u>u</u>	26	36
267	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>*a</u> * <u>c</u> * <u>a</u> * <u>c</u> * <u>u</u> * <u>u</u> * <u>c</u> * <u>g</u> * <u>u</u> * <u>g</u> * <u>g</u> <u>*u</u> * <u>u</u> * <u>u</u> * <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>*g</u> * <u>c</u> * <u>u</u>	20	34
268	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	27	59
269	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>a</u> <u>c</u> <u>a</u> <u>c</u> <u>u</u> <u>u</u> <u>c</u> <u>g</u> <u>u</u> <u>g</u> <u>g</u> <u>*u</u> * <u>u</u> * <u>u</u> * <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	45	60
270	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>*a</u> * <u>c</u> * <u>a</u> * <u>c</u> * <u>u</u> * <u>u</u> * <u>c</u> * <u>g</u> * <u>u</u> * <u>g</u> * <u>g</u> <u>u</u> <u>u</u> <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	16	43
271	<u>C</u> 3- <u>c</u> <u>u</u> <u>u</u> <u>a</u> <u>u</u> <u>a</u> <u>u</u> <u>c</u> <u>c</u> <u>a</u> <u>*a</u> * <u>c</u> * <u>a</u> * <u>c</u> * <u>u</u> * <u>u</u> * <u>c</u> * <u>g</u> * <u>u</u> * <u>g</u> * <u>g</u> <u>*u</u> * <u>u</u> * <u>u</u> * <u>u</u> <u>a</u> <u>g</u> <u>a</u> <u>g</u> <u>c</u> <u>u</u> <u>a</u> <u>u</u> <u>g</u> <u>c</u> <u>u</u> -C3	22	45

SEQ ID No.	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No 2	Cleavage % tracrRNA SEQ ID No. 100
272	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	63	57
273	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	59	60
274	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	63	63
275	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	64	62
276	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	0	1
277	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	5	16
278	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	64	61
279	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	64	63
280	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	30	49
281	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	56	60
282	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	53	61
283	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	0	3
284	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	0	2
285	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	2	8
286	cuuauauccaac <u>ac</u> cuucgugguuuuagagcuaugc <u>u</u>	48	61
287	<u>a</u> * <u>u</u> * <u>a</u> *uccaacac <u>u</u> ucgugguuuuagagcua <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
288	<u>u</u> * <u>a</u> * <u>u</u> *ccaacac <u>u</u> ucgugguuuuagagcua <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0
289	+A*+T* <u>a</u> *uccaacac <u>u</u> ucgugguuuuagagcua <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	2	14
290	+T*+A* <u>u</u> *ccaacac <u>u</u> ucgugguuuuagagcua <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>	0	0

Oligonucleotide sequences are shown 5'-3'. Uppercase = DNA; Lowercase = RNA; Underlined = 2'-O-methyl RNA; Italics = 2'-fluoro RNA; +a, +c, +t, +g = LNA; C3 = C3 spacer (propanediol modifier); * = phosphorothioate internucleotide linkage; ZEN = naphthyl-azo modifier; InvT = inverted-dT. The relative functional activity of each

species is indicated by the % cleavage in a T7EI heteroduplex assay when the indicated crRNA is paired with the indicated tracrRNA. ND = not determined.

[00106] Some kind of chemical modification is usually necessary for synthetic nucleic acids to function well in an intracellular environment due to the presence of exonucleases and endonucleases that degrade unmodified oligonucleotides. A wide range of modifications have been described that confer nuclease resistance to oligonucleotides. The precise combination and order of modifications employed that works well for a given application can vary with sequence context and the nature of the protein interactions required for biological function. Extensive prior work has been done relating to chemical modification of antisense oligonucleotides (which interact with RNase H1) and siRNAs (which interact with DICER, AGO2, and other proteins). It is expected that chemical modification will improve function of the CRISPR crRNA:tracrRNA complex. However, it is not possible to predict what modifications and/or pattern of modifications will be compatible with association of the RNAs with Cas9 in a functional way. The present invention defines minimal, moderate, and extensive chemical modification patterns for the crRNA that retain high levels of function to direct Cas9 mediated gene editing in mammalian cells. The survey in Example 7 was performed targeting a single site in the human *HPRT1* gene. Note that modification patterns of the 20 base 5'-end protospacer guide domain of the crRNA that perform well may vary with sequence context. However, it is likely that modification patterns of the 3'-end tracrRNA binding domain that perform well as defined herein will be affected when the sequence of the adjacent protospacer domain changes when different sites are targeted, so the 3'-domain modification patterns shown here will be "universal".

[00107] The results in Table 7 demonstrate that extensive modification is tolerated throughout the 5' and 3' ends of the crRNA. Modification of certain select positions within internal domains of the crRNA lead to reduced activity or totally blocks activity, likely due to altered structure of the folded RNA and/or blocking of protein contact points with the 2'-OH of key RNA residues by the 2'OMe modification. For example, compound SEQ ID No. 204 has 21/36 residues modified with 2'OMe RNA (58%) and retains full activity compared with the unmodified sequence. Compound SEQ ID No.

239 has 30/36 residues modified with 2'OMe RNA (83%) and retains full activity compared with the unmodified sequence. Both of these compounds also have 3 phosphorothioate (PS) modified internucleotide linkages at the 5'- and 3'-ends, which provides additional protection against exonuclease attack. In contrast, SEQ ID No. 165 has only 4/36 residues modified with 2'OMe RNA (11%) yet has totally lost activity.

[00108] Large blocks of sequence were tolerant to 2'OMe modification at the 5'-end and 3'-end of the crRNA, however modification of certain residues in the central portion of the molecule led to inactivation. To further investigate which individual residues can be modified using 2'OMe RNA within the central domain of the crRNA, a single base modification 2'OMe RNA 'walk' was done (SEQ ID Nos. 272-286). Specific residues (positions within the crRNA) were identified that led to large reductions or complete loss of activity. Using the 36 base crRNA SEQ ID No. 239 as model and numbering from the 5'-end of the sequence, substitution of 2'OMe RNA for the natural RNA of residues U15 and U16 lead to substantial loss of activity and residue U19 led to a moderate loss of activity (FIG. 7). These 3 sites lie within the target-specific protospacer guide domain, so sequence varies with target (residues 15, 16, and 19, FIG. 7). It is possible that in certain sequence contexts that these sites will be tolerant to modification. Within the universal tracrRNA-binding domain (residues 21-36), substitution of 2'OMe RNA for the natural RNA of residues U22, U23, and U24 led to substantial loss of activity. Given that this domain does not change with sequence context, it is likely that these sites will not vary in modification tolerance as target sequence changes. Sequence-specific effects of modification in the 20-base target-specific protospacer guide domain are studies in greater detail in Example 10.

[00109] Antisense oligonucleotide are often made with complete PS modification, where every internucleotide linkage is phosphorothioate modified. This extensive level of modification is possible because the protein effector molecule RNase H1 tolerates the PS modification in the ASO when forming a functional substrate/enzyme complex. On the other hand, siRNAs do not tolerate full PS modification; extensive PS modification disrupts productive interaction with the effector protein AGO2. Limited PS end modification of the crRNA can be done with no loss of activity (SEQ ID Nos. 177, 178, 239, etc., have 3 PS linkages on each end). End-modification is desirable as this adds additional protection from exonuclease attack. PS modification of select internal sites

may also be tolerated and may provide additional protection from endonuclease attack. Using SEQ ID No. 264 as a base modification pattern, internal linkages were PS modified in the tracrRNA-binding domain (SEQ ID No. 265), in the 3'-end of the protospacer guide domain (seed region) (SEQ ID No. 266), or both regions (SEQ ID No. 267). Increasing level of PS modification led to reduced functional activity, with SEQ ID No. 267 having ~50% the activity of the less modified SEQ ID No. 264 variant. SEQ ID No 267 has 21 out of 35 internucleotide linkages modified and will be stable to nuclease exposure. In cases where exposure to a high nuclease environment is needed (such as direct IV administration for research or therapeutic indications), this highly modified variant may actually show higher activity than the less modified variants, which will be degraded more quickly.

[00110] There are experimental settings where the PS modification contributes to chemical toxicity. In this case use of other methods to block exonuclease attack are desirable. The crRNA can have a C3 spacer (propanediol modifier) or a ZEN (naphthyl-azo modifier) placed on either or both the 5'-end and 3'-end to block exonuclease attack, obviating the need the PS modification. This strategy can be employed to eliminate the PS-end block modification (See SEQ ID Nos. 179-186). This strategy can be used to reduce PS content of more highly modified crRNA variants. SEQ ID No. 271 has the internal protospacer domain and tracrRNA binding domain PS-modified in the same pattern as SEQ ID No. 267, yet employs only 15 PS internucleotide linkages (instead of 21) and shows improved activity. Therefore combination of non-base end-blocks with internal PS modification may be used to increase nuclease stability while maintaining high activity.

EXAMPLE 8

[00111] The following example demonstrates improved potency of the modified CRISPR crRNAs and tracrRNAs of the present invention. Examples 2-7 employed transfection of crRNA:tracrRNA complexes into human HEK-Cas9 cells at 30 nM concentration. Experimental testing had previously shown that this dose represented the upper shoulder of the dose response curve such that using higher doses of RNA did not improve gene editing efficiency but use of lower doses resulted lower gene editing efficiency. Those measurements were done using unmodified RNAs. The present

example re-examines the dose response of new optimized chemically modified RNAs of the present invention compared with unmodified RNAs and demonstrates that chemical modification (i.e., nuclease stabilization) results in more potent compounds which can be used at lower dose.

[00112] Example 5 demonstrated that the truncated guide RNAs of the present invention performed superior to WT RNAs at 12 sites in the human *HPRT1* gene. Four of these sites (38087, 38231, 38133, and 38285) were chosen for comparison of unmodified vs. modified RNA in the present example. Unmodified crRNAs were paired with the unmodified tracrRNA (SEQ ID No. 2) at a 1:1 molar ratio. Unmodified crRNAs were paired with the modified tracrRNA (SEQ ID No. 100) at a 1:1 molar ratio. Modified crRNAs were paired with the modified tracrRNA (SEQ ID No. 100) at a 1:1 molar ratio. Sequences are shown in Table 8. RNAs were transfected into HEK-Cas9 cells as described previously at 30 nM, 10 nM, and 3 nM concentrations. Cells were incubated for 48 hours at 37°C, then were processed for DNA and studied for evidence of gene editing activity comparing cleavage rates at the *HPRT1* locus in the T7EI mismatch endonuclease assay, with quantitative measurement of products done using the Fragment Analyzer as previously described. Results are shown in Table 8.

Table 8: Increased potency of modified vs. unmodified crRNA:tracrRNA complexes to direct Cas9-mediated gene editing in mammalian cells.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	30 nM Cleavage %	10 nM Cleavage %	3 nM Cleavage %
		tracrRNA Sequence			
38087 Un-cr Un-tr	56	aauuauggggaauacuaggaguuuuagagcuau cu	80	76	35
	2	agcauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcuuu			
38087 Un-cr Mod-tr	56	aauuauggggaauacuaggaguuuuagagcuau cu	83	76	50
	100	a*g*c <u>auagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> u*u			
38087 Mod-cr Mod-tr	445	<u>a*a*u</u> *uauuggggaauacuaggaguuuuagagcu au*g*c <u>*u</u>	77	77	54
	100	a*g*c <u>auagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> u*u			
38231 Un-cr	69	uuuuguaauuaacagcuugcguuuuagagcuau cu	31	4	0

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	30 nM Cleavage %	10 nM Cleavage %	3 nM Cleavage %
		tracrRNA Sequence			
Un-tr	2	agcauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcuuu			
38231 Un-cr Mod-tr	69	uuuuguaauuaacagcuugcguuuuagagcuau cu	45	14	1
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			
38231 Mod-cr Mod-tr	446	<u>u</u> * <u>u</u> * <u>u</u> * <u>uguaauuaacagcuugcguuuuagagcu</u> <u>au</u> * <u>g</u> * <u>c</u> * <u>u</u>	48	25	4
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			
38133 Un-cr Un-tr	78	ggucacuuuaacacacccaguuuuagagcuau cu	73	61	27
	2	agcauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcuuu			
38133 Un-cr Mod-tr	78	ggucacuuuaacacacccaguuuuagagcuau cu	74	61	37
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			
38133 Mod-cr Mod-tr	447	<u>g</u> * <u>g</u> * <u>u</u> * <u>cacuuuaacacacccaguuuuagagcu</u> <u>au</u> * <u>g</u> * <u>c</u> * <u>u</u>	75	66	55
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			
38285 Un-cr Un-tr	48	cuuauauccaacacuucgugguuuuagagcuau cu	66	16	2
	2	agcauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcuuu			
38285 Un-cr Mod-tr	48	cuuauauccaacacuucgugguuuuagagcuau cu	67	16	5
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			
38285 Mod-cr Mod-tr	178	<u>c</u> * <u>u</u> * <u>u</u> * <u>auauccaacacuucgugguuuuagagcu</u> <u>au</u> * <u>g</u> * <u>c</u> * <u>u</u>	62	60	26
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguu</u> <u>aucaacuugaaaaaguggcaccgagucggugcu*</u> <u>u</u> * <u>u</u>			

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA; Underlined = 2'-O-methyl RNA; * = phosphorothioate internucleotide linkage. Unmodified crRNA = Un-cr. Unmodified tracrRNA = Un-tr. Modified crRNA = Mod-cr. Modified tracrRNA = Mod-tr. The relative functional activity of each species is indicated by the % cleavage in a T7EI heteroduplex assay for each dose studied.

[00113] In general, modification of the crRNA and tracrRNA had a small impact on gene editing efficiency when the RNAs were transfected at high dose where the RNAs are present in excess. At lower doses, the modified reagents showed improved potency and, in some cases, markedly improved potency. The degree of improvement varied with site. The very potent site 38087 showed highly efficiency gene editing at the 30 nM and 10 nM doses with all crRNA/tracrRNA variants tested, but at the 3 nM use of the modified tracrRNA (with either of the crRNAs) showed improved activity. A low potency site, such as 38231, showed improved gene editing efficiency even at the highest dose tested (30 nM) using the modified RNAs. Modification of the tracrRNA alone showed benefit, but the greatest benefit was realized when both the crRNA and tracrRNA were modified. FIG. 8 shows a schematic of one effective modified crRNA (SEQ ID No. 178) paired with modified tracrRNA (SEQ ID No. 100), specific for *HPRT1* site 38285. FIG. 9 shows a schematic of a more highly modified pair that is also highly functional, crRNA (SEQ ID No. 239) paired with modified tracrRNA (SEQ ID No. 134), also specific for *HPRT1* site 38285.

[00114] The present example employed transfection of the crRNA:tracrRNA complex into HEK-Cas9 cells, where Cas9 protein is constitutively expressed. Therefore transfected RNAs can bind Cas9 protein immediately, minimizing risk of degradation in the cytoplasm by nucleases. It is anticipated that the benefit of chemical modification of the crRNA and/or tracrRNA will be greater in cases where the transfected RNAs must survive exposure to cellular nucleases while Cas9 protein is being made, as occurs when using protocols where Cas9 mRNA or a Cas9 expression vector is co-transfected with the targeting RNAs, such that Cas9 is not already expressed in the cells. The benefits of using highly modified RNAs will be greatest for *in vivo* applications (such as medical therapeutics) where the RNAs may be exposed to both nucleases present in serum (following IV administration) and cellular cytoplasmic nucleases.

EXAMPLE 9

[00115] Examples 2-8 demonstrate activity of truncated and/or chemically-modified CRISPR crRNAs and/or tracrRNAs to trigger Cas9-mediated genome editing in mammalian cells that constitutively express Cas9. The present example demonstrates that the truncated, modified RNA compositions of the present invention can bind Cas9

protein and this complex can be transfected into human cells and further that transfection of the ribonuclear protein (RNP) complex is sufficient to trigger highly efficient genome editing.

[00116] Reagents specific for human *HPRT1* site 38285 were employed in the present example. Unmodified crRNA was paired with unmodified tracrRNA at a 1:1 molar ratio. Unmodified crRNA was paired with modified tracrRNA at a 1:1 molar ratio. Modified crRNA was paired with modified tracrRNA at a 1:1 molar ratio. Sequences are shown in Table 9. RNAs were transfected into unmodified HEK293 cells as described above except that a 1:1 complex of Cas9 protein (Caribou Biosciences) with crRNA:tracrRNA were employed at 10 nM concentration using increased amounts of RNAiMAX lipid transfection reagent (1.2 μ L, increased over the 0.75 μ L amount used per 100 μ L transfection in 96 well format for the 30 nM RNA-alone transfections in HEK-Cas9 cells). Cells were incubated for 48 hours at 37°C, then were processed for DNA and studied for evidence of gene editing activity comparing cleavage rates at the *HPRT1* locus in the T7EI mismatch endonuclease assay, with quantitative measurement of products done using the Fragment Analyzer as previously described. Results are shown in Table 9.

Table 9: Increased potency of modified vs. unmodified crRNA:tracrRNA complexes to direct Cas9-mediated gene editing in mammalian cells.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	10 nM Cleavage %
		tracrRNA Sequence	
38285 Un-cr Un-tr	48	cuuauauccaacacacuucgugguuuuagagcuaugcu	42
	2	agcauagcaaguuaaaauaaggcuaguccguuaucaacuugaa aaaguggcaccgagucggugcuuu	
38285 Un-cr Mod-tr	48	cuuauauccaacacacuucgugguuuuagagcuaugcu	41
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguuaucaacuug</u> <u>aaaaaguggcaccgagucggugcu</u> * <u>u</u> * <u>u</u>	
38285 Mod-cr Mod-tr	178	<u>c</u> * <u>u</u> * <u>u</u> * <u>auauccaacacacuucgugguuuuagagcuau</u> * <u>g</u> * <u>c</u> * <u>u</u>	54
	100	<u>a</u> * <u>g</u> * <u>cauagcaaguuaaaauaaggcuaguccguuaucaacuug</u> <u>aaaaaguggcaccgagucggugcu</u> * <u>u</u> * <u>u</u>	

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA; Underlined = 2'-O-methyl RNA; * = phosphorothioate internucleotide linkage. Unmodified crRNA = Un-cr. Unmodified tracrRNA = Un-tr. Modified crRNA = Mod-cr. Modified tracrRNA =

Mod-tr. The relative functional activity of each complex is indicated by the % cleavage in a T7EI heteroduplex assay for each dose studied.

[00117] All 3 CRISPR RNA complexes performed well in the RNP-transfection protocol for mammalian genome editing. The unmodified crRNA + unmodified tracrRNA pair (SEQ ID Nos. 48 and 2) and the unmodified crRNA + modified tracrRNA pair (SEQ ID Nos. 48 and 100) performed 2.5x better at 10 nM dose in the RNP protocol than in the HEK-Cas9 protocol, consistent with the less modified RNAs suffering degradation between transfection and eventual complexation with Cas9 protein in the cytoplasm or nucleus. Thus higher doses are needed for unmodified RNAs and in some settings it is likely that unmodified RNAs will fail to direct any genome editing activity. The modified crRNA + modified tracrRNA (SEQ ID NOs. 178 and 100), on the other hand, worked with high efficiency in both protocols.

[00118] The modified, truncated CRISPR RNAs of the present invention work well with direct Cas9 RNP transfection methods.

EXAMPLE 10

[00119] The chemical modification optimization studies performed in Examples 6 and 7 studied the activity of crRNAs having various modification patterns paired with a tracrRNA having various modification patterns. The tracrRNA is universal and the same sequence is employed at all target sites. It is expected that the performance of various modification patterns for the tracrRNA will be similar between different target sites. The crRNA, however, varies sequence between different target sites. In the optimized version tested in Examples 7 and 8, the 5'-20 bases of the crRNA are target-specific (i.e., the "protospacer domain") and the 3'-16 bases are universal (i.e., "the tracrRNA binding domain"). Like the tracrRNA, it is expected that the performance of various modification patterns in the universal 16 base 3'-domain of the crRNA will be similar at all target sites. However, it is possible that performance of different modification patterns may be influenced by the sequence context present in the 5'-20 base target-specific domain.

[00120] It is well established that effective modification patterns for small interfering RNAs (siRNAs) are affected by sequence context (Behlke, Oligonucleotides 18:305-320, 2008). For siRNAs, certain “limited modification” patterns can be applied to all sites, whereas for “heavy modification” it is not possible to predict which patterns will be functional for a given sequence and empiric testing is necessary. The present example studies the effect that sequence context has on the crRNA, testing different modification patterns within the 5’-20 base target-specific domain at different sites.

[00121] The modification studies in Examples 6 and 7 employed a single crRNA PAM site in the human *HPRT1* gene. The present study examines 12 sites in the human *HPRT1* gene, including the site previously examined, comparing functional performance of different modification patterns and establishes a single modification pattern that can be employed with good results at all sites. See Example 5 for other studies relating to these 12 sites.

[00122] A series of crRNAs (Table 10) were synthesized having a protospacer domain lengths of 20 bases specific to 12 sites in the human *HPRT1* gene with a 16mer universal tracrRNA binding sequence at the 3’-end. The crRNAs were made using a variety of chemical modifications, including: the ribose modifications 2’OMe RNA, the end-modifying groups propanediol spacer and naphthyl-azo modifier (N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine, or “ZEN”), an inverted-dT residue; and select internucleotide linkages with phosphorothioate modifications. A schematic representation of the different modification patterns employed is shown in FIG. 10.

[00123] The crRNAs were paired with a highly modified 67mer tracrRNA (SEQ ID No. 100). The paired crRNA:tracrRNA RNA oligonucleotides were transfected into the HEK-Cas9 cells and processed as described in Example 2. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay with quantitative measurement of products done using the Fragment Analyzer. Results are shown in Table 10 and in FIG. 11.

Table 10: Optimization of crRNA oligonucleotide modification patterns in mammalian cells across 12 target sites.

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38094	64	1	uccauuuc <u>au</u> agucuuuccg <u>uuuu</u> agagcu <u>augcu</u>	62
38231	69	1	uuuugua <u>uuu</u> aacagcuugcg <u>uuuu</u> agagcu <u>augcu</u>	35
38371	71	1	c <u>uu</u> agaga <u>uuu</u> uuug <u>u</u> agagguuuuagagcu <u>augcu</u>	66
38509	73	1	uugacuaua <u>u</u> ga <u>uu</u> acuucg <u>uuuu</u> agagcu <u>augcu</u>	71
38574	75	1	caaaacacgc <u>u</u> aaaa <u>uuu</u> guuuuagagcu <u>augcu</u>	52
38087	56	1	aa <u>uuu</u> augggg <u>uu</u> acuag <u>g</u> aguuuuagagcu <u>augcu</u>	72
38133	78	1	ggucacuuuu <u>a</u> acacacccaguuuuagagcu <u>augcu</u>	65
38285	48	1	c <u>uu</u> auauccaacac <u>u</u> ucgug <u>uuuu</u> agagcu <u>augcu</u>	62
38287	80	1	ggc <u>uu</u> auauccaacac <u>u</u> ucgguuuuagagcu <u>augcu</u>	47
38358	60	1	auuucacauaaa <u>c</u> ucuuu <u>u</u> guuuuagagcu <u>augcu</u>	59
38636	83	1	ucaa <u>uuu</u> augaggugcuggaguuuuagagcu <u>augcu</u>	27
38673	85	1	uacagcuuuuagugacua <u>u</u> guuuuagagcu <u>augcu</u>	49
38094	291	2	<u>u</u> * <u>c</u> * <u>c</u> *auuuc <u>au</u> agucuuuccg <u>uuuu</u> agagcuau * <u>g</u> * <u>c</u> * <u>u</u>	71
38231	292	2	<u>u</u> * <u>u</u> * <u>u</u> *ugua <u>uuu</u> aacagcuugcg <u>uuuu</u> agagcuau * <u>g</u> * <u>c</u> * <u>u</u>	54
38371	293	2	<u>c</u> * <u>u</u> * <u>u</u> *agaga <u>uuu</u> uuug <u>u</u> agagguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	65
38509	294	2	<u>u</u> * <u>u</u> * <u>g</u> *acuaua <u>u</u> ga <u>uu</u> acuucg <u>uuuu</u> agagcuau * <u>g</u> * <u>c</u> * <u>u</u>	78
38574	295	2	<u>c</u> * <u>a</u> * <u>a</u> *aacacgc <u>u</u> aaaa <u>uuu</u> guuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	56
38087	296	2	<u>a</u> * <u>a</u> * <u>u</u> *uauugggg <u>uu</u> acuag <u>g</u> aguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	76
38133	297	2	<u>g</u> * <u>g</u> * <u>u</u> *cacuuuu <u>a</u> acacacccaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	70
38285	178	2	<u>c</u> * <u>u</u> * <u>u</u> *auauccaacac <u>u</u> ucgug <u>uuuu</u> agagcuau * <u>g</u> * <u>c</u> * <u>u</u>	65

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38287	298	2	<u>g</u> * <u>g</u> * <u>c</u> *uuauauccaacacuucgguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	59
38358	299	2	<u>a</u> * <u>u</u> * <u>u</u> *ucacauaaaacucuuuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	73
38636	300	2	<u>u</u> * <u>c</u> * <u>a</u> * <u>a</u> uuuauaggugcuggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	29
38673	301	2	<u>u</u> * <u>a</u> * <u>c</u> *agcuuuauugugacuaauguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	60
38094	302	3	<u>u</u> * <u>c</u> * <u>c</u> * <u>a</u> uuuauagucuuuccguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	67
38231	303	3	<u>u</u> * <u>u</u> * <u>u</u> * <u>u</u> guaauuaacagcuugcguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	57
38371	304	3	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> gagaauauuuuguagagguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	65
38509	305	3	<u>u</u> * <u>u</u> * <u>g</u> * <u>a</u> cuauaaugaauacuucguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	79
38574	306	3	<u>c</u> * <u>a</u> * <u>a</u> * <u>a</u> acacgcuaaaaaauuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	52
38087	307	3	<u>a</u> * <u>a</u> * <u>u</u> * <u>u</u> auuggggauuacuaggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	76
38133	308	3	<u>g</u> * <u>g</u> * <u>u</u> * <u>c</u> acuuuuuacacacccaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	66
38285	309	3	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> uauccaacacuucgugguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	60
38287	310	3	<u>g</u> * <u>g</u> * <u>c</u> *uuauauccaacacuucgguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	56
38358	311	3	<u>a</u> * <u>u</u> * <u>u</u> * <u>u</u> ucacauaaaacucuuuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	66
38636	312	3	<u>u</u> * <u>c</u> * <u>a</u> * <u>a</u> uuuauaggugcuggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	24
38673	313	3	<u>u</u> * <u>a</u> * <u>c</u> * <u>a</u> gcuuuauugugacuaauguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	51
38094	314	4	<u>u</u> * <u>c</u> * <u>c</u> * <u>a</u> uuuauagucuuuccguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	68
38231	315	4	<u>u</u> * <u>u</u> * <u>u</u> * <u>u</u> guaauuaacagcuugcguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	53
38371	316	4	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> gagaauauuuuguagagguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	65
38509	317	4	<u>u</u> * <u>u</u> * <u>g</u> * <u>a</u> cuauaaugaauacuucguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	76
38574	318	4	<u>c</u> * <u>a</u> * <u>a</u> * <u>a</u> acacgcuaaaaaauuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	51

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38087	319	4	<u>a</u> * <u>a</u> * <u>u</u> * <u>u</u> auggggauuacuaggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	76
38133	320	4	<u>g</u> * <u>g</u> * <u>u</u> * <u>c</u> acuuuuuacacacccaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	70
38285	321	4	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> auuccaacacuucgugguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	65
38287	322	4	<u>g</u> * <u>g</u> * <u>c</u> * <u>u</u> uauauccaacacuucgguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	56
38358	323	4	<u>a</u> * <u>u</u> * <u>u</u> * <u>u</u> cacauaaaacucuuuuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	64
38636	324	4	<u>u</u> * <u>c</u> * <u>a</u> * <u>a</u> auuauaggugcuggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	23
38673	325	4	<u>u</u> * <u>a</u> * <u>c</u> * <u>a</u> gcuuuauugugacuaauguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	48
38094	326	5	<u>u</u> * <u>c</u> * <u>c</u> * <u>a</u> uuuucauagucuuuccguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	71
38231	327	5	<u>u</u> * <u>u</u> * <u>u</u> * <u>u</u> guaauuaacagcuugcguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	53
38371	328	5	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> gagaauauuuuguagagguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	69
38509	329	5	<u>u</u> * <u>u</u> * <u>g</u> * <u>a</u> cuauaauugaauacuucguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	77
38574	330	5	<u>c</u> * <u>a</u> * <u>a</u> * <u>a</u> aacacgcuaaaaaauuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	51
38087	331	5	<u>a</u> * <u>a</u> * <u>u</u> * <u>u</u> auggggauuacuaggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	80
38133	332	5	<u>g</u> * <u>g</u> * <u>u</u> * <u>c</u> acuuuuuacacacccaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	70
38285	333	5	<u>c</u> * <u>u</u> * <u>u</u> * <u>a</u> auuccaacacuucgugguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	64
38287	334	5	<u>g</u> * <u>g</u> * <u>c</u> * <u>u</u> uauauccaacacuucgguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	59
38358	335	5	<u>a</u> * <u>u</u> * <u>u</u> * <u>u</u> cacauaaaacucuuuuuguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	64
38636	336	5	<u>u</u> * <u>c</u> * <u>a</u> * <u>a</u> auuauaggugcuggaguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	25
38673	337	5	<u>u</u> * <u>a</u> * <u>c</u> * <u>a</u> gcuuuauugugacuaauguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	56
38094	338	6	<u>u</u> * <u>c</u> * <u>c</u> * <u>a</u> uuuucauagucuuuccguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	70
38231	339	6	<u>u</u> * <u>u</u> * <u>u</u> * <u>u</u> guaauuaacagcuugcguuuuagagcuau * <u>g</u> * <u>c</u> * <u>u</u>	53

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38371	340	6	<u>c*u*u*agagaauuuuguagagguuuuagagcuau</u> <u>*g*c*u</u>	68
38509	341	6	<u>u*u*g*acuauaaugaauacuucguuuuagagcuau</u> <u>*g*c*u</u>	72
38574	342	6	<u>c*a*a*aacacgc<u>au</u>aaaaauuuguuuuagagcuau</u> <u>*g*c*u</u>	51
38087	343	6	<u>a*a*u*uaugggg<u>au</u>acuaggaguuuagagcuau</u> <u>*g*c*u</u>	81
38133	344	6	<u>g*g*u*cacuuuu<u>aa</u>acacaccaguuuagagcuau</u> <u>*g*c*u</u>	71
38285	345	6	<u>c*u*u*auauccaacacuucgugguuuuagagcuau</u> <u>*g*c*u</u>	64
38287	346	6	<u>g*g*c*uuauauccaacacuucgguuuuagagcuau</u> <u>*g*c*u</u>	55
38358	347	6	<u>a*u*u*ucacauaaaacucuuuuguuuuagagcuau</u> <u>*g*c*u</u>	65
38636	348	6	<u>u*c*a*aa<u>uu</u>augaggugcuggaguuuagagcuau</u> <u>*g*c*u</u>	24
38673	349	6	<u>u*a*c*agcuuu<u>au</u>gugacuaauguuuagagcuau</u> <u>*g*c*u</u>	55
38094	350	7	<u>u*c*c*auuu<u>ca</u>uagucuuuccguuuuagagcuau</u> <u>*g*c*u</u>	73
38231	351	7	<u>u*u*u*uguaauuu<u>aa</u>cagcuugcguuuagagcuau</u> <u>*g*c*u</u>	51
38371	352	7	<u>c*u*u*agagaauuuuguagagguuuuagagcuau</u> <u>*g*c*u</u>	73
38509	353	7	<u>u*u*g*acuauaaugaauacuucguuuuagagcuau</u> <u>*g*c*u</u>	78
38574	354	7	<u>c*a*a*aacacgc<u>au</u>aaaaauuuguuuuagagcuau</u> <u>*g*c*u</u>	50
38087	355	7	<u>a*a*u*uaugggg<u>au</u>acuaggaguuuagagcuau</u> <u>*g*c*u</u>	83
38133	356	7	<u>g*g*u*cacuuuu<u>aa</u>acacaccaguuuagagcuau</u> <u>*g*c*u</u>	63
38285	357	7	<u>c*u*u*auauccaacacuucgugguuuuagagcuau</u> <u>*g*c*u</u>	63
38287	358	7	<u>g*g*c*uuauauccaacacuucgguuuuagagcuau</u> <u>*g*c*u</u>	43
38358	359	7	<u>a*u*u*ucacauaaaacucuuuuguuuuagagcuau</u> <u>*g*c*u</u>	66
38636	360	7	<u>u*c*a*aa<u>uu</u>augaggugcuggaguuuagagcuau</u> <u>*g*c*u</u>	28

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38673	361	7	<u>u*a*c*agcuuuau</u> gugacuaa <u>uguuuagagcuau</u> * <u>g*c*u</u>	61
38094	362	8	<u>u*c*c*auuuc</u> auagucuu <u>uccuguuuuagagcuau</u> * <u>g*c*u</u>	63
38231	363	8	<u>u*u*u*uguaauu</u> aacagcuugc <u>guuuuagagcuau</u> * <u>g*c*u</u>	40
38371	364	8	<u>c*u*u*agaga</u> auauu <u>uguagagguuuuagagcuau</u> * <u>g*c*u</u>	64
38509	365	8	<u>u*u*g*acuau</u> aaugaauacuuc <u>guuuuagagcuau</u> * <u>g*c*u</u>	67
38574	366	8	<u>c*a*a*aacac</u> gcauaaaaa <u>uuuguuuuagagcuau</u> * <u>g*c*u</u>	18
38087	367	8	<u>a*a*u*uaugggg</u> auuacuagga <u>guuuuagagcuau</u> * <u>g*c*u</u>	75
38133	368	8	<u>g*g*u*cacuuu</u> aaacacac <u>ccaguuuuagagcuau</u> * <u>g*c*u</u>	48
38285	369	8	<u>c*u*u*auaucca</u> acacuucgug <u>guuuuagagcuau</u> * <u>g*c*u</u>	53
38287	370	8	<u>g*g*c*uuauaucca</u> acacuucg <u>guuuuagagcuau</u> * <u>g*c*u</u>	24
38358	371	8	<u>a*u*u*ucacau</u> aaaacucuuu <u>uguuuuagagcuau</u> * <u>g*c*u</u>	56
38636	372	8	<u>u*c*a*auuuau</u> gaggugcugga <u>guuuuagagcuau</u> * <u>g*c*u</u>	22
38673	373	8	<u>u*a*c*agcuuu</u> au <u>gugacuaa</u> uguuuagagcuau * <u>g*c*u</u>	50
38094	374	9	<u>u*c*c*auuuc</u> auagucuu <u>uccuguuuuagagcuau</u> * <u>g*c*u</u>	65
38231	375	9	<u>u*u*u*uguaauu</u> aacagcuugc <u>guuuuagagcuau</u> * <u>g*c*u</u>	7
38371	376	9	<u>c*u*u*agaga</u> auauu <u>uguagagguuuuagagcuau</u> * <u>g*c*u</u>	70
38509	377	9	<u>u*u*g*acuau</u> aaugaauacuuc <u>guuuuagagcuau</u> * <u>g*c*u</u>	57
38574	378	9	<u>c*a*a*aacac</u> gcauaaaaa <u>uuuguuuuagagcuau</u> * <u>g*c*u</u>	8
38087	379	9	<u>a*a*u*uaugggg</u> auuacuagga <u>guuuuagagcuau</u> * <u>g*c*u</u>	74
38133	380	9	<u>g*g*u*cacuuu</u> aaacacac <u>ccaguuuuagagcuau</u> * <u>g*c*u</u>	38
38285	222	9	<u>c*u*u*auaucca</u> acacuucgug <u>guuuuagagcuau</u> * <u>g*c*u</u>	54

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38287	381	9	<u>g*g*c*</u> uuauauccaacacuucgguuuuagagcuau <u>*g*c*u</u>	32
38358	382	9	<u>a*u*u*</u> ucacauaaaacucuuuuguuuuagagcuau <u>*g*c*u</u>	58
38636	383	9	<u>u*c*a*</u> aaauaugaggugcuggaguuuuagagcuau <u>*g*c*u</u>	19
38673	384	9	<u>u*a*c*</u> agcuuuugugacuaauguuuagagcuau <u>*g*c*u</u>	55
38094	385	10	C3- uccauuucauagucuuuccguuuuagagcuauugcu -C3	66
38231	386	10	C3- uuuuguaauuaacagcuugcgguuuuagagcuauugcu -C3	54
38371	387	10	C3- cuuagagaauauuuuguagaggguuuuagagcuauugcu -C3	57
38509	388	10	C3- uugacuauaauugaauacuucguuuuagagcuauugcu -C3	75
38574	389	10	C3- caaaacacgcuaaaaaauuuguuuuagagcuauugcu -C3	50
38087	390	10	C3- aauuauaggggaauacuaggaguuuuagagcuauugcu -C3	71
38133	391	10	C3- ggucacuuuuuacacacccaguuuuagagcuauugcu -C3	68
38285	181	10	C3- cuuauauccaacacuucgugguuuuagagcuauugcu -C3	58
38287	392	10	C3- ggcuuauauccaacacuucgguuuuagagcuauugcu -C3	57
38358	393	10	C3- auuucacauaaaacucuuuuguuuuagagcuauugcu -C3	64
38636	394	10	C3- ucaaauuauagaggugcuggaguuuuagagcuauugcu -C3	22
38673	395	10	C3- uacagcuuuuugugacuaauguuuagagcuauugcu -C3	50
38094	396	11	ZEN- uccauuucauagucuuuccguuuuagagcuauugcu -ZEN	74
38231	397	11	ZEN- uuuuguaauuaacagcuugcgguuuuagagcuauugcu -ZEN	44
38371	398	11	ZEN- cuuagagaauauuuuguagaggguuuuagagcuauugcu -ZEN	72

HPRT1 Target site	SEQ ID No.	Mod Pattern	crRNA Sequence (5'-3')	Cleavage % tracrRNA SEQ ID No. 100
38509	399	11	ZEN- uug <u>ac</u> uauaauga <u>aa</u> uac <u>u</u> cg <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	74
38574	400	11	ZEN- caaaacacgc <u>au</u> aaaaa <u>uuu</u> g <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	57
38087	401	11	ZEN- aa <u>uu</u> auggggga <u>uu</u> acuag <u>g</u> ag <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	82
38133	402	11	ZEN- ggucac <u>uuuu</u> aacacacccag <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	73
38285	184	11	ZEN- cuuaua <u>ucca</u> acac <u>u</u> cgug <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	60
38287	403	11	ZEN- ggcuuaua <u>ucca</u> acac <u>u</u> cg <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	62
38358	404	11	ZEN- auuucacauaaaacuc <u>uuu</u> g <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	69
38636	405	11	ZEN- ucaaa <u>uu</u> augaggugcuggag <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	26
38673	406	11	ZEN- uacagcu <u>uu</u> augugacuaa <u>u</u> g <u>uuuu</u> agagcu <u>aug</u> cu -ZEN	44

Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA; Underlined = 2'-O-methyl RNA; C3 = C3 spacer (propanediol modifier); * = phosphorothioate internucleotide linkage; ZEN = naphthyl-azo modifier. The relative functional activity of each species is indicated by the % cleavage in a T7EI heteroduplex assay when the indicated crRNA is paired with the indicated tracrRNA at each of 12 sites in human *HRPT1*.

[00124] The modified crRNAs employed a fixed modification pattern in the 16-base 3'-end domain which is universal and binds the tracrRNA. Different modification pattern were tested/compared in the 5'-end domain that is target specific (i.e., sequence varies with target site). The test set comprised variants having 0, 3, 4, 6, 8, 10, 12, 13, or 14 contiguous 2'OMe RNA residues starting at the 5'-end and walking towards the 3'-end. The modification patterns avoided positions previously demonstrated to reduce functional performance of the crRNA (Example 7). Use of only non-base modifier end groups (C3 spacer or ZEN) were also tested (without additional modification). When functional activity is compared across all 12 sites in the survey, all sites tested showed

full activity when 0-10 RNA residues at the 5'-end were replaced with 2'OMe RNA residues. Only 1/12 sites showed a slight reduction in activity with 12 residues modified, however 3/12 sites showed a reduction in activity when 13 residues were modified and 4/12 sites showed a reduction in activity when 14 residues were modified. The end-modifiers (C3, ZEN) showed full activity at all sites.

[00125] The highest level of crRNA modification that showed full activity at all sites tested included Mod Patterns 6 and 7 (FIG. 10). This represents 61% and 67% of the bases in the crRNA modified with 2'OMe RNA, respectively.

n*n*nnnnnnnnnnnnnnnnnnguuuagagcuau*g*c*u Mod Pattern 6
(SEQ ID NO.:434)

n*n*nnnnnnnnnnnnnnnnnnnnguuuagagcuau*g*c*u Mod Pattern 7
(SEQ ID NO.:435)

[00126] The data in the present example also demonstrates that individual sites can be modified at higher levels and retain potency. For example, 8 of the 12 sites studied showed full activity using Mod Pattern 8, which has 72% of the residues modified. Further, example 7 demonstrates that the crRNA targeting site 38285 in HPRT1 (SEQ ID No. 239) has full activity and has 30/36 residues modified (83%, with only 6 unmodified RNA residues remaining). A base modification pattern such as Mod Pattern 6 or Mod Pattern 7 can be used as a starting point for studies to empirically ascertain the extent that a particular sequence can be modified before activity is lost. FIG. 12 shows a schematic where a Mod Pattern 6 crRNA is paired with a highly modified tracrRNA, SEQ ID No. 134.

EXAMPLE 11

[00127] The Examples herein employ the Cas9 endonuclease from *Streptococcus pyogenes*. The native amino acid sequence of *S.py.* Cas9 (SpyCas9) is shown below (SEQ ID No. 407).

[00128] The native Cas9 DNA sequence was codon optimized for expression in *E.coli* bacteria and had elements added for mammalian nuclear localization (nuclease localization signals) and aid protein purification (His-tag). The final amino-acid

sequence of the recombinant protein is shown (SEQ ID No 408). The DNA sequence employed to express the recombinant protein in *E.coli* is shown (SEQ ID No. 409).

[00129] The native Cas9 DNA sequence was codon optimized for expression in human cells and had elements added for antibody recognition (V5 epitope) and mammalian nuclear localization (nuclease localization signals, NLS) added. The final amino-acid sequence is shown (SEQ ID No. 410) and DNA sequence follows (SEQ ID No 411).

[00130] The native *S.py* Cas9 DNA sequence codon was optimized for expression in human cells and assembled as a T7 RNA polymerase expression cassette (SEQ ID No. 412). The sequence contains a T7 RNA polymerase promoter, a V5 epitope tag, a nuclear localization signal, the codon optimized Cas9 sequence, a second nuclear localization signal, and the BGH (bovine growth hormone) gene 3'-UTR element with a polyadenylation signal. Sequence of mRNA made from this expression cassette is shown (SEQ ID No. 413).

S.py Cas9 amino acid sequence (SEQ ID No. 407).

```
MDKKYSIGLDIGTNSVGWAVITDEYKVPSSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRR
YTRRKNRICYLQEIFSNEMAKVDDSSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKL
VDSTDKADLRILIYLAHMIKFRGHFLIEGDLNPDNSDVKLFILQVQTYNQLFEEENPINASGVDAKAILS
ARLSKSRRLLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDITYDDDLNLLAQIGD
QYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQS
KNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHILRRQE
DFYFPFLKDNREKIEKILTFRIPIYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNF
DKNLPNEKVLPKHSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYF
KKIECFDSVEISGVEDRFNASLGTYHDLKKI IKDKDFLDNEENEDILEDIVLTTLTFEDREMIEERLKYA
HLFDDKVMKQLKRRRYTGWRLSRKLINGIRDQKSGKTILDFLKSDFANRNFQMQLIHDDSLTFKEDIQKA
QVSGQGDSLHEHIANLAGSPAIIKKGILQTVKVVDLVKVMGRHKPENIVIEMARENQTTQKGQKNSRERMK
RIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDHDHIVPQSFLKDDSID
NKVLTRSDKNRGKSDNVPSEEVVKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVET
RQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYKVRINNYHHAHDAYLNAVVG
ALIKKYPKLESEFVYGDYKVDVRKMIKSEQEI GKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETN
GETGEIVWDKGRDFATVRKVLSPQVNIKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSP
TVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSEFKNPIDFLEAKGYKEVKKDLIIKLPKYSLELEN
GRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEQNEQKQLFVEQHKHYLDEIIIEQISEFSKR
VILADANLDKVL SAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLDTLIHQ
SITGLYETRIDLSQLGGD
```

S.py Cas9 amino acid sequence expressed from DNA codon optimized for expression in *E.coli* containing 3 NLS sequences and a purification His-tag (SEQ ID No. 408).

MGSSAPKKKRKVGIVHVPAAAMDKKYSIGLDIGTNSVGWAVITDEYKVPSSKKFKVLGNTRHSIKKNLIGAL
 LFDSETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRLSEESFLVEEDKKHERHPIFGNI
 VDEVAYHEKYPTIYHLRKKLVSDTDKADLRILIYLAHAMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYN
 QLFEEENPINASGVDAKAILSARLSKSRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKL
 QLSKDTYDDDLNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLL
 KALVRQQLPPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFD
 NGSIPHQIHLGELHAILRRQEDFYFPFLKDNREKIEKILTFRIPIYYVGPLARGNSRFAWMTRKSEETITPWN
 FEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEFYTVYNELTKVKYVTEGMRKPAFLSGEQKKAI
 VDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDI
 VLTLTLFEDREMIEERLKYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFAN
 RNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPAIIKKGILQTVKVVDELVKVMGRHKPENIVI
 EMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINR
 LSDYDVDHIVPQSFLKDDSIDNKVLTTRSDKNRGSNDNVPSEEVVKMKNYWRQLLNAKLITQRKFDNLTKA
 ERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSCLVSDFRKDFQFYK
 VREINNYHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAKYFFYSNIMNF
 FKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVLSPQVNIKKTEVQTGGFSKESILPKRNS
 DKLIARKKDWDPKKGFFDSPTVAYSVLVAKVEKGKSKLKSVEKLLGITIMERSSEFKNPIDFLEAKGY
 KEVKKDLIIKLPKYSLELENGRKRMLASAGELQKGNELALPSKYVNFYLYLASHYEKLKGPSPEDNEQKQLF
 VEQHKHYLDEIIEQISEFSKRVIILADANLDKVL SAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDT
 TIDRKRYTSTKEVLDATLIHQSIITGLYETRIDLSQLGGDAAPKKKRKVDPKKKRKVAAAALHHHHHH

S.py Cas9 DNA sequence codon optimized for expression in *E.coli* containing 3 NLS
 sequences and a purification His-tag (SEQ ID No. 409).

ATGGGCAGCAGCGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCAGCCATGGACAAAA
 GTACTCTATTGGCCTGGATATCGGGACCAACACGCGTCGGGTGGGCTGTTATCACCGACGAGTATAAAGTAC
 CTTGCAAAAAGTTCAAAGTGCTGGGCAACACCGATCGCCATTCAATCAAAAAGAACTTGATTGGTGCGCTG
 TTGTTTGACTCCGGGGAAACCGCCGAGGCGACTCGCCTTAAACGTACAGCACGTCGCCGGTACACTCGGCG
 TAAGAATCGCATTTGCTATTTGCAGGAAATCTTTAGCAACGAGATGGCAAAAGTCGATGACTCGTTTTTCC
 ACCGCTCGAGGAAAGCTTTCTGGTGGAGGAAGACAAAAAGCATGAGCGTCACCCGATCTTCGGCAACATT
 GTCGATGAAGTAGCGTATCATGAAAAATACCAACCATTTACCACTTACGCAAAAAGCTGGTGGACAGCAC
 TGACAAAGCTGATTTGCGCCTTATCTATTTAGCCCTGGCACATATGATTAAGTTTCGTGGTCACTTCCTGA
 TCGAAGGAGACTTAAATCCCAGCAACAGTGATGTTGATAAATTGTTTATTCAGCTTGTCCAAACTTACAAT
 CAACTGTTTCGAGGAAAACCCGATCAATGCCTCCGGTGTGGATGCAAAAGCCATTTTAAAGTGACGCTTAG
 CAAGTCCCGTCGCTTAGAAAACCTTATCGCGCAGCTGCCCAGGAGAAAAAGAAATGGTTTGTGGGAACC
 TTATTGCCTTGAGCTTAGGCCTCACCCGAATTTCAAAGTAATTTGATCTTGCAGAAGACGCCAAATTA
 CAACTGTGCAAGGATACTTATGATGACGATCTCGATAATCTGTTAGCGCAGATTGGTGACCAATACGCCGA
 TCTTTTTCTGGCGGCTAAAAATCTGAGCGACGCCATCTTGCTTTCCGATATTCTCCGCGTTAACACCGAAA
 TCACGAAAGCGCCTCTTAGTGCCAGCATGATTAACGTTATGATGAACACCACCAGGACCTGACCTTACTC
 AAAGCGTTGGTTCGCCAGCAACTGCCAGAGAAGTACAAAGAAATCTTCTTTGATCAGTCAAAGAATGGTTA
 TGCCGGCTATATTGACGGGGGTGCAAGCCAAGAGGAATTCTACAAATTTATCAAGCCTATTCTGGAGAAAA
 TGGATGGCACCAGAGATTATTGGTGAAGCTTAACCGTGAAGACCTCCTGCGGAAACAGCGCACATTCGAT
 AATGGTTGATCCACACCAAATCCATTTGGGGGAGTTACACGCTATTTTTCGTCGCCAGGAAGACTTTTA
 CCCTTTCTGAAGGATAACCGGGAGAAAAATTGAGAAGATCCTTACCTTTTCGTATTCCGTATTACGTAGGCC
 CCTTAGCACGGGGTAATAGCCGTTTCGCGTGGATGACACGGAAGTCGGAAGAGACGATCACCCCGTGAAC
 TTCGAAGAGGTAGTCGACAAGGGCGCATCAGCGCAGTCTTTTATTGAACGTATGACGAATTTTCGATAAAAA
 CTTGCCCAATGAGAAGGTGCTTCCGAAACATTCTTGTATATGAATATTTTACAGTTTACAACGAGCTGA
 CCAAGGTAAATACGTGACGGAAGGAATGCGCAAGCCCGCTTTTCTTAGCGGTGAGCAAAAAAAGGCGATC
 GTCGACCTGTTATTCAAACGAATCGTAAGGTGACTGTAAAGCAACTCAAAGAAGATTACTTCAAAGAT

TGAGTGCTTCGACAGCGTCGAAATCTCTGGGGTAGAGGATCGGTTTAAACGCAAGTTTAGGTACCTACCATG
 ACCTGCTTAAATCATTAAGGATAAAGACTTCTTAGATAATGAAGAGAACGAAGATATTCTCGAGGACATC
 GTCTTGACGTTAACCTTATTTGAGGATCGTGAAATGATTGAGGAACGCCTCAAACTTATGCCACCTGTT
 CGACGATAAGGTGATGAAGCAGCTGAAACGTCGGCGCTACACAGGATGGGGCCGCTTGAGTCGCAAACTTA
 TTAACGGAATCCGTGACAAGCAATCCGGCAAAACGATTCTGGATTTCTTGAAGTCGGACGGATTTGCTAAT
 CGCAACTTCATGCAGTTGATCCATGATGACTCCCTGACTTTTAAAGAGGATATTCAAAGGCGCAGGTTAG
 TGGTCAAGGCGACAGCTTACACGAACACATCGCAAAATTTGGCTGGTTCGCCGGCCATTAAAAAGGGGATCC
 TCCAGACCGTGAAAGTTGTAGATGAGCTTGTTAAGGTCATGGGTCGTCATAAGCCCGAAAACATCGTGATT
 GAAATGGCGCGGGAGAATCAAACGACCCAGAAAGGACAAAAGAATAGCCGTGAACGGATGAAGCGGATCGA
 GGAAGGCATTAAAGAGCTGGGGTCTCAAATCTTGAAGGAACACCCTGTGGAGAACACTCAGCTCCAAATG
 AAAAATTTTACCTGTACTATTTGCAGAACGGACGCGATATGTACGTGGACCAAGAGTTGGATATTAATCGG
 CTGAGTGACTACGACGTTGATCATATCGTCCCGCAGAGCTTCCTCAAAGACGATTCTATTGACAATAAGGT
 ACTGACGCGCTCTGATAAAAAACCGTGGTAAGTCGGACAACGTGCCCTCCGAAGAGGTTGTGAAAAAGATGA
 AAAATTATTGGCGCCAGCTTTTAAACGCGAAGCTGATCACACAACGTAAATTCGATAATTTGACCAAGGCT
 GAACGGGGTGGCCTGAGCGAGTTAGATAAGGCAGGATTTATTAAACGCCAGTTAGTGGAGACTCGTCAAAT
 CACCAAACATGTCGCGCAGATTTTGGACAGCCGGATGAACACCAAGTACGATGAAAATGACAACTGATCC
 GTGAGGTGAAAGTCATTACTCTGAAGTCCAAATTAGTTAGTGATTTCCGGAAGGACTTTCAATTCTACAAA
 GTCCGTGAAATTAATAACTATCATCACGCACATGACGCGTACCTGAATGCAGTGGTTGGGACCGCCCTTAT
 CAAGAAATATCCTAAGCTGGAGTCGGAGTTTGTCTATGGCGACTATAAGGTATACGATGTTTCGCAAAATGA
 TTGCGAAATCTGAGCAGGAGATCGGTAAGGCAACCGCAAAATATTTCTTTTACTCAAACATTATGAATTTT
 TTTAAGACAGAAATCACTCTGGCCAACGGGGAGATTCGCAAACGTCCGTTGATCGAAACAAACGGCGAGAC
 TGGCGAAATTGTTTGGGACAAAGGGCGTGATTTGCGGACGGTGCGCAAGGTACTGAGCATGCCTCAAGTCA
 ATATTGTTAAGAAAACCGAAGTGCAGACGGGCGGGTTTCCAAAGGAAAGCATCTTACCCAAACGTAATTCA
 GATAAACTTATTGCACGCAAAAAAGGACTGGGATCCGAAAAAGTATGGAGGCTTCGACAGTCCAACCGTAGC
 CTACTCTGTTCTCGTTGTAGCGAAAAGTAGAAAAAGGGTAAATCCAAGAACTGAAATCTGTCAAGGAGTTGC
 TTGGAATCACCATTATGGAGCGTAGCTCCTTCGAGAAGAACCCGATTGACTTTCTGGAAGCCAAAGGATAT
 AAAGAGGTCAAGAAAGATCTTATCATTAAGCTGCCTAAGTATTAATCTTCGAGCTGGAAAATGGTCGTAA
 ACGCATGCTCGCTTCTGCCGGCGAGTTGCAGAAAGGGCAATGAATTAGCACTTCCATCAAAGTACGTTAACT
 TCCTGTATTTGGCCAGCCATTACGAGAACTGAAGGGGTCTCCAGAGGACAACGAACAGAAACAATTATTT
 GTAGAGCAGCACAAGCATTATCTTGATGAAATCATTGAGCAAATTTCCGAATTCAGTAAACGCGTAATCCT
 GGCCGATGCAAACCTCGACAAGGTGCTGAGCGCTTACAATAAGCATCGCGACAAACCTATCCGTGAGCAGG
 CTGAAAATATCATTACCTGTTACATTAACGAACCTGGGCGCTCCGGCCGCTTTTAAATATTTGACACG
 ACAATCGACCGTAAGCGCTATACCAGTACGAAAGAAGTGTGGATGCGACCTTATTACCAGTCAATTAC
 AGGATTATATGAGACCCGTATCGACCTTAGCCAATTAGGTGGGGATGCGGCCCCGAAGAAAAAACGCAAAG
 TGGATCCGAAGAAAAAACGCAAAGTGGCGGCCGCACTCGAGCACCACCACCACCACCTGA

S.py Cas9 amino acid sequence expressed from DNA codon optimized for expression in human cells containing a V5 epitope tag and 2 NLS sequences (SEQ ID No. 410).

MGKPIPNPLLGLDSTAPKKRKVGVIHGVPADKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVL
 GNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHHR
 LEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLLIYLALAHMIKFR
 GHFLIEGDLNPDNSDVKLFIQLVQTYNQLFEEENPINASGVDAKAILSARLSKSRRLLENLIAQLP
 GEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDITYDDDLNLLAQIGDQYADLFLAAKN
 LSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYA
 GYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNRDILLRKQRTFDNGSIPHQIHLGELHAILRR
 QEDFYFPFLKDNREKIEKILTFRIPIYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQS
 FIERMTNFDKNLPNEKVLPHKSLLEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKT
 NRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIV

LTTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKS
DGFANRNFQMQLIHDDSLTFKEDIQKAQVSGQDSLHEHIANLAGSPAIAKKGILQTVKVDELVKV
MGRHKPENIVIEARENQTTQKGQKNSRERMKRIEEGKELGSQILKEHPVENTQLQNEKLYLYY
LQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGSNDNVPSEEVVKMK
NYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDE
NDKLIREVKVIITLKSCLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGITALIKKYPKLESEFVY
GDYKVYDVRKMIKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWD
KGRDFATVRKVLSPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVA
YSVLVVAKEVEKSKKLKSVKELLGITIMERSSSFENPIDFLEAKGYKEVKKDLIIKLPKYSLFE
LENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEQKQLFVEQHKHYLDEII
EQISEFSKRVLADANLDKVL SAYNKHDKPIREQAENI IHLFTLTNLGAPAAFKYFDTTIDRKR
YTSTKEVL DATLIHQSI TGLYETRIDLSQLGGDSRADPKKKRKVEFHHTGLVDPSSVPSLSLNR

S.py Cas9 DNA sequence codon optimized for expression in human cells containing a
V5 epitope tag and 2 NLS sequences (SEQ ID No. 411).

ATGGGCAAGCCCATCCCTAACCCCTGTTGGGGCTGGACAGCACCGCTCCCAAAAAGAAAAGGAAGGTGGG
CATTCACGGCGTGCCTGCGGCCGACAAAAAGTACAGCATCGGCCTTGATATCGGCACCAATAGCGTGGGCT
GGGCCGTTATCACAGACGAATACAAGGTACCCAGCAAGAAGTTCAAGGTGCTGGGGAATACAGACAGGCAC
TCTATCAAGAAAAACCTTATCGGGGCTCTGCTGTTTGACTCAGGCGAGACCGCCGAGGCCACCAGGTTGAA
GAGGACCGCAAGGCGAAGGTACACCCGGAGGAAGAACAGGATCTGCTATCTGCAGGAGATCTTCAGCAACG
AGATGGCCAAGGTGGACGACAGCTTCTTCCACAGGCTGGAGGAGAGCTTCTTGTGCGAGGAGGATAAGAAG
CACGAACGACACCCCATCTTCGGCAACATAGTCGACGAGGTGCTTATCACGAGAAGTACCCACCATCTA
CCACCTGCGAAAGAAATTGGTGGATAGCACCGATAAAGCCGACTTGCGACTTATCTACTTGGCTCTGGCGC
ACATGATTAAGTTCAGGGGCCACTTCTGATCGAGGGCGACCTTAACCCGACAACAGTGACGTAGACAAA
TTGTTTCATCCAGCTTGTACAGACCTATAACCAGCTGTTGAGGAAAACCTATTAACGCCAGCGGGGTGGA
TGCGAAGGCCATACTTAGCGCCAGGCTGAGCAAAAGCAGGCGCTTGGAGAACCTGATAGCCAGCTGCCCCG
GTGAAAAGAAGAACGGCCTCTTCGGTAATCTGATTGCCCTGAGCCTGGGCCTGACCCCAACTTCAAGAGC
AACTTCGACCTGGCAGAAGATGCCAAGCTGCAGTTGAGTAAGGACACCTATGACGACGACTTGGACAATCT
GCTCGCCCAAATCGGCGACCACTACGCTGACCTGTTCTCGCCGCAAGAACCTTTCTGACGCAATCCTGC
TTAGCGATATCCTTAGGGTGAACACAGAGATCACCAAGGCCCCCTGAGCGCCAGCATGATCAAGAGGTAC
GACGAGCACCATCAGGACCTGACCCTTCTGAAGGCCCTGGTGAAGGAGCAACTGCCCAGAGTACAAGGA
GATCTTTTTTCGACCAGAGCAAGAACGGCTACGCCGGCTACATCGACGGCGGAGCCAGCCAAGAGGAGTTCT
ACAAGTTCATCAAGCCCATCCTGGAGAAGATGGATGGCACCCGAGGAGCTGCTGGTGAAGCTGAACAGGGAA
GATTTGCTCCGGAAGCAGAGGACCTTTGACAACGGTAGCATCCCCACCAGATCCACCTGGGCGAGCTGCA
CGCAATACTGAGGCGACAGGAGGATTTCTACCCCTTCTCAAGGACAATAGGGAGAAAATCGAAAAGATTCT
TGACCTTCAGGATCCCCTACTACGTGGGCCCTCTTGCCAGGGGCAACAGCCGATTGCTTGGATGACAAGA
AAGAGCGAGGAGACCATCACCCCTGGAACCTCGAGGAAGTGGTGGACAAAGGAGCAAGCGCGCAGTCTTT
CATCGAACGGATGACCAATTTGACAAAAACCTGCCTAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTTT
ACGAGTACTTCACCGTGTACAACGAGCTCACCAAGGTGAAATATGTGACCGAGGGCATGCGAAAACCCGCT
TTCCTGAGCGGCGAGCAGAAGAAGGCCATCGTGGACCTGCTGTTCAAGACCAACAGGAAGGTGACCGTGAA
GCAGCTGAAGGAGGACTACTTCAAGAAGATCGAGTGCTTTGATAGCGTGGAATAAGCGGCGTGGAGGACA
GGTTCAACGCCAGCCTGGGCACCTACCACGACTTGTGTAAGATAATCAAAGACAAGGATTTCTGGATAAT
GAGGAGAACGAGGATATACTCGAGGACATCGTGCTGACTTTGACCCTGTTTGAGGACCGAGAGATGATTGA
AGAAAGGCTCAAAACCTACGCCACCTGTTGACGACAAAAGTGATGAAACAACGAAGAGACGAAGATACA
CCGGCTGGGGCAGACTGTCCAGGAAGCTCATCAACGGCATTAGGGACAAGCAGAGCGGCAAGACCATCCTG
GATTTCTGAAAGTCCGACGGCTTCGCCAACCGAACTTCATGCAGCTGATTCACGATGACAGCTTGACCTT
CAAGGAGGACATCCAGAAGGCCAGGTTAGCGGCCAGGGCGACTCCCTGCACGAACATATTGCAAACCTGG

CAGGCTCCCCGCGATCAAGAAGGGCATACTGCAGACCGTTAAGGTTGTGGACGAATTGGTCAAGGTCATG
GGCAGGCACAAGCCCGAAAACATAGTTATAGAGATGGCCAGAGAGAACCAGACCACCCAAAAGGGCCAGAA
GAACAGCCGGGAGCGCATGAAAAGGATCGAGGAGGGTATCAAGGAACTCGGAAGCCAGATCCTCAAAGAGC
ACCCCGTGGAGAATACCCAGCTCCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAACGGCAGGGACATG
TACGTTGACCAGGAGTTGGACATCAACAGGCTTTCAGACTATGACGTGGATCACATAGTGCCCCAGAGCTT
TCTTAAAGACGATAGCATCGACAACAAGGTCTTGACCCGCTCCGACAAAAACAGGGGCAAAGCGACAACG
TGCCAAGCGAAGAGGTGGTTAAAAAGATGAAGAACTACTGGAGGCAACTGCTCAACGCGAAATTGATCACC
CAGAGAAAGTTTCGATAACCTGACCAAGGCCGAGAGGGGCGGACTCTCCGAAC'TTGACAAAGCGGGCTTCAT
AAAGAGGCAGCTGGTCGAGACCCGACAGATCACGAAGCACGTGGCCCAAATCCTCGACAGCAGAATGAATA
CCAAGTACGATGAGAATGACAACTCATCAGGGAAAGTGAAAGTGATTACCCTGAAGAGCAAGTTGGTGTCC
GACTTTCGCAAAGATTTCCAGTTCTACAAGGTGAGGGAGATCAACAACCTACCACCATGCCCACGACGCATA
CCTGAACGCCGTGGTCGGCACCGCCCTGATTAAAGAGTATCCAAAGCTGGAGTCCGAATTTGTCTACGGCG
ACTACAAAGTTTACGATGTGAGGAAGATGATCGCTAAGAGCGAACAGGAGATCGGCAAGGCCACCGCTAAG
TATTTCTTCTACAGCAACATCATGAAC'TTTTCAAGACCGAGATCACACTTGCCAACGGCGAAATCAGGAA
GAGGCCGCTTATCGAGACCAACGGTGAGACCGCGAGATCGTGTGGGACAAGGGCAGGGACTTCGCCACCG
TGAGGAAAGTCTGAGCATGCCCCAGGTGAATATTGTGAAAAAACTGAGGTGCAGACAGGCGGCTTTAGC
AAGGAATCCATCCTGCCCCAAGAGGAACAGCGACAAGCTGATCGCCCGGAAGAAGGACTGGGACCCTAAGAA
GTATGGAGGCTTCGACAGCCCCACCGTAGCCTACAGCGTGCTGGTGGTCGCGAAGGTAGAGAAGGGGAAGA
GCAAGAAACTGAAGAGCGTGAAGGAGCTGCTCGGCATAACCATCATGGAGAGGTCCAGCTTTGAGAAGAAC
CCCATTGACTTTTTTGAAGCCAAGGGCTACAAAGAGGTCAAAAAGGACCTGATCATCAAAC'TCCCCAAGTA
CTCCCTGTTTGAATTGGAGAACGGCAGAAAAGAGGATGCTGGCGAGCGCTGGGGAAC'TGAAAAGGGCAACG
AACTGGCGCTGCCCAGCAAGTACGTGAATTTTCTGTACCTGGCGTCCCACTACGAAAAGCTGAAAGGCAGC
CCCGAGGACAACGAGCAGAAGCAGCTGTTCTGGAGCAGCACAAAGCATTACCTGGACGAGATAATCGAGCA
AATCAGCGAGTTCAGCAAGAGGGTGATTCTGGCCGACGCGAACCTGGATAAGGTCCTCAGCGCCTACAACA
AGCACCGAGACAAACCCATCAGGGAGCAGGCCGAGAATATCATACACCTGTTACCCCTGACAAATCTGGGC
GCACCTGCGGCATTCAAATACTTCGATACCACCATCGACAGGAAAAGGTACACTAGCACTAAGGAGGTGCT
GGATGCCACCTTGATCCACAGTCCATTACCGGCCTGTATGAGACCAGGATCGACCTGAGCCAGCTTGGAG
GCGACTCTAGGGCGGACCCAAAAAAGAAAAGGAAGGTGGAATTCACCACACTGGACTAGTGGATCCGAGC
TCGGTACCAAGCTTAAGTTTAAACCGCTGA

S.py Cas9 DNA sequence codon optimized for expression in human cells as a T7 RNA polymerase expression cassette (SEQ ID No. 412). The sequence contains a T7 RNA polymerase promoter, a V5 epitope tag, a nuclear localization signal, the codon optimized Cas9 sequence, a second nuclear localization signal, and the BGH (bovine growth hormone) gene 3'-UTR element with a polyadenylation signal.

TAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACGGGGCCCTCTAGACTCGAGCGGCCGC
CACCATGGGCAAGCCCATCCCTAACCCCTGTTGGGGCTGGACAGCACCGCTCCCAAAAAGAAAAGGAAGG
TGGGCATTACGGCGTGCCTGCGGCCGACAAAAAGTACAGCATCGGCCTTGATATCGGCACCAATAGCGTG
GGCTGGGCCGTTATCACAGACGAATACAAGGTACCCAGCAAGAAGTTCAAGGTGCTGGGGAATACAGACAG
GCACTCTATCAAGAAAAACCTTATCGGGGCTCTGCTGTTTGACTCAGGCGAGACCGCCGAGGCCACCAGGT
TGAAGAGGACCGCAAGGCGAAGGTACACCCGAGGAAGAACAGGATCTGCTATCTGCAGGAGATCTTCAGC
AACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGGCTGGAGGAGAGCTTCCTTGTGAGGAGGATAA
GAAGCACGAACGACCCCCATCTTCGGCAACATAGTCGACGAGGTGCTTATCACGAGAAGTACCCACCA
TCTACCACCTGCGAAAGAAATTGGTGGATAGCACCGATAAAGCCGACTTGCGACTTATCTACTTGGCTCTG
GCGCACATGATTAAAGTTTCAGGGGCCACTTCTGATCGAGGGCGACCTTAACCCGACAACAGTGACGTAGA
CAAATTGTTTCATCCAGCTTGTACAGACCTATAACCAGCTGTTTCAGGAAAACCTATTAAACGCCAGCGGGG
TGGATGCGAAGGCCATACTTAGCGCCAGGCTGAGCAAAAGCAGGCGCTTGGAGAACCTGATAGCCAGCTG

CCCGGTGAAAAGAAGAACGGCCTCTTCGGTAATCTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAA
GAGCAACTTCGACCTGGCAGAAGATGCCAAGCTGCAGTTGAGTAAGGACACCTATGACGACGACTTGGACA
ATCTGCTCGCCCCAATCGGCGACCACTACGCTGACCTGTTCTCGCCGCAAGAACCTTTCTGACGCAATC
CTGCTTAGCGATATCCTTAGGGTGAACACAGAGATCACCAAGGCCCCCTGAGCGCCAGCATGATCAAGAG
GTACGACGAGCACCATCAGGACCTGACCCTTCTGAAGGCCCTGGTGAGGCAGCAACTGCCCGAGAAGTACA
AGGAGATCTTTTTTCGACCAGAGCAAGAACGGCTACGCCGGCTACATCGACGGCGGAGCCAGCCAAGAGGAG
TTCTACAAGTTTCATCAAGCCCATCCTGGAGAAGATGGATGGCACCGAGGAGCTGCTGGTGAAGCTGAACAG
GGAAGATTTGCTCCGGAAGCAGAGGACCTTTGACAACGGTAGCATCCCCACCAGATCCACCTGGGCGAGC
TGCACGCAATACTGAGGCGACAGGAGGATTTCTACCCCTTCTCAAGGACAATAGGGAGAAAATCGAAAAG
ATTCTGACCTTCAGGATCCCCTACTACGTGGGCCCTCTTGCCAGGGGCAACAGCCGATTTCGCTTGATGAC
AAGAAAGAGCGAGGAGACCATCACCCCTGGAACTTCGAGGAAGTGGTGGACAAAGGAGCAAGCGCGCAGT
CTTTTCATCGAACGGATGACCAATTTTCGACAAAAAACCCTGCCTAACGAGAAGGTGCTGCCCAAGCACAGCCTG
CTTTACGAGTACTTCACCGTGTACAACGAGCTCACCAAGGTGAAATATGTGACCGAGGGCATGCGAAAACC
CGCTTTCTGAGCGGCGAGCAGAAGAAGGCCATCGTGGACCTGCTGTTCAAGACCAACAGGAAGGTGACCG
TGAAGCAGCTGAAGGAGGACTACTTCAAGAAGATCGAGTGCTTTGATAGCGTGGAATAAGCGGCGTGGAG
GACAGGTTCAACGCCAGCCTGGGCACCTACCACGACTTGTTGAAGATAATCAAAGACAAGGATTTCTTGGA
TAATGAGGAGAACGAGGATATACTCGAGGACATCGTGCTGACTTTGACCCGTGTTTGAGGACCGAGAGATGA
TTGAAGAAAGGCTCAAAAACCTACGCCACCTGTTGACGACAAAAGTGATGAAACAACCTGAAGAGACGAAGA
TACACCGGCTGGGGCAGACTGTCCAGGAAGCTCATCAACGGCATTAGGGACAAGCAGAGCGGCAAGACCAT
CCTGGATTTCTGAAGTCCGACGGCTTCGCCAACCGAACTTCATGCAGCTGATTACGATGACAGCTTGA
CCTTCAAGGAGGACATCCAGAAGGCCAGGTTAGCGGCCAGGGCGACTCCCTGCACGAACATATTGCAAAC
CTGGCAGGCTCCCCTGCGATCAAGAAGGGCATACTGCAGACCGTTAAGGTTGTGGACGAATTGGTCAAGGT
CATGGGCAGGCACAAGCCCGAAAAACATAGTTATAGAGATGGCCAGAGAGAACCAGACCACCCAAAAGGGCC
AGAAGAACAGCCGGGAGCGCATGAAAAGGATCGAGGAGGGTATCAAGGAACTCGGAAGCCAGATCCTCAAA
GAGCACCCCGTGGAGAATACCCAGCTCCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAACGGCAGGGA
CATGTACGTTGACCAGGAGTTGGACATCAACAGGCTTTTCAAGTATGACGTGGATCACATAGTGCCCCAGA
GCTTTCTTAAAGACGATAGCATCGACAACAAGGTCTGACCCGCTCCGACAAAAACAGGGGCAAAAGCGAC
AACGTGCCAAGCGAAGAGGTGGTTAAAAAGATGAAGAACTACTGGAGGCAACTGCTCAACGCGAAATTGAT
CACCCAGAGAAAGTTTCGATAACCTGACCAAGGCCGAGAGGGGCGGACTCTCCGAACCTTGACAAAGCGGGCT
TCATAAAGAGGCAGCTGGTTCGAGACCCGACAGATCACGAAGCACGTGGCCCAAATCCTCGACAGCAGAATG
AATACCAAGTACGATGAGAATGACAACTCATCAGGGAAGTGAAAGTGATTACCCTGAAGAGCAAGTTGGT
GTCCGACTTTTCGAAAGATTTCCAGTTCTACAAGGTGAGGGAGATCAACAACCTACCACCATGCCACGACG
CATACTGAACGCCGTGGTCCGACCCGCCCTGATTAAGAAGTATCCAAAGCTGGAGTCCGAATTTGTCTAC
GGCGACTACAAAGTTTACGATGTGAGGAAGATGATCGCTAAGAGCGAACAGGAGATCGGCAAGGCCACCGC
TAAGTATTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATCACACTTGCCAACGGCGAAATCA
GGAAGAGGCCGCTTATCGAGACCAACGGTGAGACCGGCGAGATCGTGTGGGACAAGGGCAGGGACTTCGCC
ACCGTGAGGAAAGTCTGAGCATGCCCCAGGTGAATATTTGTAAAAAACTGAGGTGCAGACAGGCGGCTT
TAGCAAGGAATCCATCCTGCCCAAGAGGAACAGCGACAAGCTGATCGCCCGGAAGAAGGACTGGGACCCTA
AGAAGTATGGAGGCTTCGACAGCCCCACCGTAGCCTACAGCGTGCTGGTGGTCCGGAAGGTAGAGAAGGGG
AAGAGCAAGAACTGAAGAGCGTGAAGGAGCTGCTCGGCATAACCATCATGGAGAGGTCCAGCTTTGAGAA
GAACCCCATTGACTTTTTTGAAGCCAAGGGCTACAAAGAGGTCAAAAAGGACCTGATCATCAAACCTCCCA
AGTACTCCCTGTTTTGAATTGGAGAACGGCAGAAAAGAGGATGCTGGCGAGCGCTGGGGAACCTGCAAAAGGGC
AACGAACTGGCGCTGCCCAGCAAGTACGTGAATTTTCTGTACCTGGCGTCCCACTACGAAAAGCTGAAAGG
CAGCCCCGAGGACAACGAGCAGAAGCAGCTGTTCTGTTGAGCAGCACAAGCATTACCTGGACGAGATAATCG
AGCAAATCAGCGAGTTTCAGCAAGAGGGTGATTCTGGCCGACGCAACCTGGATAAGGTCTCAGCGCCTAC
AACAAGCACCGAGACAAACCATCAGGGAGCAGGCCGAGAATATCATAACCTGTTTACCCTGACAAATCT
GGGCGCACCTGCGGCATTCAAATACTTCGATACCACCATCGACAGGAAAAGGTACACTAGCACTAAGGAGG
TGCTGGATGCCACCTTGATCCACCAGTCCATTACCGGCCGTGATGAGACCAGGATCGACCTGAGCCAGCTT
GGAGGCGACTCTAGGGCGGACCCAAAAAAGAAAAGGAAGGTGGAATTCACCACACTGGACTAGTGGATCC
GAGCTCGGTACCAAGCTTAAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTG
TTGTTTGCCCCCTCCCCGTGCCTTCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCTTAATAAAAT

GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAA
GGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGGC

S.py Cas9 mRNA (SEQ ID No. 413) as made from the expression cassette (SEQ ID No. 412). The sequence contains a V5 epitope tag, a nuclear localization signal, the codon optimized Cas9 sequence, a second nuclear localization signal, and the BGH (bovine growth hormone) gene 3'-UTR element and poly-A tail.

GGGAGACCCAAGCUGGCUAGCGUUUAAACGGGCCCUCUAGACUCGAGCGGCCGCCACCAUGGGCAAGCCCA
UCCCUAACCCCCUGUUGGGGCUGGACAGCACCGCUCCCAAAAAGAAAGGAAGGUGGGCAUUCACGGCGUG
CCUGCGGCCGACAAAAAGUACAGCAUCGGCCUUGAUUACGGCACCAUAGCGUGGGCUGGGCCGUUACAC
AGACGAAUACAAGGUACCCAGCAAGAAGUUAAGGUGCUGGGGAAUACAGACAGGCACUCUAUCAAGAAAA
ACCUUAUCGGGGCUCUGCUGUUGACUCAGGCGAGACCGCCGAGGCCACCAGGUUGAAGAGGACCGCAAGG
CGAAGGUACACCCGGAGGAAGAACAGGAUCUGCUAUCUGCAGGAGAUUCUACGCAACGAGAUGGCCAAGGU
GGACGACAGCUUCUUCACAGGCUGGAGGAGAGCUUCCUUGUCGAGGAGGAUAAGAAGCACGAACGACACC
CCAUCUUCGGCAACAUAGUCGACGAGGUCGCUUAUCACGAGAAGUACCCACCAUCUACCACCUGCCAAAG
AAAUUGGUGGAUAGCACCGAUAAAAGCCGACUUGCGACUUAUCUACUUGGCUCUGGGCGCAUUAUUAAGUU
CAGGGGCCACUUCUGAUCGAGGGCGACCUUAACCCCGACAACAGUGACGUAGACAAAUUGUUAUCCAGC
UUGUACAGACCUAUAACCAGCUGUUCGAGGAAAACCUUAUAACGCCAGCGGGUGGAUGCGAAGGCCAUUA
CUUAGCGCCAGGCUGAGCAAAAGCAGGCGCUUGGAGAACCUGAUAGCCCAGCUGCCCGGUGAAAAGAAGAA
CGGCCUCUUCGUAUUCUGAUUUGCCUGAGCCUGGGCCUGACCCCCAACUUAAGAGCAACUUCGACCUGG
CAGAAGAUUGCCAAGCUGCAGUUGAGUAAGGACACCUAUGACGACGACUUGGACAAUCUGCUCGCCCAAUC
GGCGACCAGUACGCUGACCUGUUCUUGCCGCCAAGAACCUUUCUGACGCAUCCUGCUUAGCGAUUCCU
UAGGGUGAACACAGAGAUACCAAGGCCCCCCUGAGCGCCAGCAUGAUCAAGAGGUACGACGAGCACCAUC
AGGACCUGACCCUUCUGAAGGCCCCUGGUGAGGCAGCAACUGCCCGAGAAGUACAAGGAGAUUUUUUCGAC
CAGAGCAAGAACGGCUACGCCGGCUACAUCGACGGCGGAGCCAGCCAAGAGGAGUUCUACAAGUUCAUCAA
GCCAUCCUGGAGAAGAUUGGAUGGCACCGAGGAGCUGCUGGUGAAGCUGAACAGGGAAGAUUUGCUCGGGA
AGCAGAGGACCUUUGACAACGGUAGCAUCCCCACCAGAUCCACCUGGGCGAGCUGCACGCAUACUGAGG
CGACAGGAGGAUUUCUACCCCUUCCUCAAGGACAAUAGGGAGAAAAUCGAAAAGAUUCUGACCUUCAGGAU
CCCCUACUACGUGGGGCCUCUUGCCAGGGGCAACAGCCGAUUCGCUUGGAUGACAAGAAAGAGCGAGGAGA
CCAUCACCCCCUGGAACUUCGAGGAAGUGGUGGACAAAGGAGCAAGCGCGCAGUCUUUAUCGAACGGAUG
ACCAAUUUCGACAAAAACCUGCCUAACGAGAAGGUGCUGCCCAAGCACAGCCUGCUUUAACGAGUACUUCAC
CGUGUACAACGAGCUCACCAAGGUGAAAUAUGUGACCGAGGGCAUGCGAAAACCCGCUUUCUGAGCGGCG
AGCAGAAGAAGGCCAUUCGUGGACCUGCUGUUAAGACCAACAGGAAGGUGACCGUGAAGCAGCUGAAGGAG
GACUACUUAAGAAGAUUCGAGUGCUUUGAUAGCGUGGAAAUAAGCGGCGUGGAGGACAGGUUAACGCCAG
CCUGGGCACCUACCACGACUUGUUGAAGAUAAUCAAAGACAAGGAUUUCCUGGAUAAUAGGAGAAACGAGG
AUUAUCUCGAGGACAUUCGUGCUGACUUGACCCUGUUGAGGACCAGAGAUUAUGAAGAAAGGCUCAAA
ACCUACGCCACCUGUUCGACGACAAAGUGAUGAAACAACUGAAGAGACGAAGAUACACCGGCUGGGGCAG
ACUGUCCAGGAAGCUCAUCAACGGCAUUAAGGACAAGCAGAGCGGCAAGACCAUCCUGGAUUCUGAAGU
CCGACGGCUUCGCCAACCGAAAACUUAUGCAGCUGAUUCACGAUGACAGCUUGACCUUAAGGAGGACAUUC
CAGAAGGCCCAGGUUAGCGGCCAGGGCGACUCCUGGCACGAACAUUUGCAAACCUUGGCAGGCUCCCCUGC
GAUCAAGAAGGGCAUACUGCAGACCGUUAAGGUUGUGGACGAAUUGGUCAAGGUCAUGGGCAGGCACAAGC
CCGAAAACAUAGUUAUAGAGAUUGGCCAGAGAGAACAGACCACCCAAAAGGGCCAGAAGAACAGCCGGGAG
CGAUGAAAAGGAUCGAGGAGGGUAUCAAGGAACUCGGAAGCCAGAUCCUCAAAGAGCACCCCGUGGAGAA
UACCCAGCUCCAGAACGAGAAGCUGUACCUGUACUACCUGCAGAACGGCAGGGACAUGUACGUUGACCAGG
AGUUGGACAUCAACAGGCUUUCAGACUAUGACGUGGAUCACAUAGUGCCCCAGAGCUUUCUUAAGACGAU
AGCAUCGACAACAAGGUCCUGACCCGCUCCGACAAAAACAGGGGCAAAAGCGACAACGUGCCAAGCGAAGA
GGUGGUUAAAAAGAUAGAAGAACUACUGGAGGCAACUGCUCAACCGGAAUUGAUCACCCAGAGAAAGUUCG
AUAACCUGACCAAGGCCGAGAGGGGCGACUCUCCGAACUUGACAAAGCGGGCUUCAUAAAGAGGCAGCUG
GUCGAGACCCGACAGAUACGAAGCACGUGGCCCAAUCCUCGACAGCAGAAUGAAUACCAAGUACGAUGA

GAAUGACAAACUCAUCAGGGGAAGUGAAAAGUGAUUACCCUGAAGAGCAAGUUGGUGUCCGACUUUCGCAAAG
 AUUUCCAGUUCUACAAGGUGAGGGGAGAUCAACAACUACCACCAUGCCCACGACGCAUACCUGAACGCCGUG
 GUCGGCACCGCCUGAUUAAGAAGUAUCCAAAGCUGGAGUCCGAAUUUGUCUACGGCGACUACAAAGUUUA
 CGAUGUGAGGAAGAUGAUCGCUAAGAGCGAACAGGAGAUCCGGCAAGGCCACCGCUAAGUAUUUCUUCUACA
 GCAACAUCAUGAACUUUUUCAAGACCGAGAUACACUUGCCAACGGCGAAAUCAGGAAGAGGCCGCUUUAUC
 GAGACCAACGGUGAGACCGGCGAGAUUCGUGUGGGACAAGGGCAGGGACUUCGCCACCGUGAGGAAAGUCCU
 GAGCAUGCCCCAGGUGAAUAUUGUGAAAAAAACUGAGGUGCAGACAGGCGGCUUUAGCAAGGAAUCCAUC
 UGCCCCAAGAGGAACAGCGACAAGCUGAUCGCCCCGGAAGAAGGACUGGGACCCUAAGAAGUAUGGAGGCUUC
 GACAGCCCCACCGUAGCCUACAGCGUGCUGGUGGUCGCGAAGGUAGAGAAGGGGAAGAGCAAGAAACUGAA
 GAGCGUGAAGGAGCUGCUCGGCAUAACCAUCAUGGAGAGGUCCAGCUUUGAGAAGAACCCCAUUGACUUUU
 UGGAAGCCAAGGGCUACAAAGAGGUCAAAAAGGACCUGAUCAUCAAACUCCCCAAGUACUCCUGUUUGAA
 UUGGAGAACGGCAGAAAGAGGAUGCUGGCGAGCGCUGGGGAACUGCAAAGGGCAACGAACUGGCGCUGCC
 CAGCAAGUACGUGAAUUUUCUGUACCUGGCGUCCACUACGAAAAGCUGAAAGGCAGCCCCGAGGACAACG
 AGCAGAAGCAGCUGUUCGUGGAGCAGCACAAGCAUUACCUGGACGAGAUAAUCGAGCAAAUCAGCGAGUUC
 AGCAAGAGGGUGAUUCUGGCCGACGCGAACCUGGAUAAAGGUCCUAGCGCCUACAACAAGCACCAGAGACAA
 ACCCAUCAGGGAGCAGGCCGAGAAUAUCAUACACCGUUCACCCUGACAAAUCUGGGCGCACCUGCGGCAU
 UCAAAUACUUCGAUACCACCAUCGACAGGAAAAGGUACACUAGCACUAAGGAGGUGCUGGAUGCCACCUUG
 AUCCACCAGUCCAUAUACCGGCCUGUAUGAGACCAGGAUCGACCUGAGCCAGCUUGGAGGCGACUCUAGGGC
 GGACCCAAAAAAGAAAAGGAAGGUGGAAUUCACCACACUGGACUAGUGGAUCCGAGCUCGGUACCAAGCU
 UAAGUUUAAACCGCUGAUCAGCCUCGACUGUGCCUUCUAGUUGCCAGCCAUCUGUUGUUUGCCCCUCCCCC
 GUGCCUUCUUGACCCUGGAAGGUGCCACUCCACUGUCCUUUCCUAAUAAAUGAGGAAAUUGCAUCGCA
 UUGUCUGAGUAGGUGUCAUUCUAUUCUGGGGGUGGGGUGGGGCAGGACAGCAAGGGGGAGGAUUGGGAAG
 ACAAUAGCAGGCAUGCUGGGGAUGCGGUGGGCUCUAUGGC - polyA

EXAMPLE 12

[00131] The following example demonstrates reduced stimulation of the innate immune system in mammalian cells by the truncated chemically modified crRNA:tracrRNA complexes of the present invention when compared with unmodified IVT sgRNAs.

[00132] Mammalian cells possess a variety of receptors intended to identify and respond to foreign RNAs as part of anti-viral immunity. This includes receptors such as TLR-3, TLR-7, TLR8, RIG-I, MDA5, OAS, PKR, and others. In broad terms, RNAs that are short or contain chemical modifications present in mammalian cells (such as 2'OMe RNA) evade detection or are less stimulatory than are long, unmodified RNAs. The present example compares the level of stimulation of 2 immune response associated genes (*IFIT1* and *IFITM1*) when mammalian HEK293 cells are transfected with truncated unmodified or truncated modified crRNA:tracrRNA complexes of the present invention with a commercial IVT sgRNA (Thermo Fisher Scientific, Waltham, MA).

[00133] CRISPR guide RNAs specific to human *HPRT1* site 38285 were employed. Sequences are shown in Table 11 below. The unmodified crRNA:tracrRNA complexes (SEQ ID Nos. 48 and 2), the modified crRNA:tracrRNA complexes (SEQ ID Nos. 178 and 100) and the sgRNA (SEQ ID No. 414) were transfected into HEK-Cas9 cells at 30 nM concentration as outlined in Example 2 above. RNA was prepared 24 hours after transfection using the SV96 Total RNA Isolation Kit (Promega, Madison, WI). cDNA was synthesized using 150 ng total RNA with SuperScriptTM-II Reverse Transcriptase (Invitrogen, Carlsbad, CA) per the manufacturer's instructions using both random hexamer and oligo-dT priming. Transfection experiments were all performed a minimum of three times.

[00134] Quantitative real-time PCR was performed using 10 ng cDNA per 10 μ L reaction with ImmolaseTM DNA Polymerase (Bioline, Randolph, MA), 200 nM primers, and 200 nM probe. Cycling conditions employed were: 95°C for 10 minutes followed by 40 cycles of 2-step PCR with 95°C for 15 seconds and 60°C for 1 minute. PCR and fluorescence measurements were done using an ABI PrismTM 7900 Sequence Detector (Applied Biosystems Inc., Foster City, CA). All reactions were performed in triplicate using 2-color multiplexing. Expression data were normalized against an average of two internal control genes. Copy number standards were linearized cloned amplicons for all assays. Unknowns were extrapolated against standards to establish absolute quantitative measurements. Housekeeping internal control normalization assays were *HPRT1* (primers and probe SEQ ID Nos. 415-417) and *SFRS9* (primers and probe SEQ ID Nos. 418-420). Immune activation pathway assays were *IFITM1* (primers and probe SEQ ID Nos. 421-423) and *IFIT1* (primers and probe SEQ ID Nos. 424-426). The results were normalized using non-transfected cells as baseline and are shown in FIG. 13.

Table 11. Nucleic acid reagents employed in immune activation experiments in Example 12.

SEQ ID No.	Reagent	Sequence
48	Unmodified crRNA	cuuauauccaacacacuucgugguuuuagagcuaugcu
2	Unmodified tracrRNA	agcauagcaaguuaaaaauaaggcuaguccguaucaacuugaaaaaguggcacgagucggugcuuu
178	Modified crRNA	<u>c</u> * <u>u</u> * <u>u</u> *auauccaacacacuucgugguuuuagagcua <u>u</u> * <u>g</u> * <u>c</u> * <u>u</u>

SEQ ID No.	Reagent	Sequence
100	Modified tracrRNA	<u>a</u> * <u>g</u> *cauagcaaguuaaaaaaaggcuaguccguaucaacuugaaaaaguggcaccgagucggugcu* <u>u</u> * <u>u</u>
414	IVT sgRNA	ppp-gcuuauauccaacacucgugguuuuagagcuagaaauagcaaguuaa aaauaaggcuaguccguaucaacuugaaaaaguggcaccgagucggugcuuu uuuu
415	Hs HPRT F517	GACTTTGCTTTCCTTGGTCAG
416	Hs HPRT R591	GGCTTATATCCAACACTTCGTGGG
I ¹	Hs HPRT P554	FAM-ATGGTCAAG (ZEN) GTCGCAAGCTTGCTGGT-ZEN
418	Hs SFRS9 F569	TGTGCAGAAGGATGGAGT
419	Hs SFRS9 R712	CTGGTGCTTCTCTCAGGATA
II ²	Hs SFRS9 P644	HEX-TGGAATATG (ZEN) CCCTGCGTAAACTGGA-ZEN
421	Hs IFITM1 For	CTCTTCTTGAACTGGTGCTGTCTG
422	Hs IFITM1 Rev	CAGGATGAATCCAATGGTCATGAGG
III ³	Hs IFITM1 Probe FAM	FAM-AAGTGCCTG (ZEN) AACATCTGGGCCCTGATT-ZEN
424	Hs IFIT1 For	CCATTGTCTGGATTTAAGCGG
425	Hs IFIT1 Rev	GCCACAAAAAATCACAAGCCA
IV ⁴	Hs IFIT1 Probe HEX	HEX-TTTCCTTTGC (ZEN) TTCCCCTAAGGCAGGCTG-ZEN

¹ Compound I is an oligonucleotide having the formula SEQ ID NO: 417-(ZEN)-SEQ ID NO: 441.

² Compound II is an oligonucleotide having the formula SEQ ID NO: 420-(ZEN)-SEQ ID NO: 442.

³ Compound III is an oligonucleotide having the formula SEQ ID NO: 423-(ZEN)-SEQ ID NO: 443.

⁴ Compound IV is an oligonucleotide having the formula SEQ ID NO: 426-(ZEN)-SEQ ID NO: 444.

Oligonucleotide sequences are shown 5'-3'. Uppercase = DNA; Lowercase = RNA; Underlined = 2'-O-methyl RNA; * = phosphorothioate internucleotide linkage; ppp = triphosphate; ZEN = naphthyl-azo modifier, dark quencher; FAM = 6-carboxyfluorescein; HEX = hexachlorofluorescein.

[00135] Treatment with the unmodified or chemically modified truncated crRNA:tracrRNA complex did not lead to detectable increases in IFIT1 or IFITM1 expression over baseline. In contrast, treatment with the longer IVT sgRNA led to a 45-

fold induction of IFITM1 and a 220-fold induction of IFIT1. Thus, significant stimulation of the innate immune system occurred using the sgRNA that was absent using the short crRNA:tracrRNA complexes of the present invention.

EXAMPLE 13

[00136] The following example combines modification patterns identified in Examples 6 and 7 as being particularly efficacious to demonstrate new highly modified crRNA and tracrRNA compositions that perform with high efficiency in mammalian CRISPR genome editing applications.

[00137] A series of crRNAs and tracrRNAs (Table 12) were synthesized having chemical modifications as indicated. The crRNAs employed a 20 base protospacer domain targeting the same site in the human *HPRT1* gene (38285) at the 5'-end with a 16 base tracrRNA binding domain at the 3'-end. The tracrRNAs were synthesized having chemical modifications as indicated, using the 67 nucleotide or 62 nucleotide truncated versions of the tracrRNA sequence. The crRNAs and tracrRNAs listed in Table 12 were paired as indicated and transfected into the HEK-Cas9 cells at 30 nM concentration and processed as described in previous Examples. Relative gene editing activities were assessed by comparing cleavage rates in the *HPRT1* gene using the T7EI mismatch endonuclease cleavage assay, with quantitative measurement of products done using the Fragment Analyzer.

[00138] Table 12: Activity of highly modified crRNA:tracrRNA complexes to direct Cas9-mediated gene editing in mammalian cells.

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Cleavage %
		tracrRNA Sequence	
1	448	<i>c*u*u*auauccaacacuucgugguuuuagagcuau*g*c*u</i>	57
	2	<i>agcauagcaaguuaaaauaaggcuaguccguaucaacuugaa aaaguggcaccgagucggugcuuu</i>	
2	448	<i>c*u*u*auauccaacacuucgugguuuuagagcuau*g*c*u</i>	58
	100	<i>a*g*cauagcaaguuaaaauaaggcuaguccguaucaacuug aaaaaguggcaccgagucggugcu<u>*u*u</u></i>	
3	48	<i>cuuauauccaacacuucgugguuuuagagcuau</i>	58

cr/tracrRNA pair	SEQ ID No.	crRNA Sequence	Cleavage %
		tracrRNA Sequence	
	449	<i>a*g*cauagcaaguu^uaaaauaaggcuaguccguuaucaacuug aaaaaguggcaccgagucggugcu*u*u</i>	
4	48	cuuauauccaacacuu^cgugguuuuagagcuau^gcu	57
	450	<i>a*g*cauagcaaguu^uaaaauaaggcuaguccguuaucaacuug aaaaaguggcaccgagucggugcu*u*u</i>	
5	48	cuuauauccaacacuu^cgugguuuuagagcuau^gcu	65
	451	<u><i>a*g*cauagcaaguu^uaaaauaaggcuaguccguuaucaacuug aaaaaguggcaccgagucg*g*u</i></u>	

[00139] Oligonucleotide sequences are shown 5'-3'. Lowercase = RNA; Underlined = 2'-O-methyl RNA; Lowercase italic = 2'F RNA; * = phosphorothioate internucleotide linkage. The relative functional activity of each complex is indicated by the % cleavage in a T7EI heteroduplex assay for each dose studied.

[00140] The crRNA:tracrRNA pairs #1 and #2 show that a highly 2'F RNA modified crRNA (SEQ ID No. 448, which has 22/36 residues modified, or 61%) is highly functional when paired with either an unmodified tracrRNA (SEQ ID No. 2) or a highly 2'OMe modified tracrRNA (SEQ ID No. 100). The crRNA:tracrRNA pairs #3 and #4 show that tracrRNA compositions having moderate (SEQ ID No. 450, with 19/67 residues modified, or 28%) or high (SEQ ID No. 449, with 46/67 residues modified, or 69%) levels of 2'F RNA modification are highly functional. Information derived from Example 6 (in particular, the 2'OMe "walk", SEQ ID Nos. 144-162) was used to identify specific residues that can be modified within the internal domain of the tracrRNA (see Fig. 6). The crRNA:tracrRNA pair #5 demonstrates that an extremely highly modified tracrRNA, which in this case was a truncated 62 nucleotide design (SEQ ID No. 451, having 51/62 residues modified with 2'OMe RNA, or 82%), has high potency in triggering CRISPR genome editing in mammalian cells. Therefore, the original 89 RNA nucleotide wild-type tracrRNA has been optimized herein to a form that has as little as 11 RNA residues remaining (11/62), thereby significantly reducing risk of RNA-based activation of the mammalian innate immune system and reducing the nuclease-susceptible RNA content of the tracrRNA to a minimal level.

[00141] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00142] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00143] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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[00144] In a first aspect, the invention relates to an isolated tracrRNA selected from the group of SEQ ID NOS: 30-33, 37-40, and 50-53, wherein the isolated tracrRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.

[00145] In a second aspect, the invention relates to an isolated tracrRNA selected from the group consisting of SEQ ID NOS:19-21 and 449-451, wherein the isolated tracrRNA is active in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein endonuclease system.

CLAIMS:

1. An isolated tracrRNA selected from the group of SEQ ID NOS: 30-33, 37-40, and 50-53, wherein the isolated tracrRNA displays activity in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) endonuclease system.
2. An isolated tracrRNA selected from the group consisting of SEQ ID NOS:19-21 and 449-451, wherein the isolated tracrRNA is active in a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein endonuclease system.

Integrated DNA Technologies, Inc.

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

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cuuauccaacacucgugguuuuaga--gcuaugcuguuuug (SEQ ID NO.:46)
||||||| |||||||||||||
c--ggauaaaaauugaacgaucgacaaacuuaccaagguug
u| ||
a| ||
guccguaucaacuug
|||| a
|||| a
agccacggugaaa
g |||||
ucggugcuuuuuu (SEQ ID NO.:18)

FIG. 1

cuuauccaacacucgugguuuuaga--gcuag
||||| a
||||| a
c-ggaauaaaauugaacgaua
u| |
a| |
guccguuaucaacug
|||| a
|||| a
agccacggugaaa
g |||||
ucggugcuuu (SEQ ID NO.:428)

FIG. 2

SEQ ID No.	tracrRNA Sequence (5'-3')	Cleavage (%)
18	GUUGGAACCAUUCAAAAACAGCAAGUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	38
30	CAAAAACAGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUU	26
31	AACAGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU	32
2	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU	57
32	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU	47
33	CAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU	27
34	AGCAUAGCAAGUUAUUUUUA	0
35	AGCAUAGCAAGUUAUUUUUA AACUUGAAAAAGUGGCACCGAGUCGGUGCU	0
36	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC	53
37	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUG	56
38	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGU	56
39	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGG	53
40	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG CCGAGUCGG	5
41	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCC AACUUGAAAAAGUGGCACCGAGUCGGUGCU	0
42	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCC AACUUGAAAAAGUGGCACCGAGUCGG	0
43	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCC AACUUGAAAAAGUG CCGAGUCGG	0
44	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUG	0
45	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGUUAUCA GCACCGAGUCGGUGCU	0
427	AGCAUAGCAAGUUAUUUUUAAGGCUAGUCCGU CAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU	4

FIG. 3

SEQ ID No.	crRNA and tracrRNA Sequences 5'-3'	Length
46	<u>CUUAUAUCCAAACACUUCUGUGUUUAGAGCTAUGCTGURUG</u>	42 base crRNA
47	<u>CUUAUAUCCAAACACUUCUGUGUUUAGAGCTAUGCTGURU</u>	39 base crRNA
48	<u>CUUAUAUCCAAACACUUCUGUGUUUAGAGCTAUGCU</u>	36 base crRNA
49	<u>CUUAUAUCCAAACACUUCUGUGUUUAGAGCTAUG</u>	34 base crRNA
18	<u>GUUGGAAUCCAAACACAGCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUUUUUU</u>	89 base tracrRNA
50	<u>CAAAACACGCUAGSCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUUUU</u>	74 base tracrRNA
51	<u>AACACGCUAGSCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUUUU</u>	70 base tracrRNA
2	<u>AGCAUAGSCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUUUU</u>	67 base tracrRNA
52	<u>AGCAUAGSCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUU</u>	65 base tracrRNA
53	<u>CAGAGSCAAGUUHAAAUAUAGGCTAGUCCGUHAUCAACTUGAAAAAGUGGCACCCAGUCGCGUCUU</u>	63 base tracrRNA

FIG. 4

cuuauccaacacucgugguuuaga--gcuaugcu (SEQ ID NO.:48)
||||||| |||||||
c-ggaauaaaauugaacgauacga
u| ||
a| ||
guccguaucaacug
||||| a
||||| a
agccacggugaaa
g |||||||
ucggugcuuu (SEQ ID NO.: 2)

FIG. 5

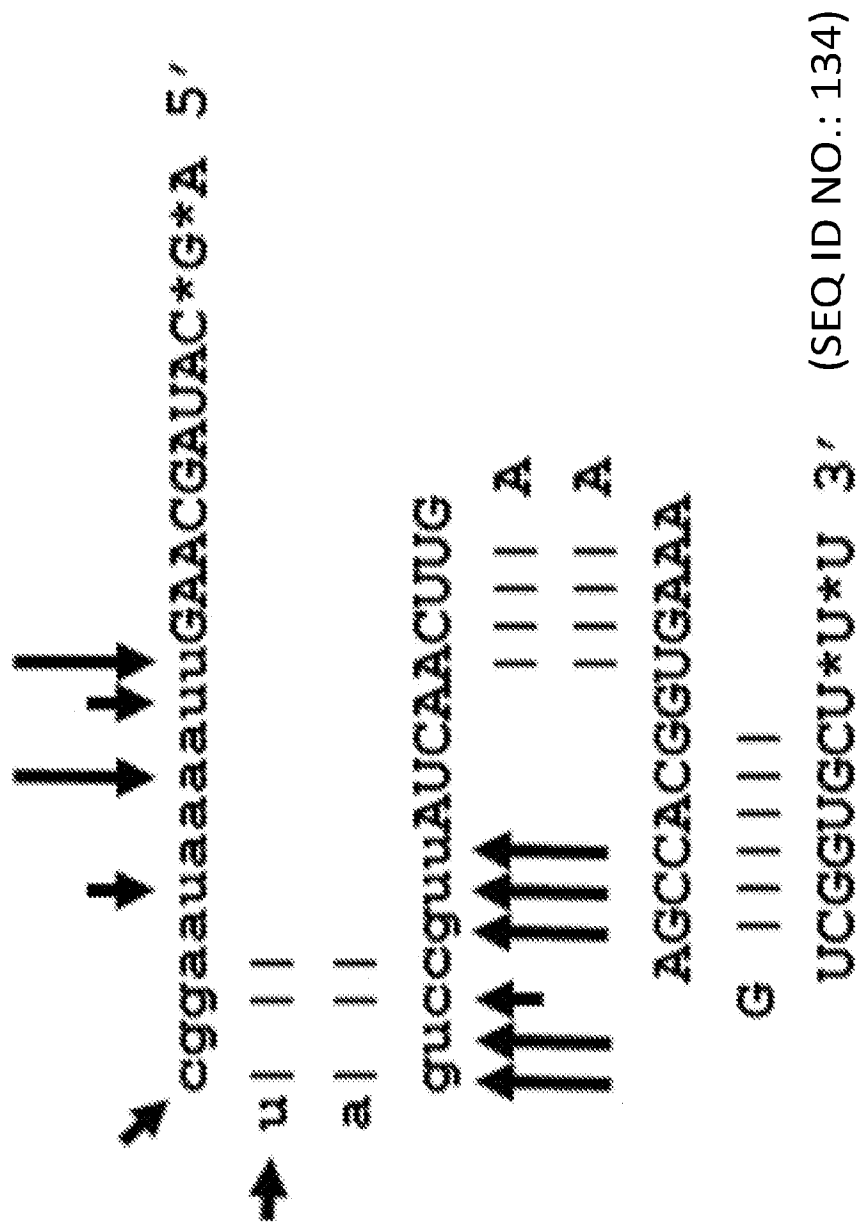


FIG. 6

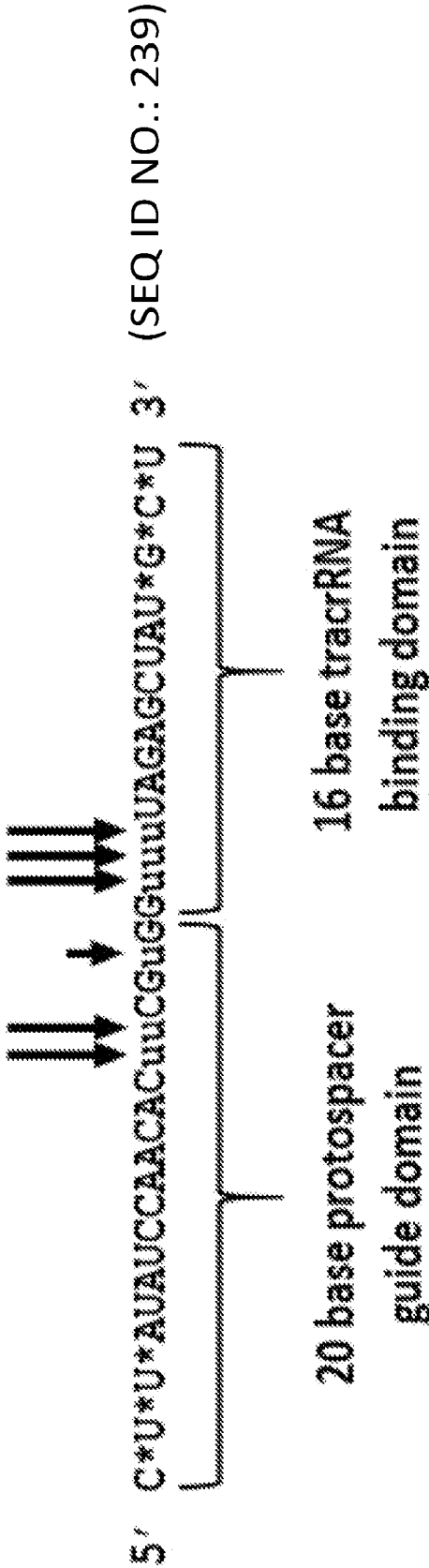


FIG. 7

C*U*U*auauccaacacuucgugguuuuaga--gcuau*G*C*U (SEQ ID NO.: 178)
||||| ||||| |||
c-ggauaaaaaugaACGAUA C*G*A
u| ||
a| ||
ggucguancaaCUUG
|||| A
|||| A
AGCCACGGUGAAA
G |||||
UCGGUGCU*U*U (SEQ ID NO.: 100)

FIG. 8

C*U*U*AUAUCCAACACACuucGuGGuuuUAGA--GCUAU*G*C*U (SEQ ID NO.: 446)
||||||| ||||| |||
c-ggaauaaaaauGAACGAUA C*G*A
u| ||
a| ||
ggucguuAUCCACUUG
|||| A
|||| A
AGCCACGGUGAAA
G |||||
UCGGUGCU*U*U (SEQ ID NO.: 134)

FIG. 9

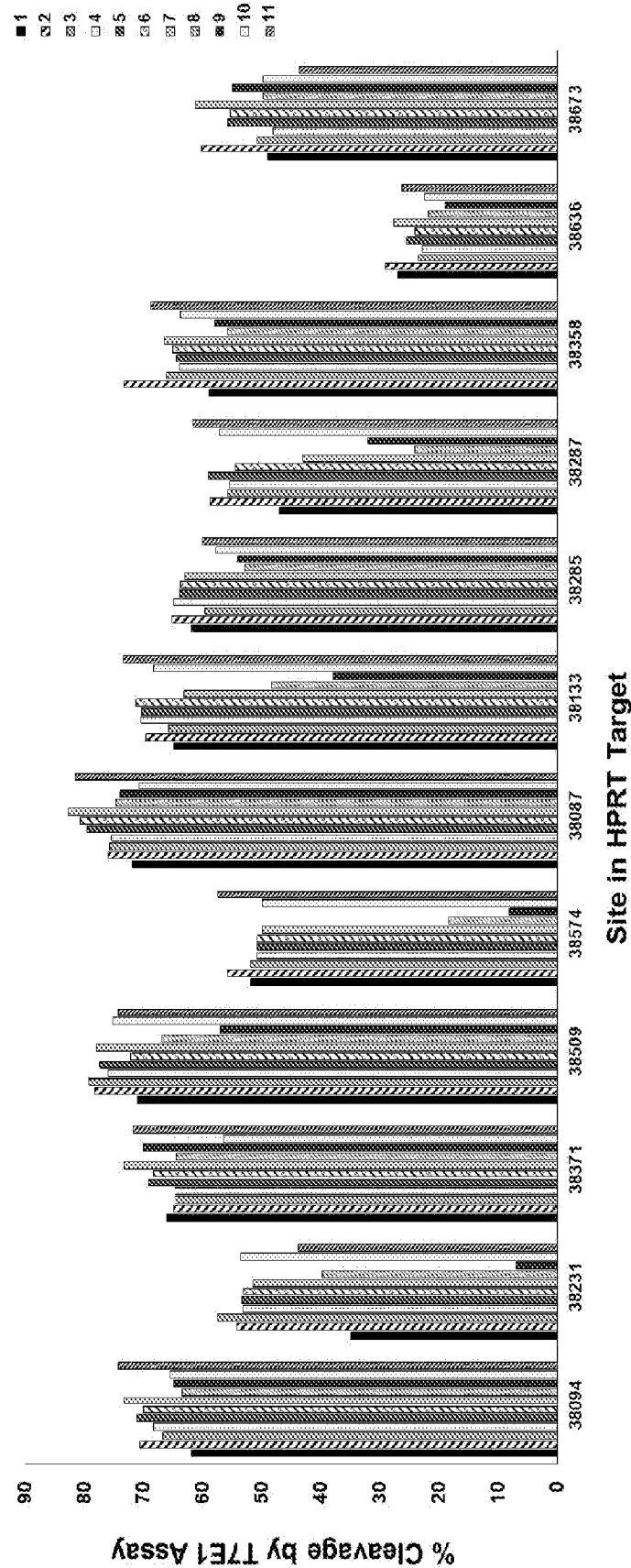


FIG. 11

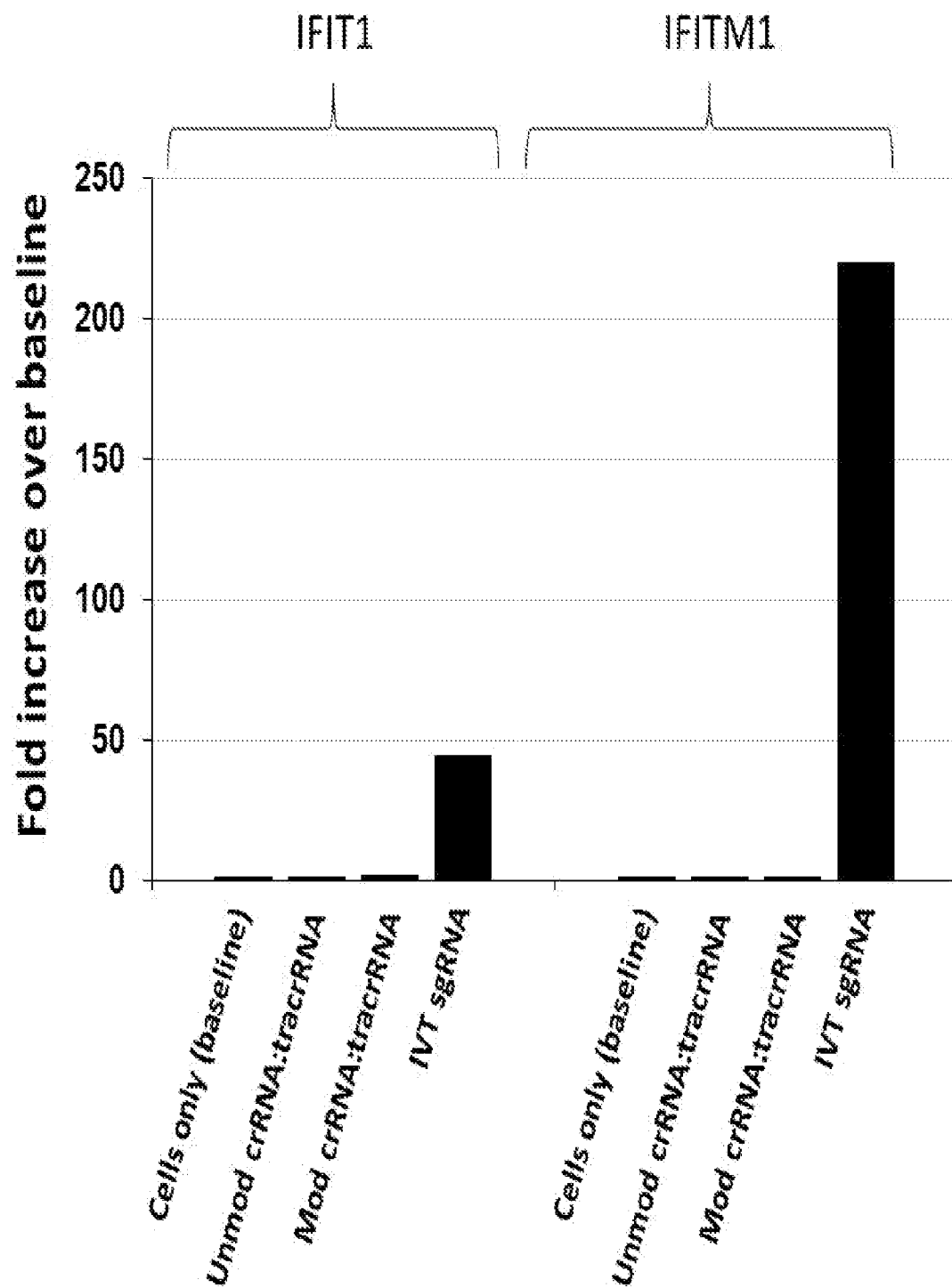


FIG. 13

SEQUENCE LISTING

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JACOBI, ASHLEY MAE
RETTIG, GARRETT RICHARD
SCHUBERT, MOLLIE SUE
BEHLKE, MARK AARON

<120> CRISPR-BASED COMPOSITIONS AND METHODS OF USE

<130> IDT01-008-PCT-AU

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<150> 62/239,546

<151> 2015-10-09

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 aaaaguggca ccgagucggu gcuuuuuuuu 89

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 gugcuuu 67

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gugcuuu 67

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 aaaaguggca ccgagucggu gcuuuuuuu 89

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gaatgtaagt aattgcttct ttttctcact cttttttcaa aacacgcata aaaatttagg 780
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<210> 29
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<210> 30
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<400> 35
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<400> 36
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gugc 64

<210> 37
 <211> 63
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<400> 37
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gug 63

<210> 38
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<400> 38
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gu 62

<210> 39
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 <400> 43
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 <210> 44
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<400> 49
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<210> 52
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<210> 53
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<210> 54
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<210> 56

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<210> 65
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<210> 66
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<400> 67
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<210> 68
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 gugcuuu 67

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 oligonucleotide

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oligonucleotide

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<220>
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Ile	Leu	Arg	Val	Asn	Thr	Glu	Ile	Thr	Lys	Ala	Pro	Leu	Ser	Ala	Ser
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Met	Ile	Lys	Arg	Tyr	Asp	Glu	His	His	Gln	Asp	Leu	Thr	Leu	Leu	Lys
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Ala	Leu	Val	Arg	Gln	Gln	Leu	Pro	Glu	Lys	Tyr	Lys	Glu	Ile	Phe	Phe
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Gln	Glu	Glu	Phe	Tyr	Lys	Phe	Ile	Lys	Pro	Ile	Leu	Glu	Lys	Met	Asp	
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Lys	Gln	Arg	Thr	Phe	Asp	Asn	Gly	Ser	Ile	Pro	His	Gln	Ile	His	Leu	
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Phe	Asp	Lys	Asn	Leu	Pro	Asn	Glu	Lys	Val	Leu	Pro	Lys	His	Ser	Leu	
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Lys	Ala	Ile	Val	Asp	Leu	Leu	Phe	Lys	Thr	Asn	Arg	Lys	Val	Thr	Val	
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Lys	Gln	Leu	Lys	Glu	Asp	Tyr	Phe	Lys	Lys	Ile	Glu	Cys	Phe	Asp	Ser	
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Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg Leu
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Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys Asp
835 840 845

Asp Ser Ile Asp Asn Lys Val Leu Thr Arg Ser Asp Lys Asn Arg Gly
850 855 860

Lys Ser Asp Asn Val Pro Ser Glu Glu Val Val Lys Lys Met Lys Asn
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Tyr Trp Arg Gln Leu Leu Asn Ala Lys Leu Ile Thr Gln Arg Lys Phe
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Asp Asn Leu Thr Lys Ala Glu Arg Gly Gly Leu Ser Glu Leu Asp Lys
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Ala Gly Phe Ile Lys Arg Gln Leu Val Glu Thr Arg Gln Ile Thr Lys
915 920 925

His Val Ala Gln Ile Leu Asp Ser Arg Met Asn Thr Lys Tyr Asp Glu
930 935 940

Asn Asp Lys Leu Ile Arg Glu Val Lys Val Ile Thr Leu Lys Ser Lys
945 950 955 960

Leu Val Ser Asp Phe Arg Lys Asp Phe Gln Phe Tyr Lys Val Arg Glu
965 970 975

Ile Asn Asn Tyr His His Ala His Asp Ala Tyr Leu Asn Ala Val Val
980 985 990

Gly Thr Ala Leu Ile Lys Lys Tyr Pro Lys Leu Glu Ser Glu Phe Val
995 1000 1005

Tyr Gly Asp Tyr Lys Val Tyr Asp Val Arg Lys Met Ile Ala Lys

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Lys	Val 1085	Leu	Ser	Met	Pro	Gln 1090	Val	Asn	Ile	Val	Lys 1095	Lys	Thr	Glu	
Val	Gln 1100	Thr	Gly	Gly	Phe	Ser 1105	Lys	Glu	Ser	Ile	Leu 1110	Pro	Lys	Arg	
Asn	Ser 1115	Asp	Lys	Leu	Ile	Ala 1120	Arg	Lys	Lys	Asp	Trp 1125	Asp	Pro	Lys	
Lys	Tyr 1130	Gly	Gly	Phe	Asp	Ser 1135	Pro	Thr	Val	Ala	Tyr 1140	Ser	Val	Leu	
Val	Val 1145	Ala	Lys	Val	Glu	Lys 1150	Gly	Lys	Ser	Lys	Lys 1155	Leu	Lys	Ser	
Val	Lys 1160	Glu	Leu	Leu	Gly	Ile 1165	Thr	Ile	Met	Glu	Arg 1170	Ser	Ser	Phe	
Glu	Lys 1175	Asn	Pro	Ile	Asp	Phe 1180	Leu	Glu	Ala	Lys	Gly 1185	Tyr	Lys	Glu	
Val	Lys 1190	Lys	Asp	Leu	Ile	Ile 1195	Lys	Leu	Pro	Lys	Tyr 1200	Ser	Leu	Phe	
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Leu Gln Lys Gly Asn Glu Leu Ala Leu Pro Ser Lys Tyr Val Asn
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Phe Leu Tyr Leu Ala Ser His Tyr Glu Lys Leu Lys Gly Ser Pro
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Glu Asp Asn Glu Gln Lys Gln Leu Phe Val Glu Gln His Lys His
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Tyr Leu Asp Glu Ile Ile Glu Gln Ile Ser Glu Phe Ser Lys Arg
1265                               1270                      1275

Val Ile Leu Ala Asp Ala Asn Leu Asp Lys Val Leu Ser Ala Tyr
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Asn Lys His Arg Asp Lys Pro Ile Arg Glu Gln Ala Glu Asn Ile
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Ile His Leu Phe Thr Leu Thr Asn Leu Gly Ala Pro Ala Ala Phe
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Lys Tyr Phe Asp Thr Thr Ile Asp Arg Lys Arg Tyr Thr Ser Thr
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Lys Glu Val Leu Asp Ala Thr Leu Ile His Gln Ser Ile Thr Gly
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Leu Tyr Glu Thr Arg Ile Asp Leu Ser Gln Leu Gly Gly Asp
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<210> 408

<211> 1416

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
polypeptide

<400> 408

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Thr	Asn	Ser	Val	Gly	Trp	Ala	Val	Ile	Thr	Asp	Glu	Tyr	Lys	Val	Pro	
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Ala	Thr	Arg	Leu	Lys	Arg	Thr	Ala	Arg	Arg	Arg	Tyr	Thr	Arg	Arg	Lys	
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Ala	Leu	Ala	His	Met	Ile	Lys	Phe	Arg	Gly	His	Phe	Leu	Ile	Glu	Gly	
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Asp	Leu	Asn	Pro	Asp	Asn	Ser	Asp	Val	Asp	Lys	Leu	Phe	Ile	Gln	Leu	
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Val	Gln	Thr	Tyr	Asn	Gln	Leu	Phe	Glu	Glu	Asn	Pro	Ile	Asn	Ala	Ser	
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Thr	Leu	Leu	Lys	Ala	Leu	Val	Arg	Gln	Gln	Leu	Pro	Glu	Lys	Tyr	Lys
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Glu	Lys	Met	Asp	Gly	Thr	Glu	Glu	Leu	Leu	Val	Lys	Leu	Asn	Arg	Glu
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Phe	Tyr	Pro	Phe	Leu	Lys	Asp	Asn	Arg	Glu	Lys	Ile	Glu	Lys	Ile	Leu	
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Thr	Phe	Arg	Ile	Pro	Tyr	Tyr	Val	Gly	Pro	Leu	Ala	Arg	Gly	Asn	Ser	
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Arg	Phe	Ala	Trp	Met	Thr	Arg	Lys	Ser	Glu	Glu	Thr	Ile	Thr	Pro	Trp	
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Asn	Phe	Glu	Glu	Val	Val	Asp	Lys	Gly	Ala	Ser	Ala	Gln	Ser	Phe	Ile	
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Glu	Arg	Met	Thr	Asn	Phe	Asp	Lys	Asn	Leu	Pro	Asn	Glu	Lys	Val	Leu	
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<210> 409

<211> 4251

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide

<400> 409

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<211> 1429

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic
polypeptide

<400> 410

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Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe Lys Val
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 <211> 4290
 <212> DNA
 <213> Artificial Sequence

<220>
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<210> 412

<211> 4601

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide

<400> 412

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<211> 4584

<212> RNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic polynucleotide

<400> 413

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ggacccaaaa aagaaaagga agguggaauu ccaccacacu ggacuagugg auccgagcuc	4320
gguaccaagc uuaaguuaa accgcugauc agccucgacu gugccuucua guugccagcc	4380
aucuguuguu ugccccuccc ccgugccuuc cuugaccug gaaggugcca cuccacug	4440
ccuuuccuaa uaaaugagg aaauugcauc gcauugucug aguagguguc auucuauc	4500
gggggguggg guggggcagg acagcaaggg ggaggauugg gaagacaaua gcaggcaugc	4560
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<220>
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ccguuaucaa cuagaaaaag uggcaccgag ucggugcuuu uuuu	104

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<210> 416
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<210> 417
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<220>
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atggtcaag 9

<210> 418
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oligonucleotide

<400> 418
tgtgcagaag gatggagt 18

<210> 419
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<213> Artificial Sequence

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ctggtgcttc tctcaggata

20

<210> 420

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<212> DNA

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<220>

<223> 3' ZEN modifier

<400> 420

tggaatatg

9

<210> 421

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24

<210> 422

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<400> 422

caggatgaat ccaatggtca tgagg

25

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<400> 423
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9

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21

<210> 425
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<400> 425
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21

<210> 426
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cuuu 64

<210> 428
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<400> 428
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cguaaucaac uugaaaaagu ggcaccgagu cggugcuuu 99

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36

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<223> 2'-O-methyl nucleotide

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<220>
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36

<210> 433
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oligonucleotide

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<223> a, c, u, g, unknown or other

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<223> Phosphorothioate linkage

<400> 434

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36

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 <400> 436
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36

<210> 438
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36

<210> 439

<211> 36

<212> RNA

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<223> 5' ZEN modifier

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<222> (1)..(20)

<223> a, c, u, g, unknown or other

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<400> 439

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36

<210> 440

<211> 36

<212> RNA

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<400> 440
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36

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<400> 441
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17

<210> 442
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aacatctggg ccctgatt	18
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ttcccctaag gcaggctg	18
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<220>
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<400> 445
 aaauaugggg auuacuagga guuuuagagc uaugcu

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<210> 446
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 <222> (1)..(3)
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<400> 446
uuuuguaauu aacagcuugc guuuuagagc uaugcu

36

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<400> 447
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36

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 <222> (33)..(36)
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<400> 448
 cuuauaucca acacuucgug guuuuagagc uaugcu

36

<210> 449
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<220>
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<222> (65)..(67)
<223> Phosphorothioate linkage

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gugcuuu                                                                    67

<210> 450
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gugcuuu                                                                    67

<210> 451
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gu 62