



US 20170175142A1

(19) **United States**

(12) **Patent Application Publication**
ZHANG et al.

(10) **Pub. No.: US 2017/0175142 A1**

(43) **Pub. Date: Jun. 22, 2017**

(54) **CRISPR-CAS SYSTEMS AND METHODS
FOR ALTERING EXPRESSION OF GENE
PRODUCTS**

filed on Jan. 2, 2013, provisional application No. 61/791,409, filed on Mar. 15, 2013, provisional application No. 61/835,931, filed on Jun. 17, 2013, provisional application No. 61/842,322, filed on Jul. 2, 2013.

(71) Applicants: **The Broad Institute Inc.**, Cambridge, MA (US); **Massachusetts Institute of Technology**, Cambridge, MA (US); **The Rockefeller University**, New York, NY (US)

Publication Classification

(51) **Int. Cl.**

C12N 15/90 (2006.01)

C12N 15/11 (2006.01)

C12N 9/22 (2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/907*** (2013.01); ***C12N 9/22*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 2800/80*** (2013.01); ***C12N 2310/10*** (2013.01)

(72) Inventors: **Feng ZHANG**, Cambridge, MA (US); **Luciano MARRAFFINI**, New York, NY (US)

(21) Appl. No.: **15/217,489**

(22) Filed: **Jul. 22, 2016**

Related U.S. Application Data

(63) Continuation of application No. 14/324,960, filed on Jul. 7, 2014, which is a continuation of application No. 14/183,429, filed on Feb. 18, 2014, now Pat. No. 8,771,945, which is a continuation of application No. 14/054,414, filed on Oct. 15, 2013, now Pat. No. 8,697,359.

(60) Provisional application No. 61/736,527, filed on Dec. 12, 2012, provisional application No. 61/748,427,

(57)

ABSTRACT

The invention provides for systems, methods, and compositions for altering expression of target gene sequences and related gene products. Provided are vectors and vector systems, some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells and methods for utilizing the CRISPR-Cas system.

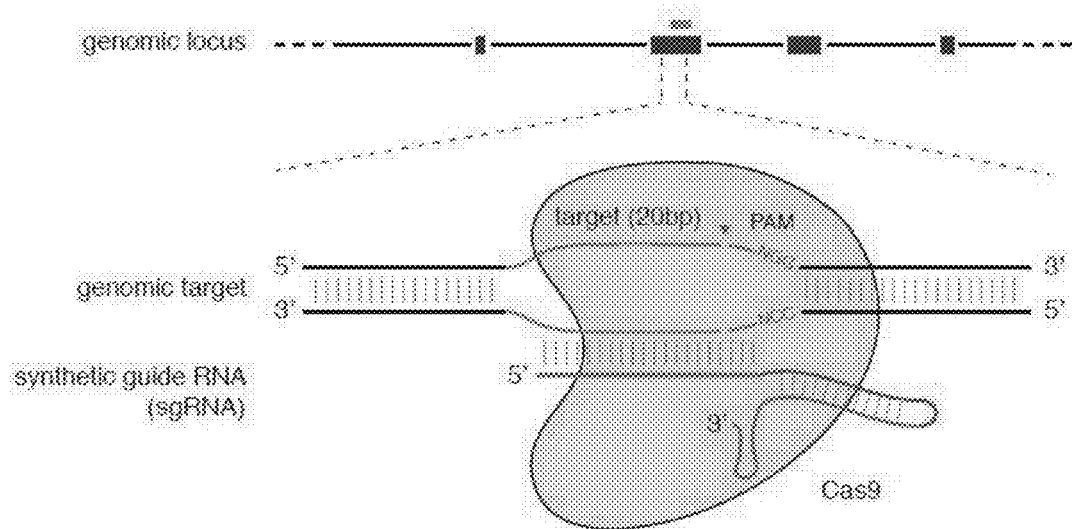


FIG. 1

A

Streptococcus pyogenes SF370 CRISPR locus 1

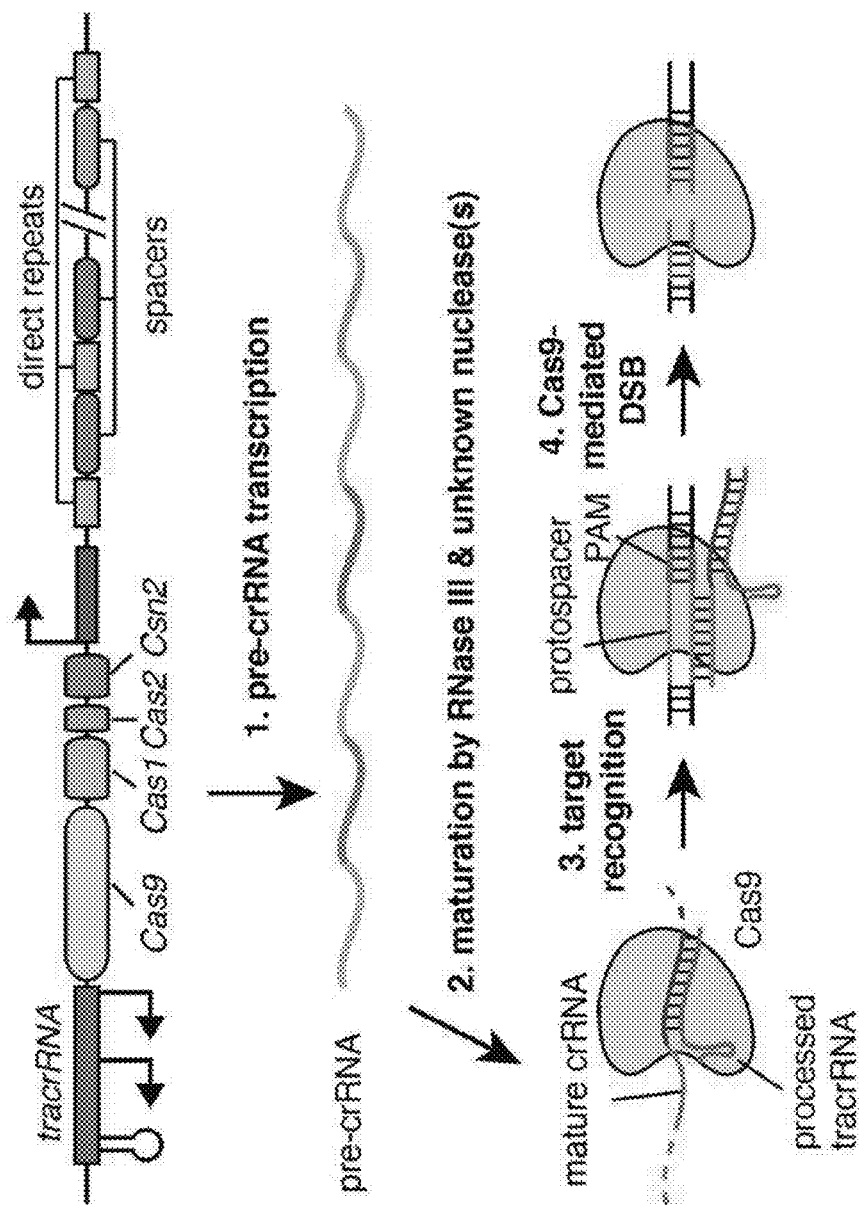


FIG. 2A

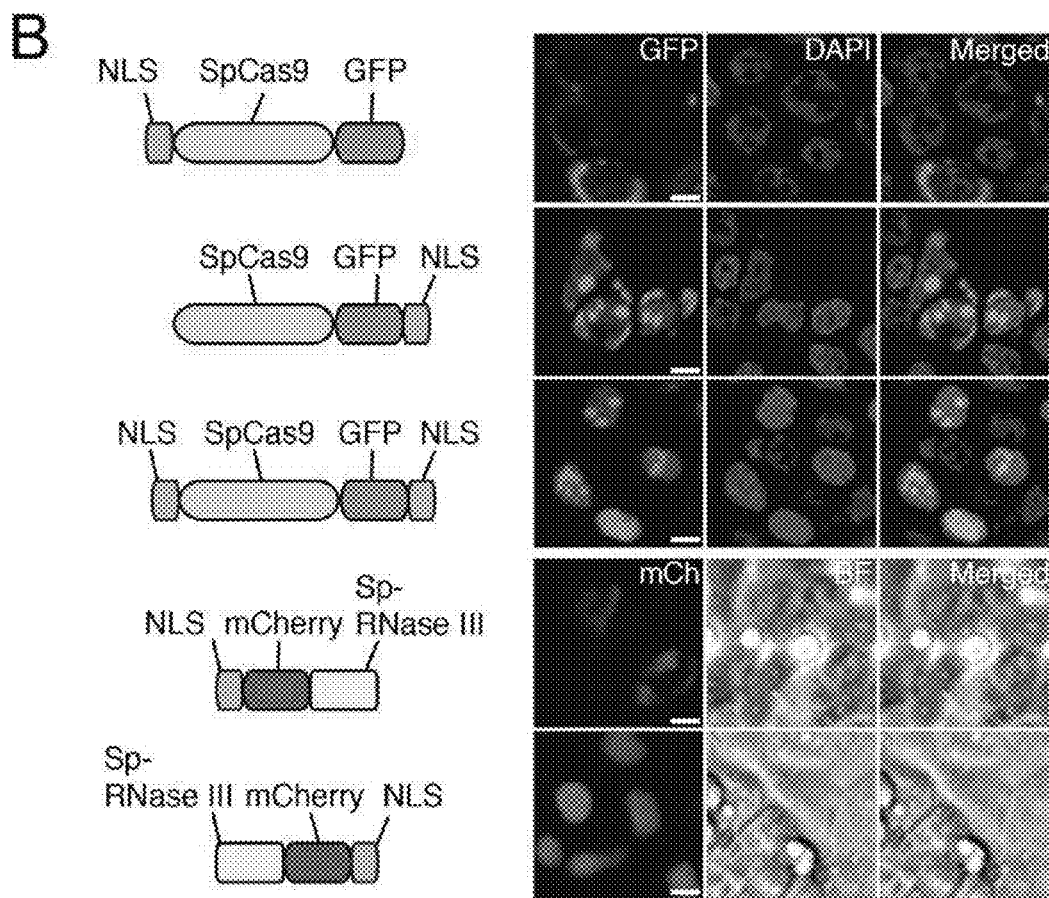
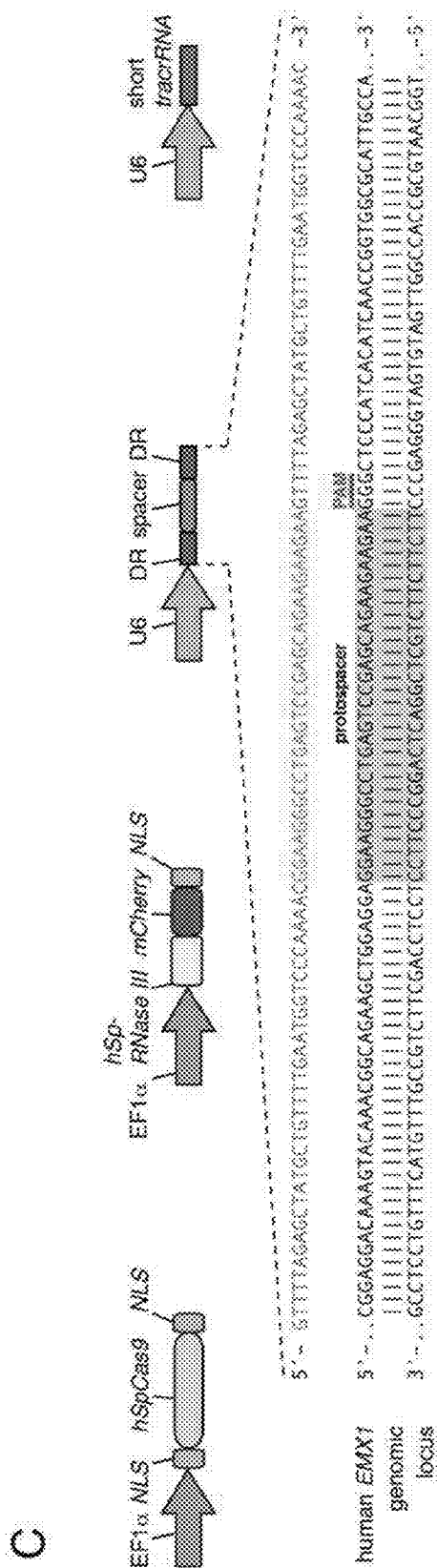


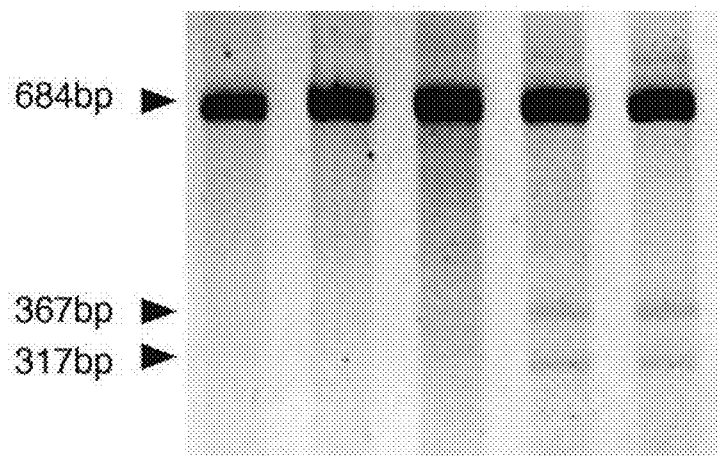
FIG. 2B



2025.10.01

D

| | | | | | |
|----------------|---|---|---|---|---|
| 2xNLS-SpCas9 | + | + | + | + | + |
| SpRNAse III | - | + | + | - | + |
| short tracrRNA | - | + | - | + | + |
| DR-EMX1-DR | + | - | + | + | + |

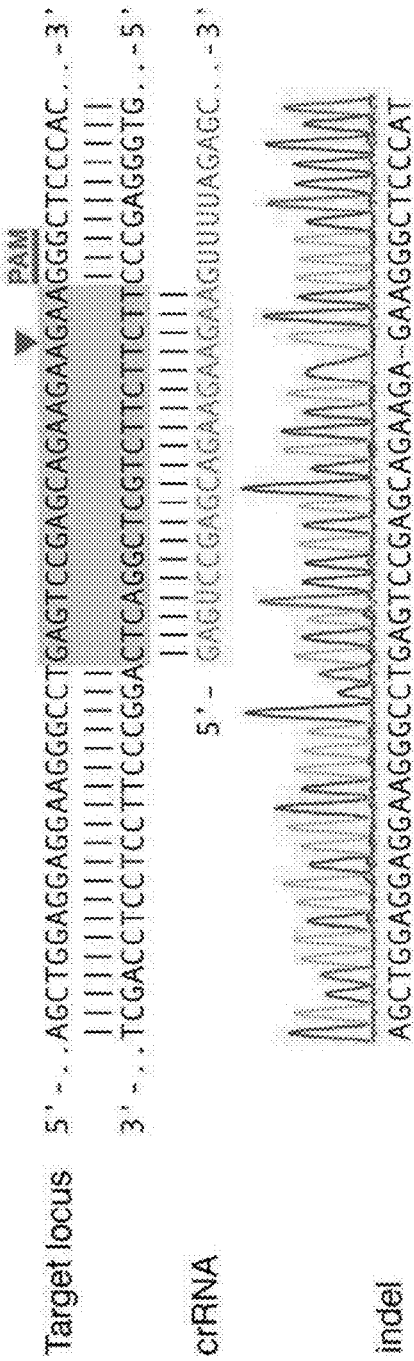


indel (%):

4.7 5.0

FIG. 2D

E



F

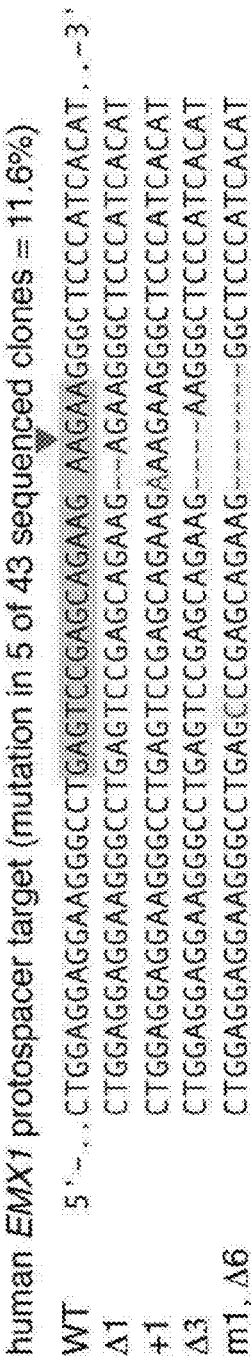


FIG. 2E-F

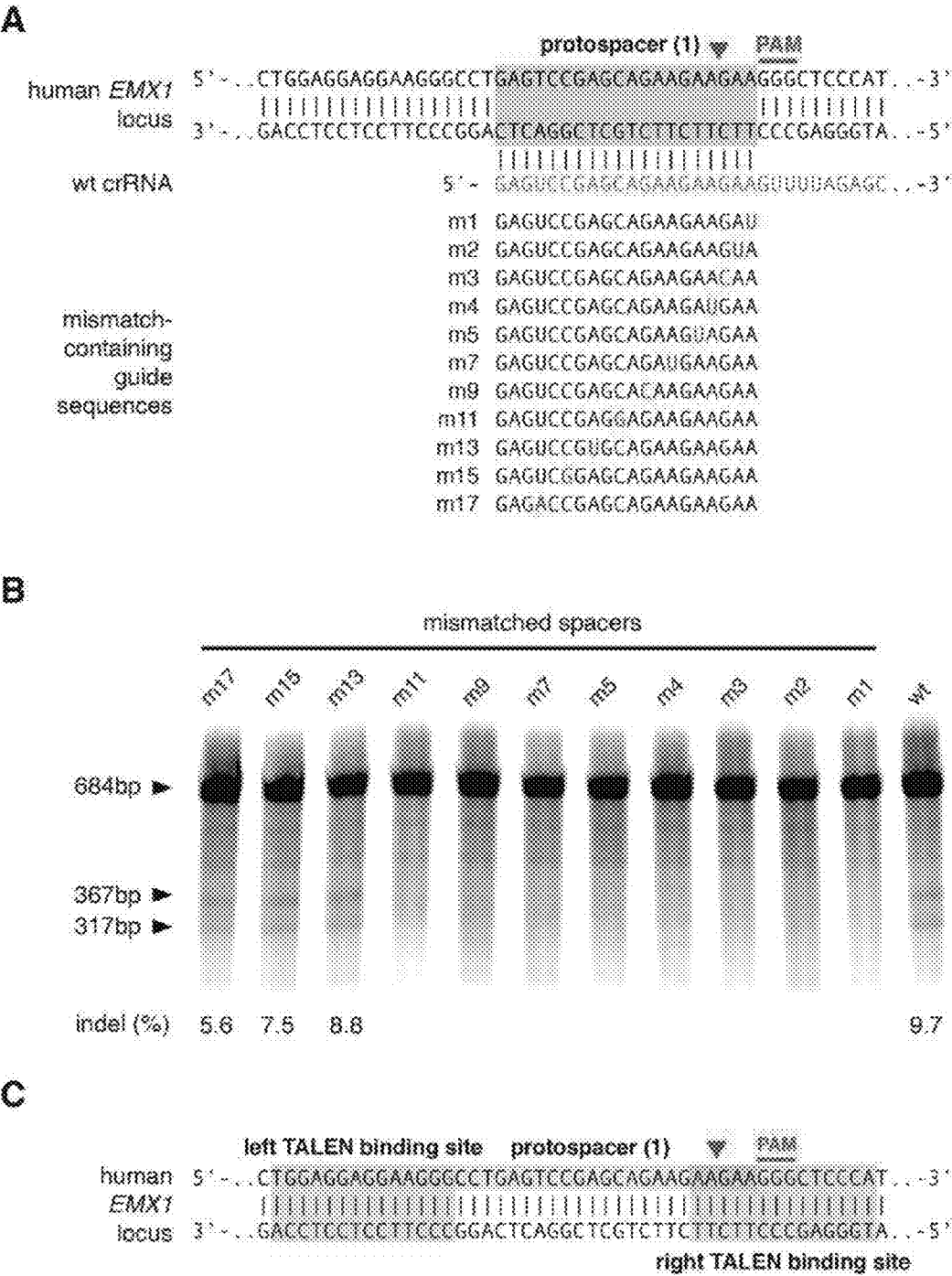


FIG. 3A-C

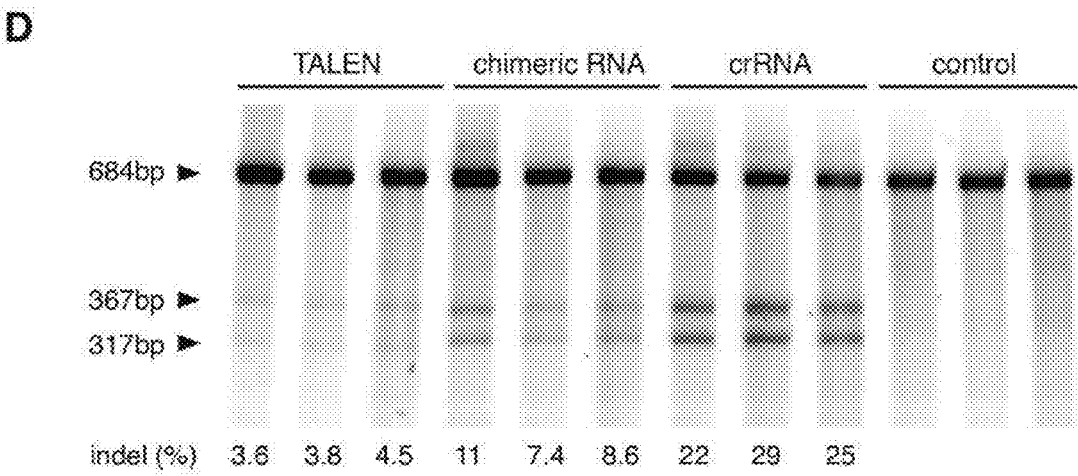


FIG. 3D

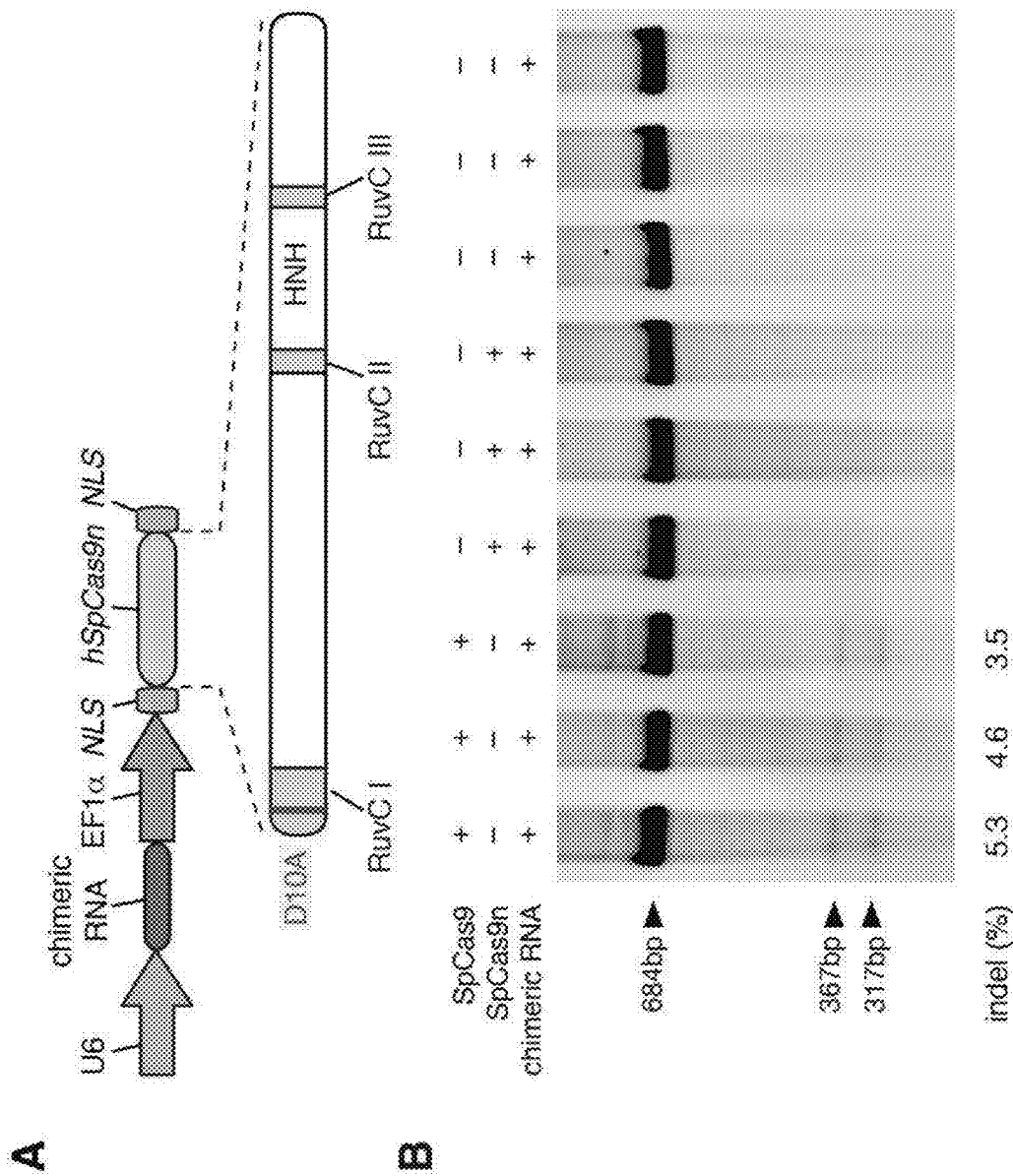


FIG. 4A-B

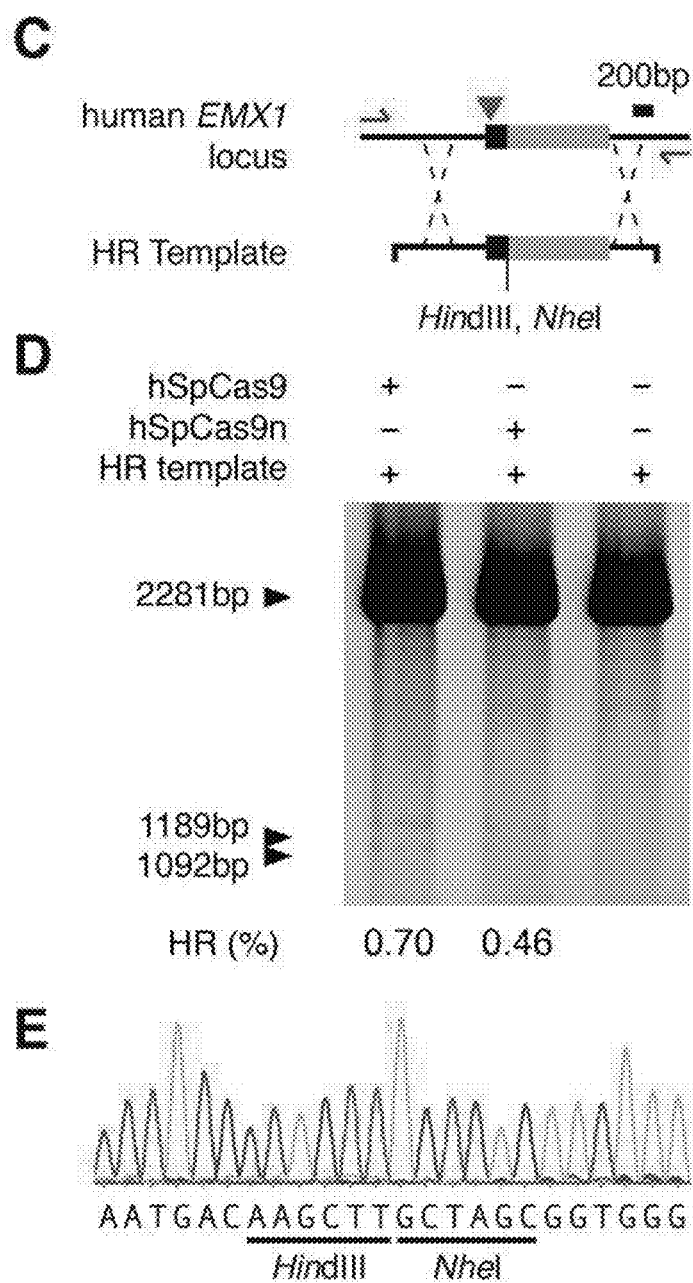


FIG. 4C-E

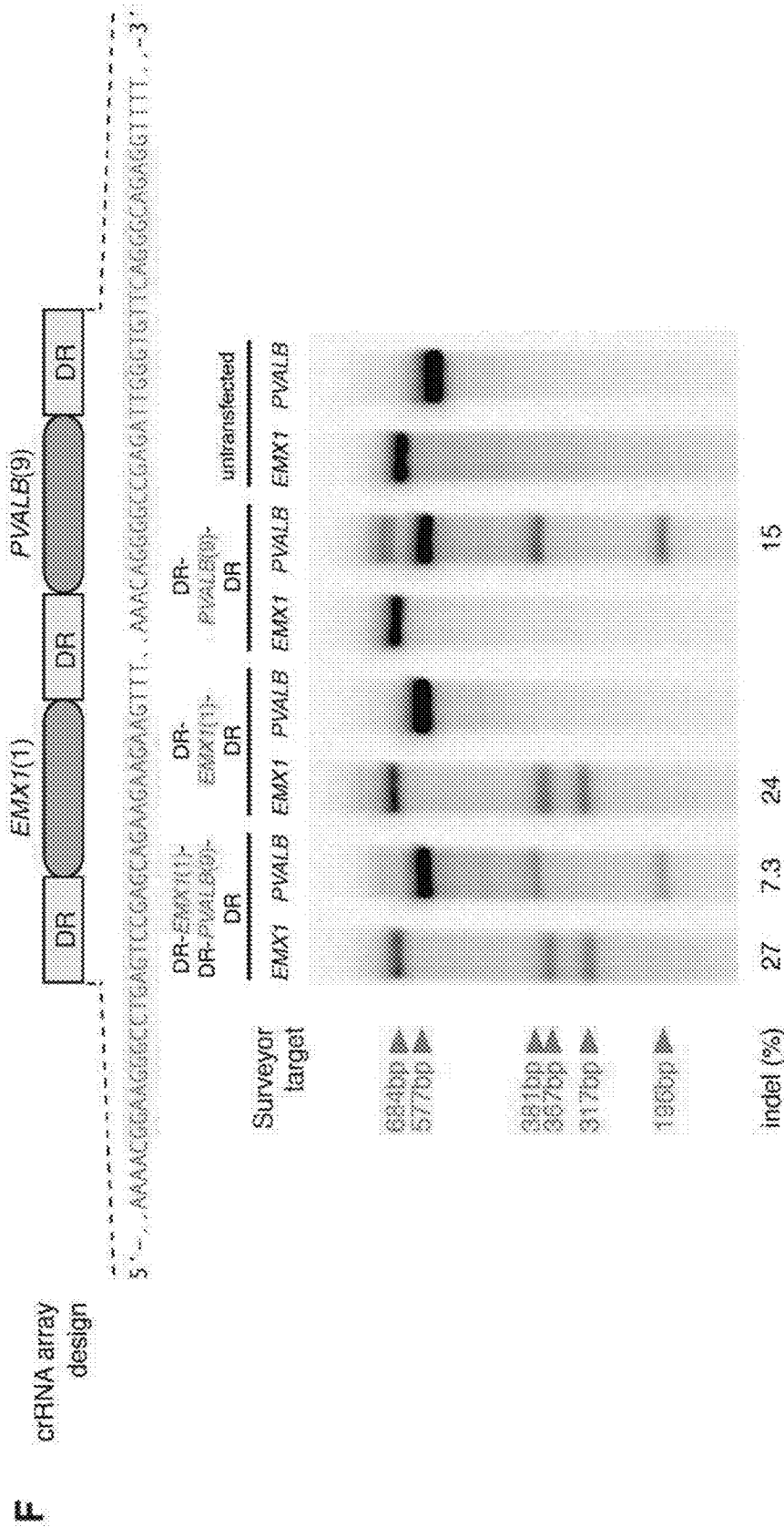


FIG. 4F

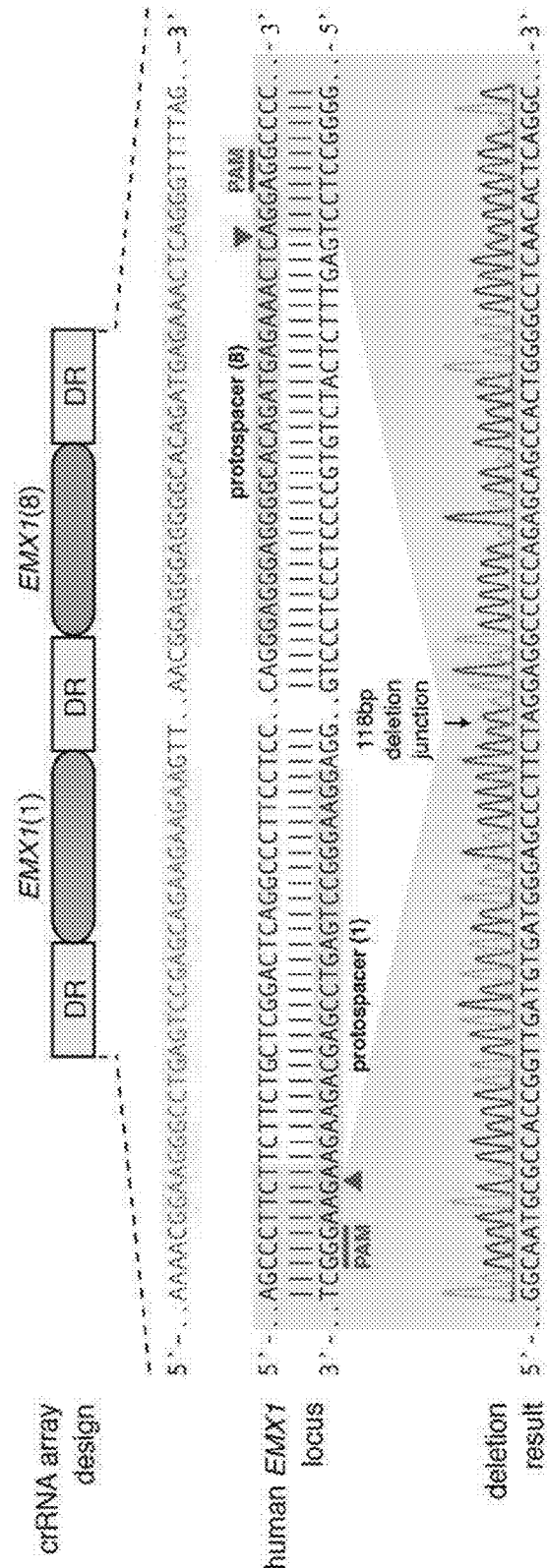


FIG. 4G

| Cas9 | target species | gene | protospacer ID | protospacer sequence (5' to 3') | PAM | strand | cell line tested | % indel (pre-crRNA + tracrRNA) | % indel (chimeric RNA) |
|---|----------------|-------|----------------|---------------------------------|----------|--------|------------------|--------------------------------|------------------------|
| <i>S. pyogenes</i> SF370 type II | Homo sapiens | EMX1 | 1 | GGAGGGCCGTGAGTCCGAGCAGAGAGAA | GCG | + | 293FT | 20 ± 1.8 | 6.7 ± 0.62 |
| | | | 2 | CATTGGAGGTGACATGATGCTCTCCCAT | TGG | - | 293FT | 2.1 ± 0.31 | N.D. |
| | | | 3 | GGACATGGATGTCACCTCCCACTAGGG | TGG | + | 293FT | 14 ± 1.1 | N.D. |
| | | | 4 | CATCGATGCTCTCCCATTTGGCTGCTTCG | TGG | - | 293FT | 11 ± 1.7 | N.D. |
| | | | 5 | TTGGTGGCAATGGCCACCGGTTGATGGA | TGG | - | 293FT | 4.3 ± 0.48 | 2.1 ± 0.51 |
| | | | 6 | TGTTGGCAATGGCCACCGGTTGATGAT | GCG | - | 293FT | 4.0 ± 0.66 | 0.41 ± 0.25 |
| | | | 7 | TCCAGCTTCTGCCGTTTGATCTTGCTTC | GCG | - | 293FT | 1.5 ± 0.12 | N.D. |
| | | | 8 | GGAGGAGGGGCACAGATGAGAACTCAGG | AGG | - | 293FT | 7.8 ± 0.83 | 2.3 ± 1.2 |
| CRISPR | Homo sapiens | PVALB | 9 | AGGGGCCGAGATTGGGTGTCAGGGCAGAG | AGG | + | 293FT | 21 ± 2.6 | 6.5 ± 0.32 |
| | | | 10 | ATGCAGAGGGTGGCGAGAGGGGCCGAGAT | TGG | + | 293FT | N.D. | N.D. |
| | | | 11 | GGTGGCAGAGGGGCCGAGATTGGGTGTTTC | AGG | + | 293FT | N.D. | N.D. |
| <i>Mus musculus</i> | Th | | 12 | CAGCAGTGAAGTCCATTAGCTAATGTCAT | AGG | - | Neuro2A | 27 ± 4.3 | 4.1 ± 2.2 |
| | | | 13 | AATGCATAGGGTATCCACCCACAGGTGCAG | GCG | - | Neuro2A | 4.8 ± 1.2 | N.D. |
| | | | 14 | ACACACATGGGAAAGCCCTCTGGGCCAGGA | AGG | + | Neuro2A | 11.3 ± 1.3 | N.D. |
| | | | | | | | | | |
| <i>S. thermophilus</i> LMD-9 CRISPR1 | Homo sapiens | EMX1 | 15 | GGAGGAGGTAGTATACAGAAACACAGAA | GTAGAAAT | - | 293FT | 14 ± 0.88 | N.T. |
| | | | 16 | AGCATGTAGAGGATCACAGAAACTCAGCA | CTAGAAA | - | 293FT | 7.8 ± 0.77 | N.T. |

FIG. 5

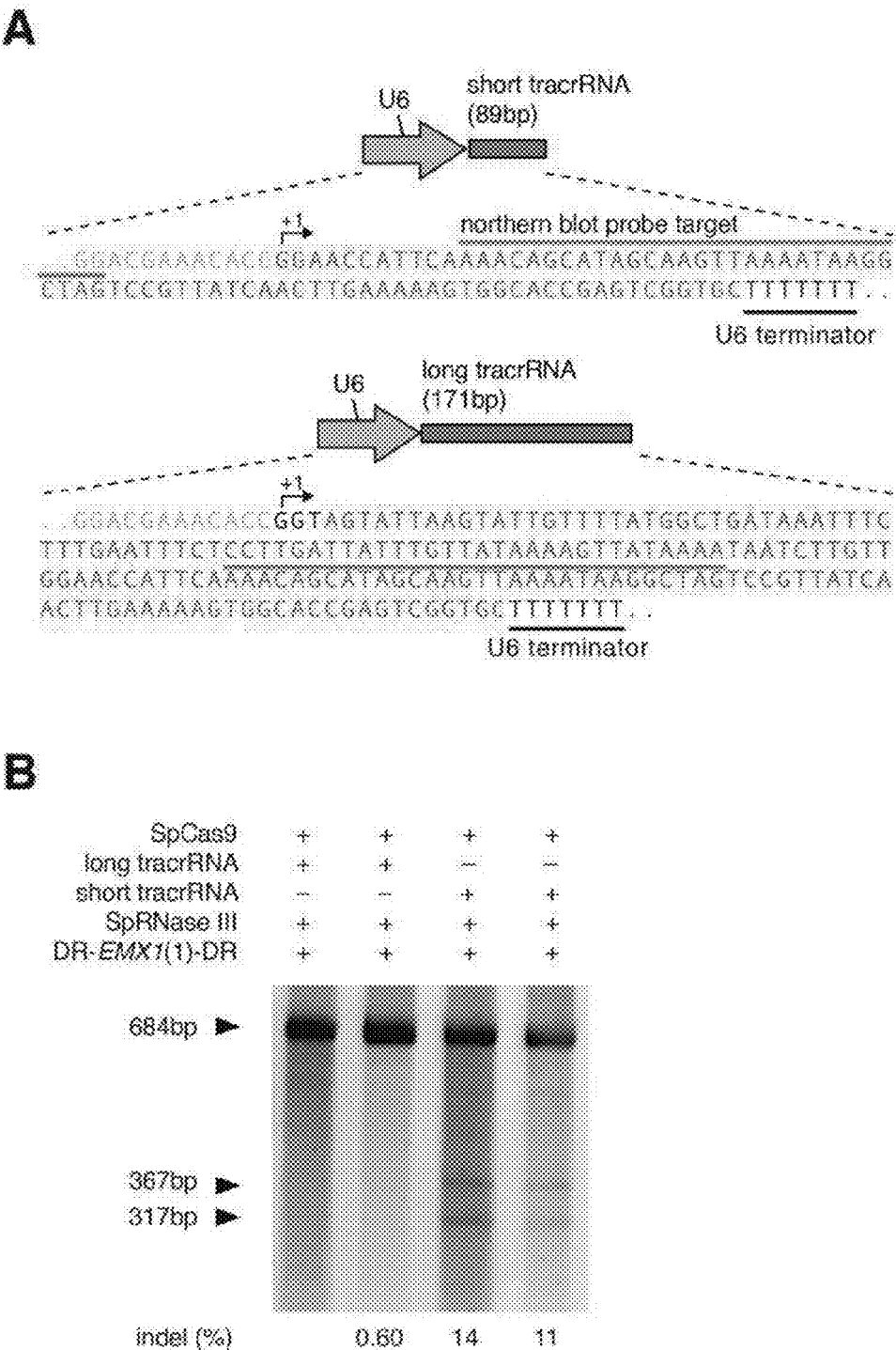


FIG. 6A-B

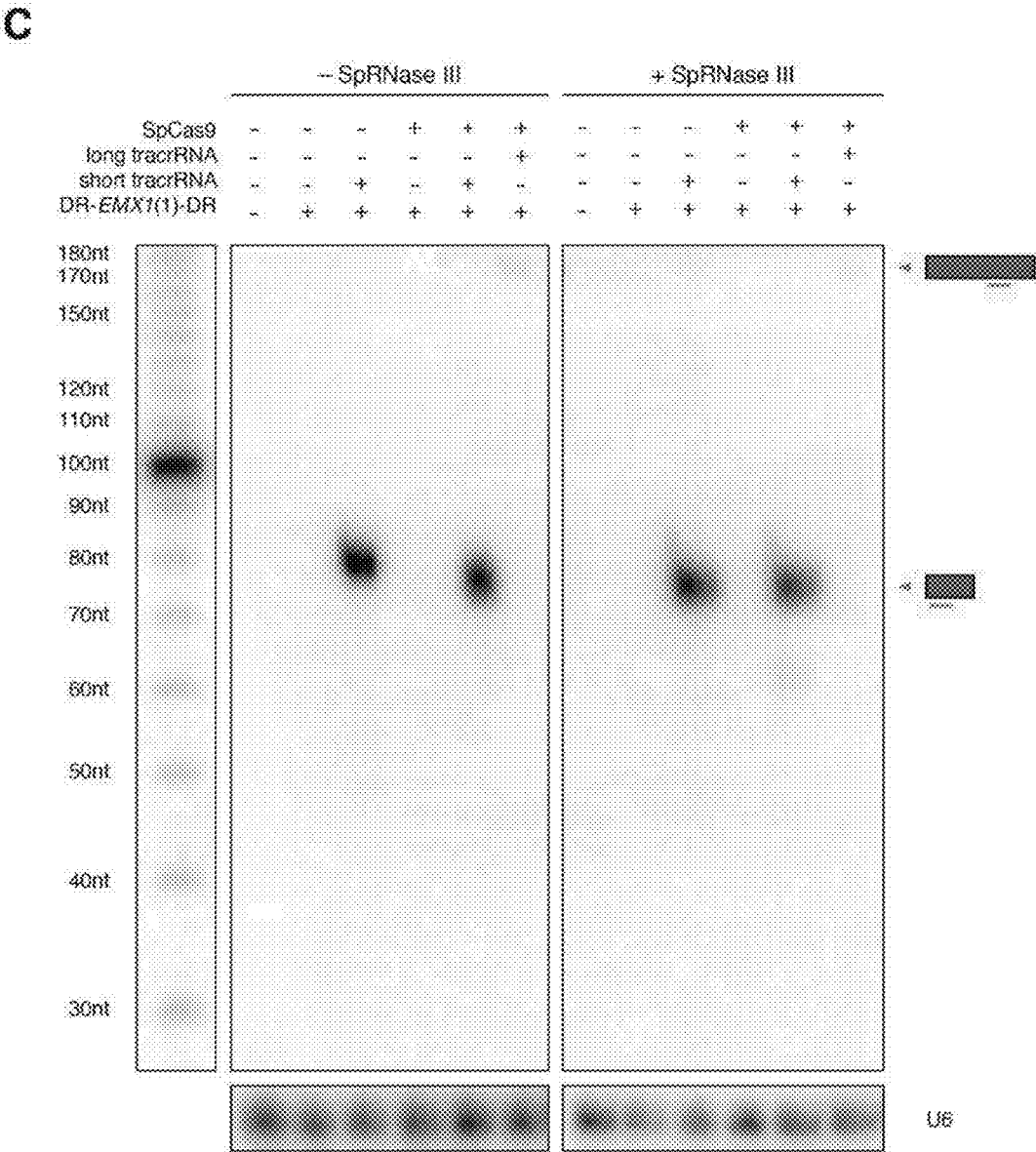
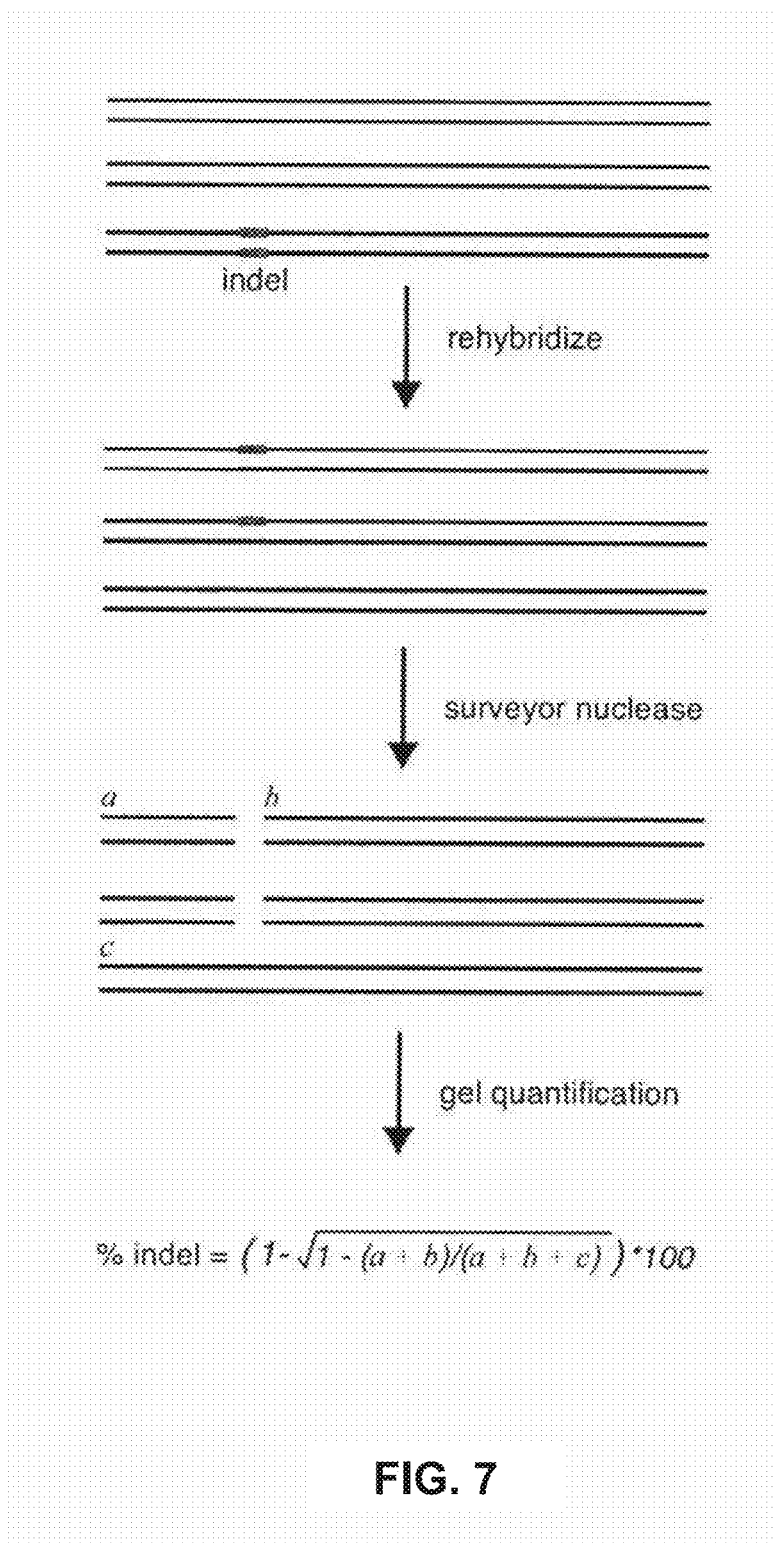


FIG. 6C



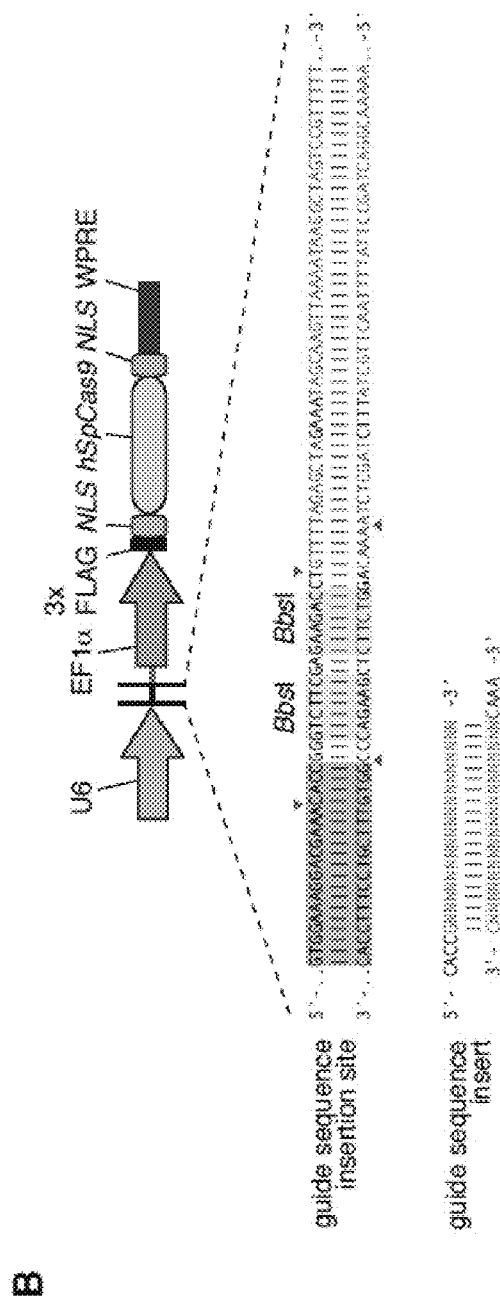
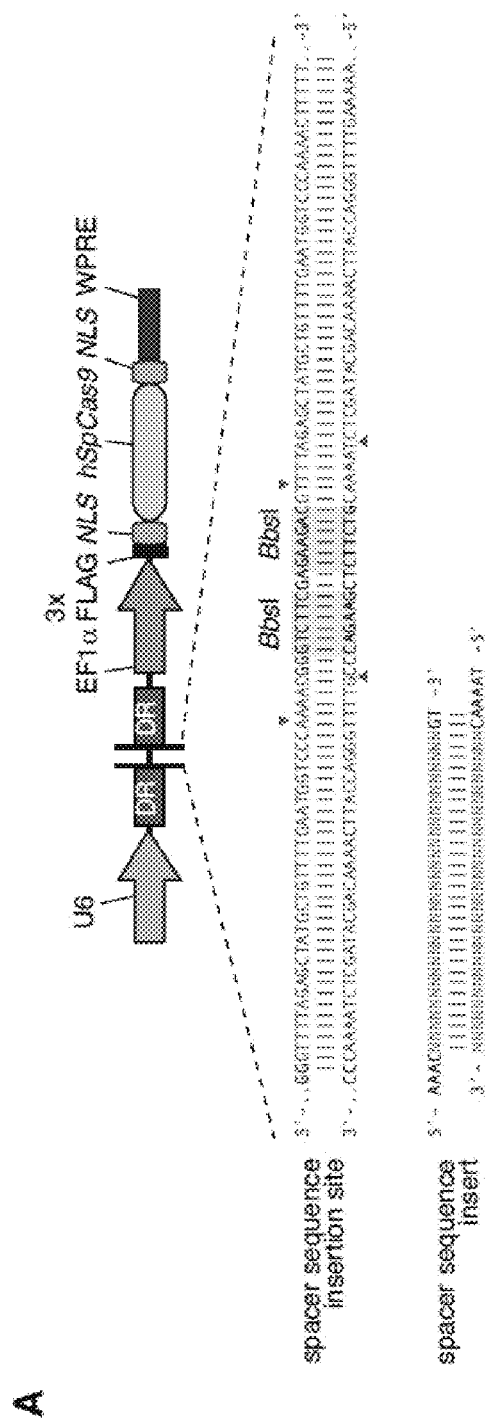


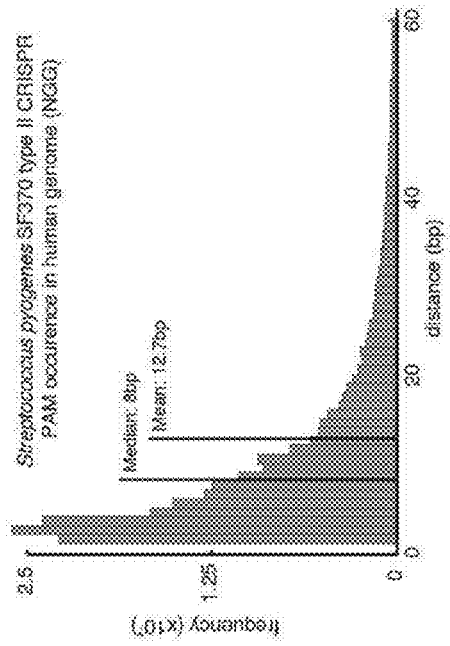
FIG. 8A-B

C

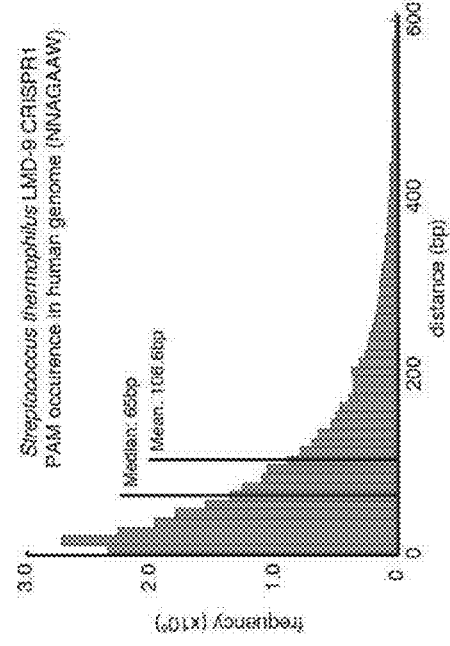
| NGG | | NNAGAAW | |
|-----|--------|---------|--------|
| Chr | median | mean | median |
| 1 | 7 | 12.8 | 67 |
| 2 | 8 | 12.7 | 64 |
| 3 | 8 | 13.0 | 63 |
| 4 | 9 | 14.0 | 61 |
| 5 | 8 | 13.1 | 63 |
| 6 | 8 | 13.1 | 63 |
| 7 | 8 | 12.4 | 64 |
| 8 | 8 | 12.8 | 64 |
| 9 | 7 | 13.9 | 65 |
| 10 | 7 | 12.1 | 66 |
| 11 | 7 | 12.0 | 65 |
| 12 | 8 | 12.4 | 65 |
| 13 | 8 | 13.6 | 62 |
| 14 | 8 | 12.0 | 65 |
| 15 | 7 | 11.5 | 68 |
| 16 | 7 | 11.7 | 74 |
| 17 | 6 | 10.3 | 76 |
| 18 | 8 | 13.4 | 63 |
| 19 | 6 | 9.4 | 82 |
| 20 | 7 | 11.1 | 72 |
| 21 | 7 | 13.4 | 64 |
| 22 | 6 | 9.2 | 85 |
| X | 8 | 13.2 | 63 |
| Y | 8 | 29.2 | 62 |
| | | | mean |
| | | | 115.8 |
| | | | 100.8 |
| | | | 98.5 |
| | | | 94.5 |
| | | | 97.9 |
| | | | 98.5 |
| | | | 102.9 |
| | | | 100.9 |
| | | | 120.5 |
| | | | 107.0 |
| | | | 105.8 |
| | | | 103.5 |
| | | | 94.6 |
| | | | 101.5 |
| | | | 107.7 |
| | | | 136.8 |
| | | | 127.9 |
| | | | 101.8 |
| | | | 145.4 |
| | | | 121.8 |
| | | | 111.4 |
| | | | 140.3 |
| | | | 99.0 |
| | | | 223.7 |

FIG. 9A-C

A



B



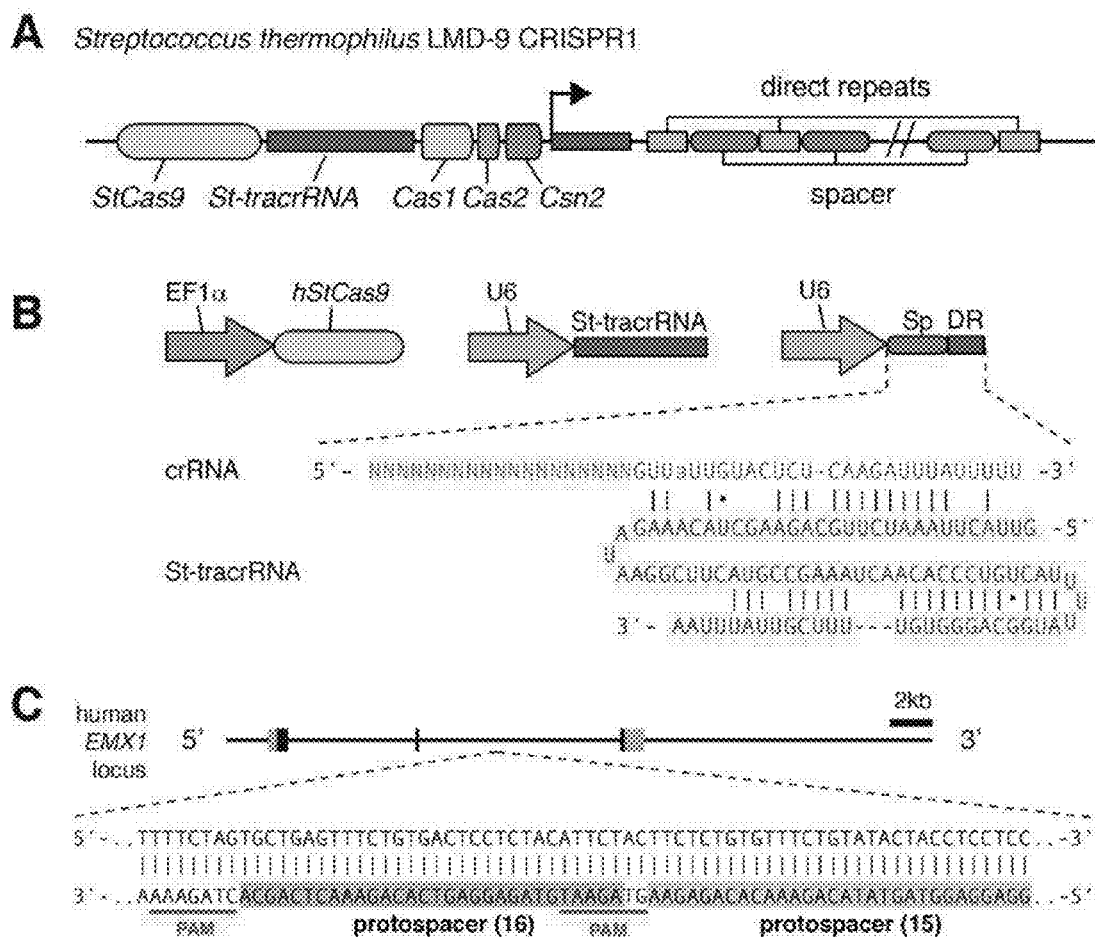


FIG. 10A-C

D

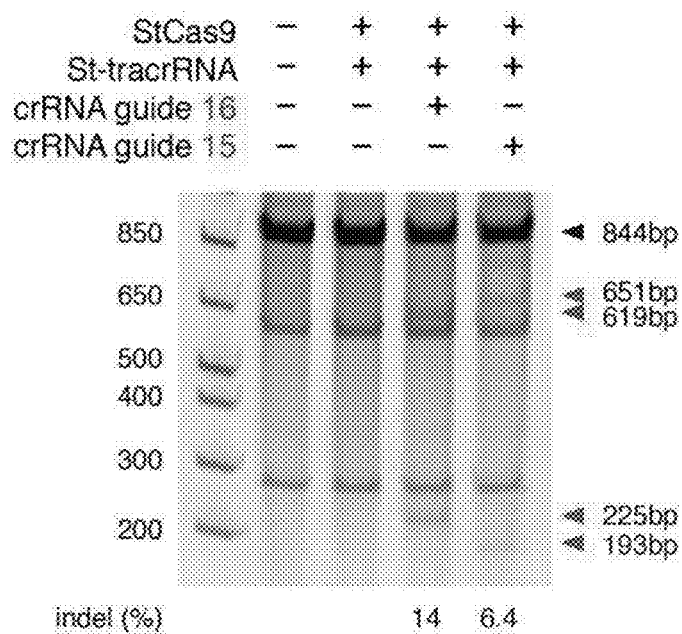
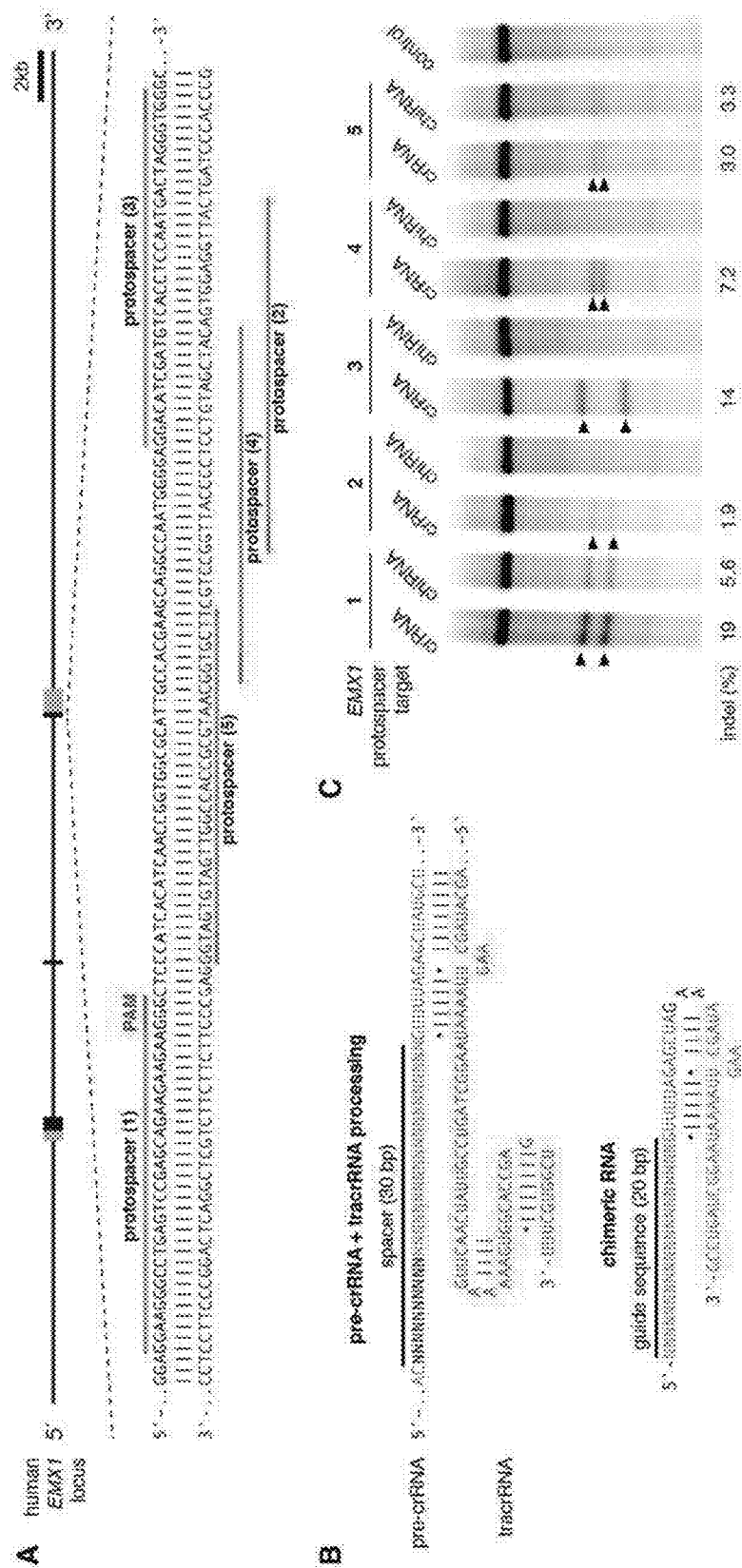


FIG. 10D



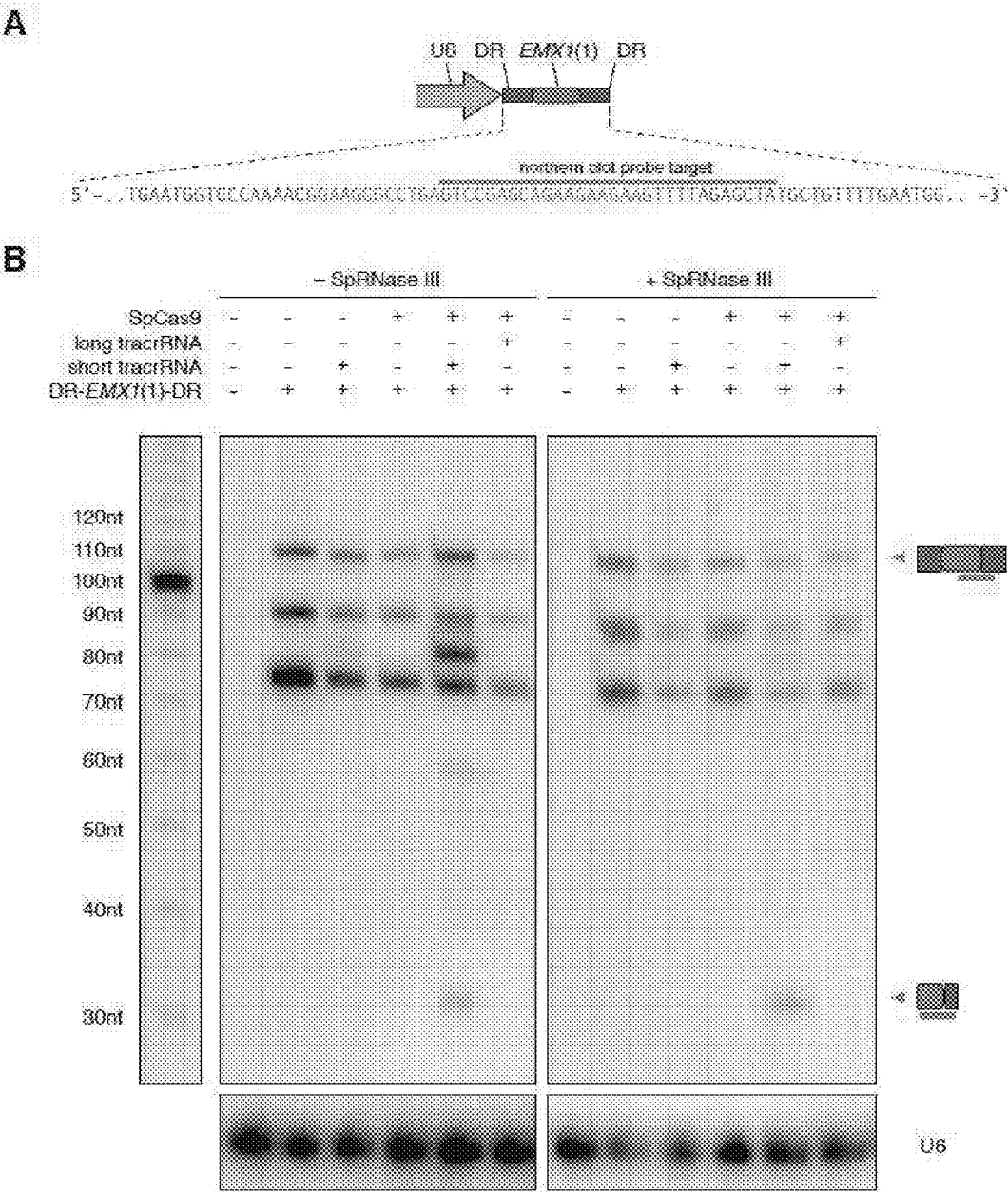


FIG. 12A-B

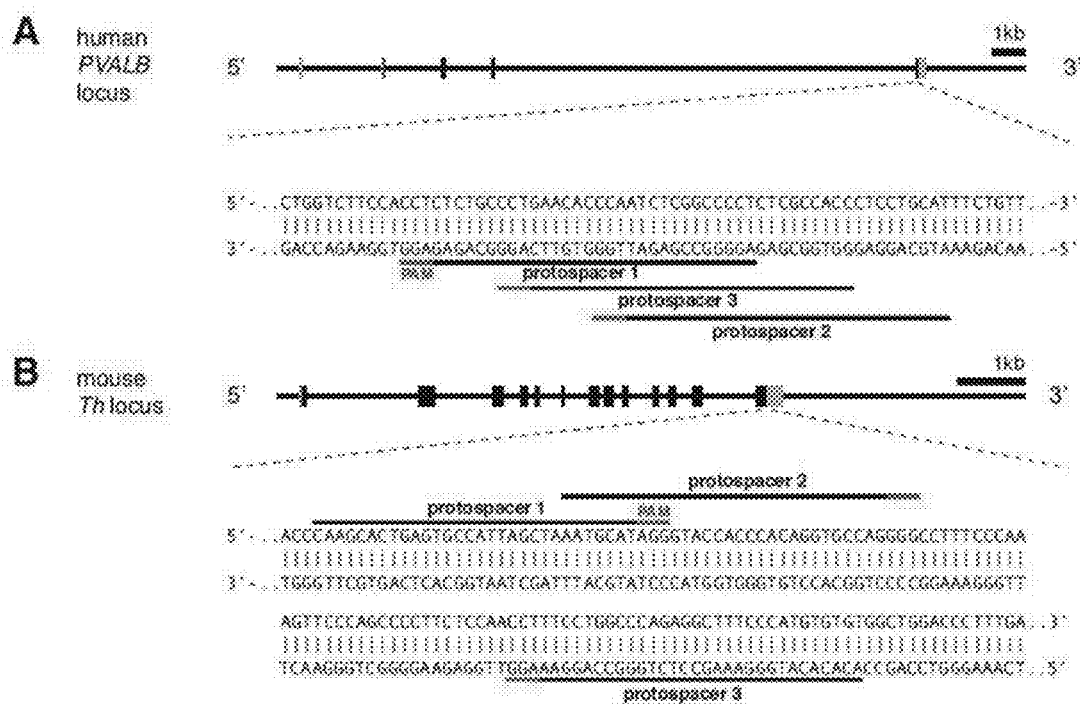


FIG. 13A-B

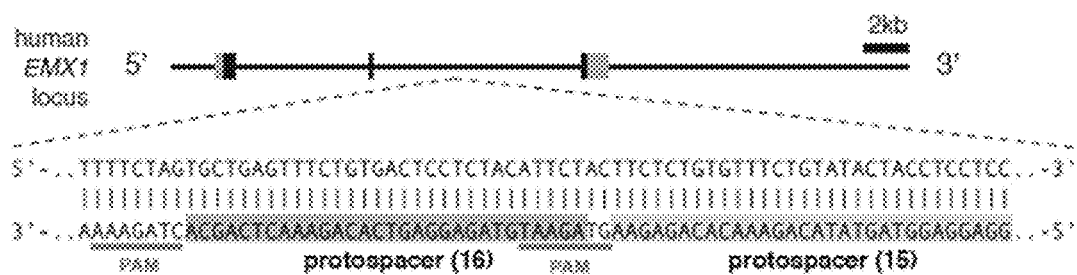


FIG. 14

| Primer name | Assay | Genomic Target | Primer sequence |
|--------------------|-------------------------------|-------------------|--------------------------------|
| Sp-EMX1-F | SURVEYOR assay, sequencing | <i>EMX1</i> | AAAACCACCCCTTCTCTCTGGC |
| Sp-EMX1-R | SURVEYOR assay, sequencing | <i>EMX1</i> | GGAGATTGGAGACACGGAGAG |
| Sp-PVALB-F | SURVEYOR assay, sequencing | <i>PVALB</i> | CTGGAAAGCCAATGCCTGAC |
| Sp-PVALB-R | SURVEYOR assay, sequencing | <i>PVALB</i> | GGCAGCAAACCTCCTTGTCCT |
| Sp-Th-F | SURVEYOR assay, sequencing | <i>Th</i> | GTGCTTTGCAGAGGCC TACC |
| Sp-Th-R | SURVEYOR assay, sequencing | <i>Th</i> | CCTGGAGCGCATGCAGTAGT |
| St-EMX1-F | SURVEYOR assay, sequencing | <i>EMX1</i> | ACCTTCTGTGTTTCCACCATTC |
| St-EMX1-R | SURVEYOR assay, sequencing | <i>EMX1</i> | TTGGGGAGTGCACAGACTTC |
| Sp-EMX1- RFLP-F | RFLP, sequencing | <i>EMX1</i> | GGCTCCCTGGGTTCAAAGTA |
| Sp-EMX1- RFLP-R | RFLP, sequencing | <i>EMX1</i> | AGAGGGGTCTGGATGTCGTAA |
| Pb_EMX1_sp1 | Northern Blot Probe | Not applicable | TAGCTCTAAAACCTTCTTCTCTGCTCGGAC |
| Pb_tracrRNA | Northern Blot Probe | Not applicable | CTAGCCTTATTTTAACTTGCTATGCTGTTT |

FIG. 15

a

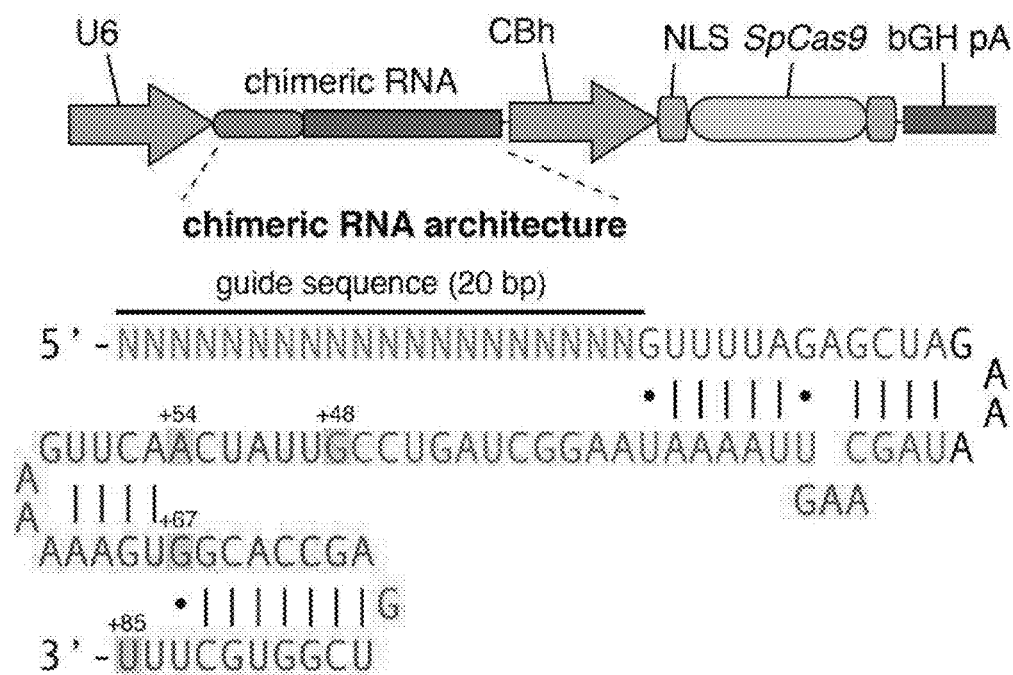


FIG. 16A

b

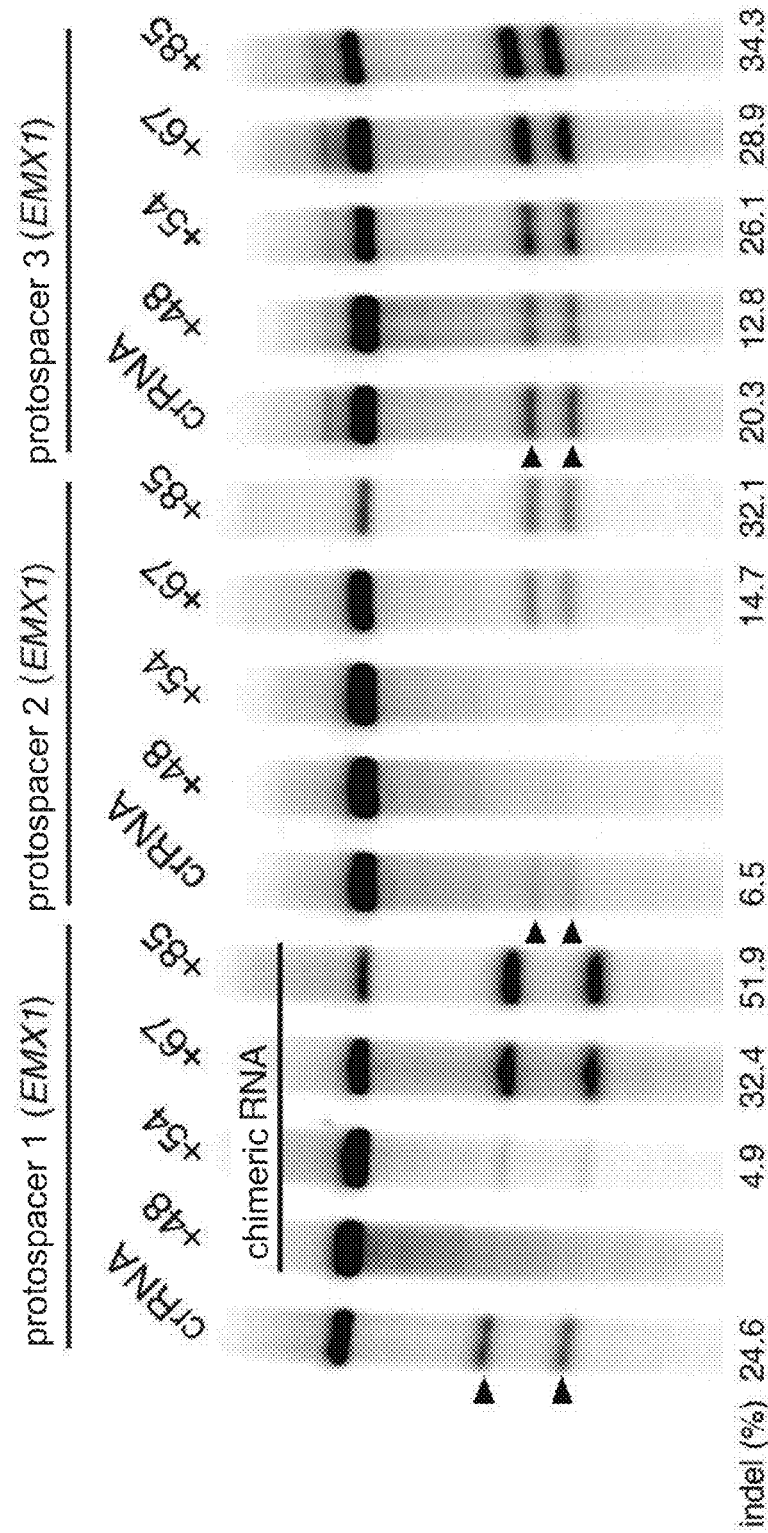


FIG. 16B

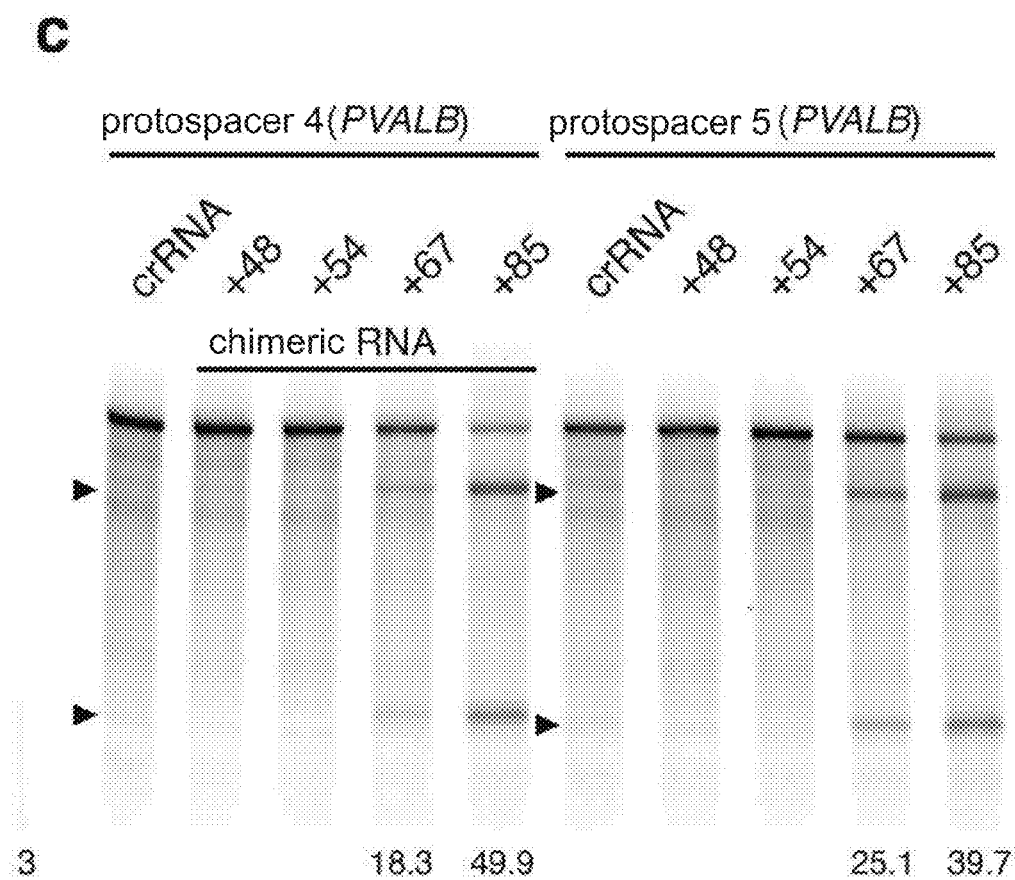


FIG. 16C

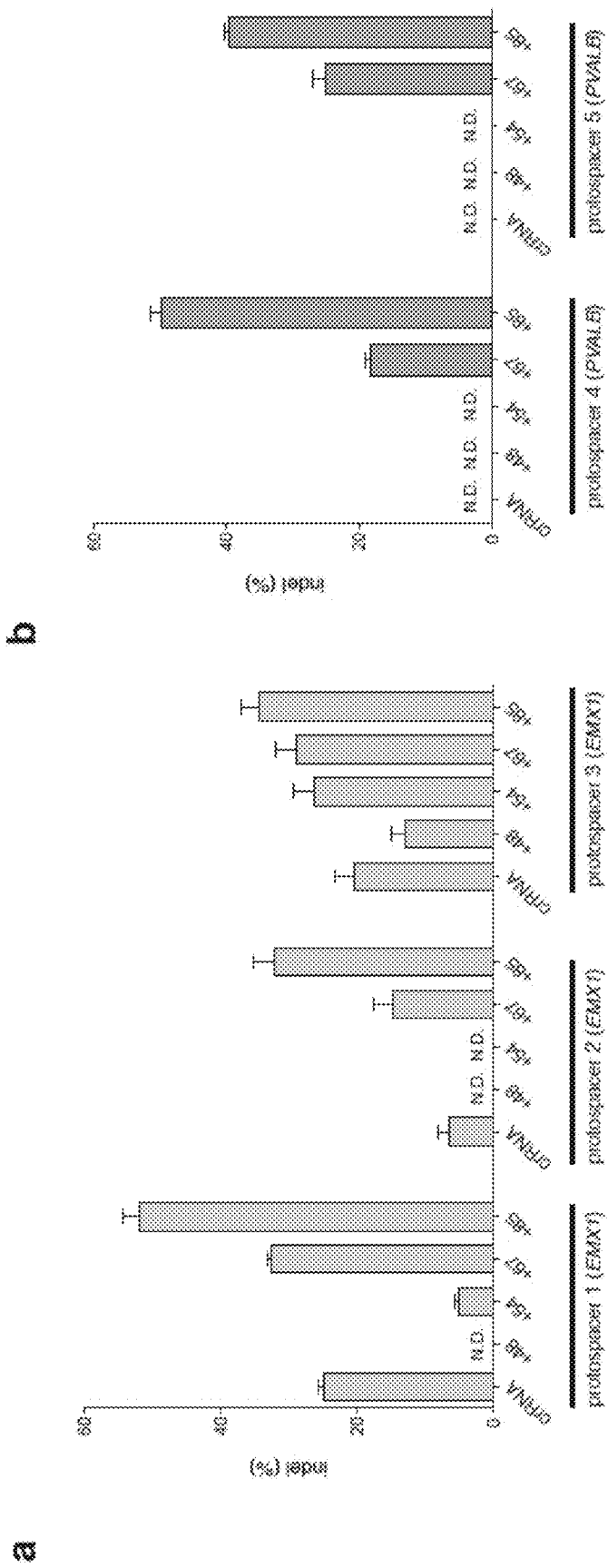


FIG. 17A-B



3. Results

222

2

65

U

2002

7

16

6502

33067

3

chr12:2,614,008-2,675,748:61,741 bp

enter position, gene symbol or search terms

8

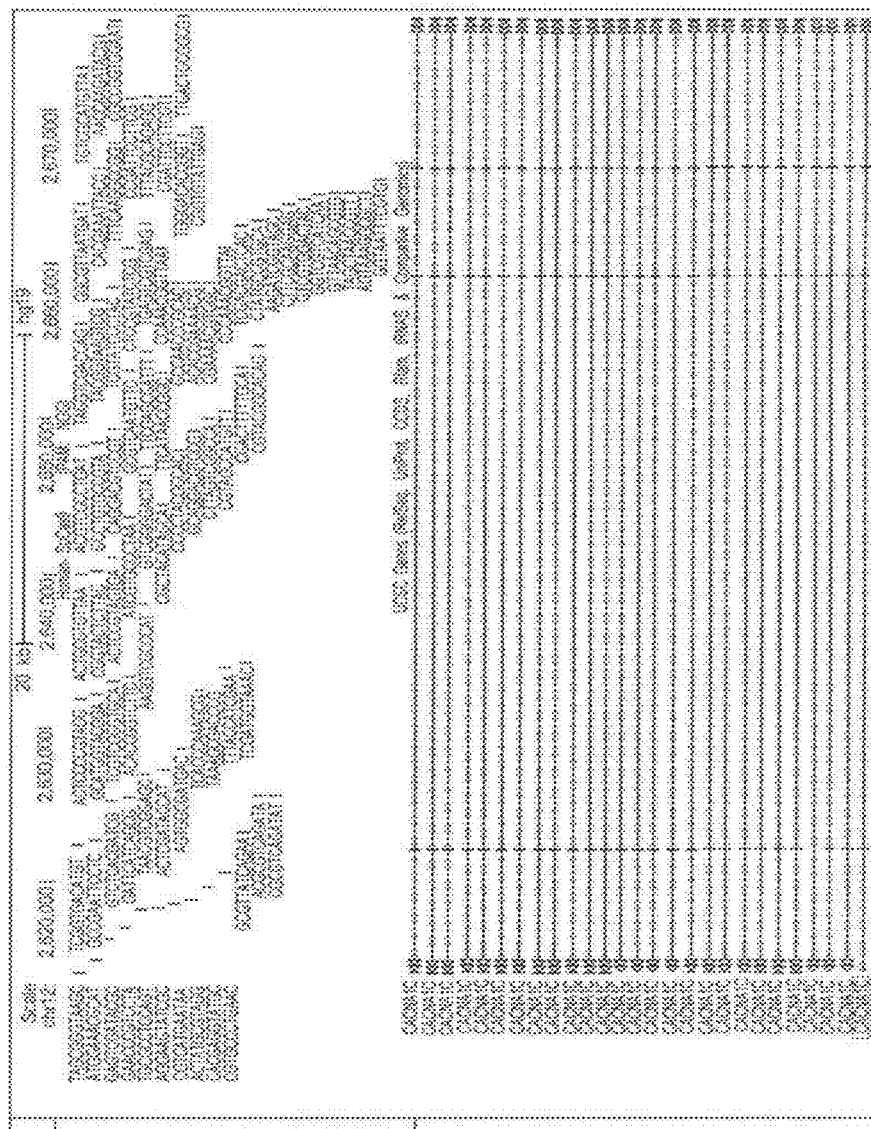
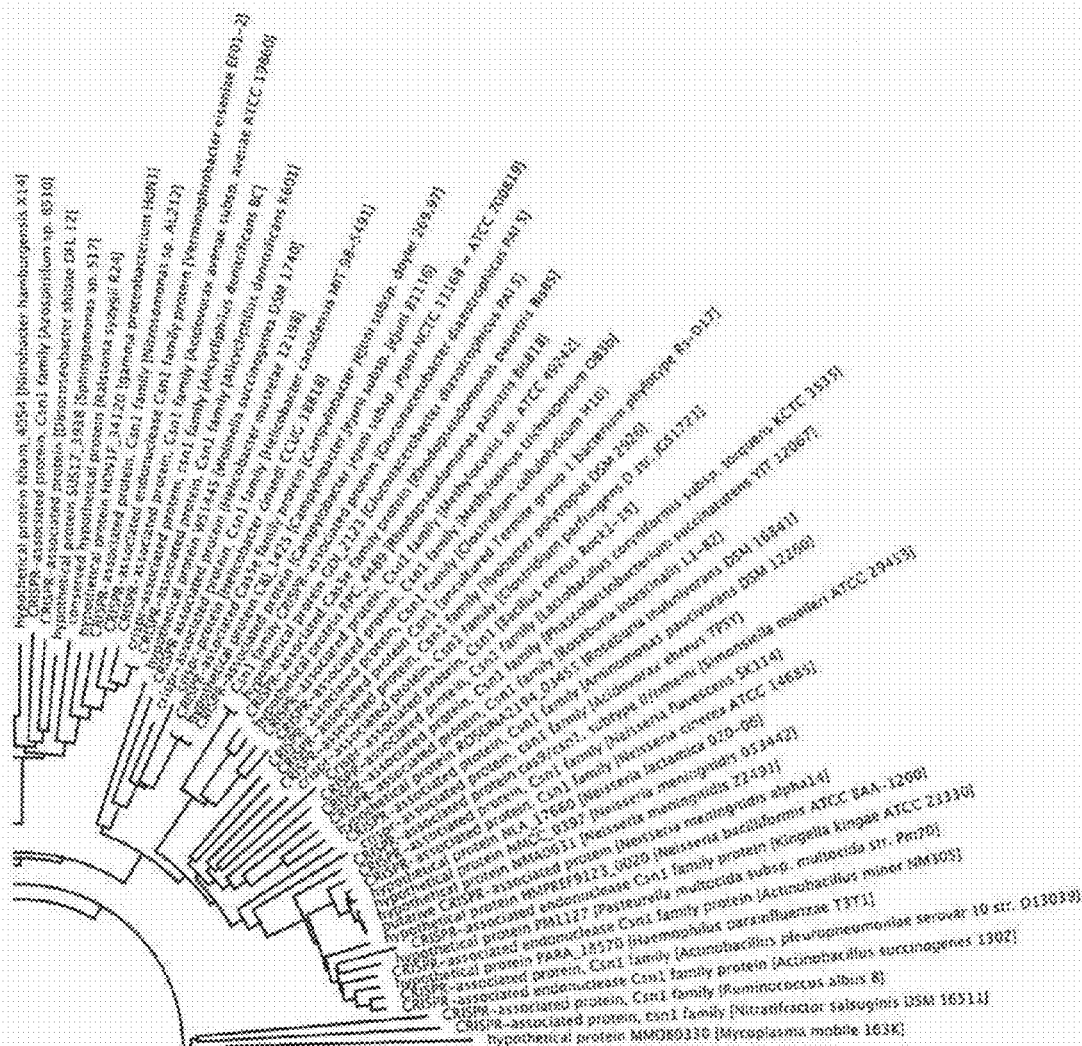
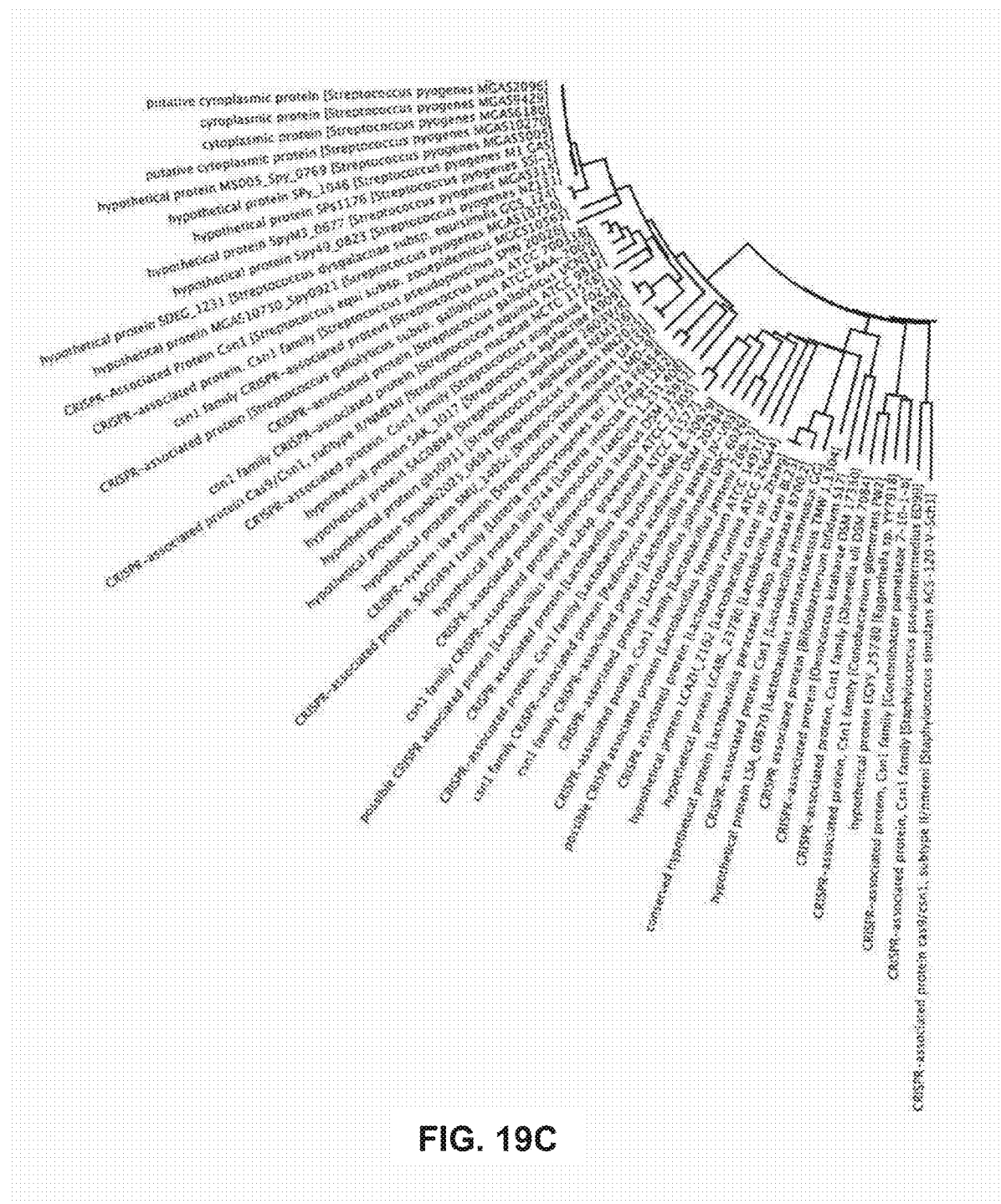
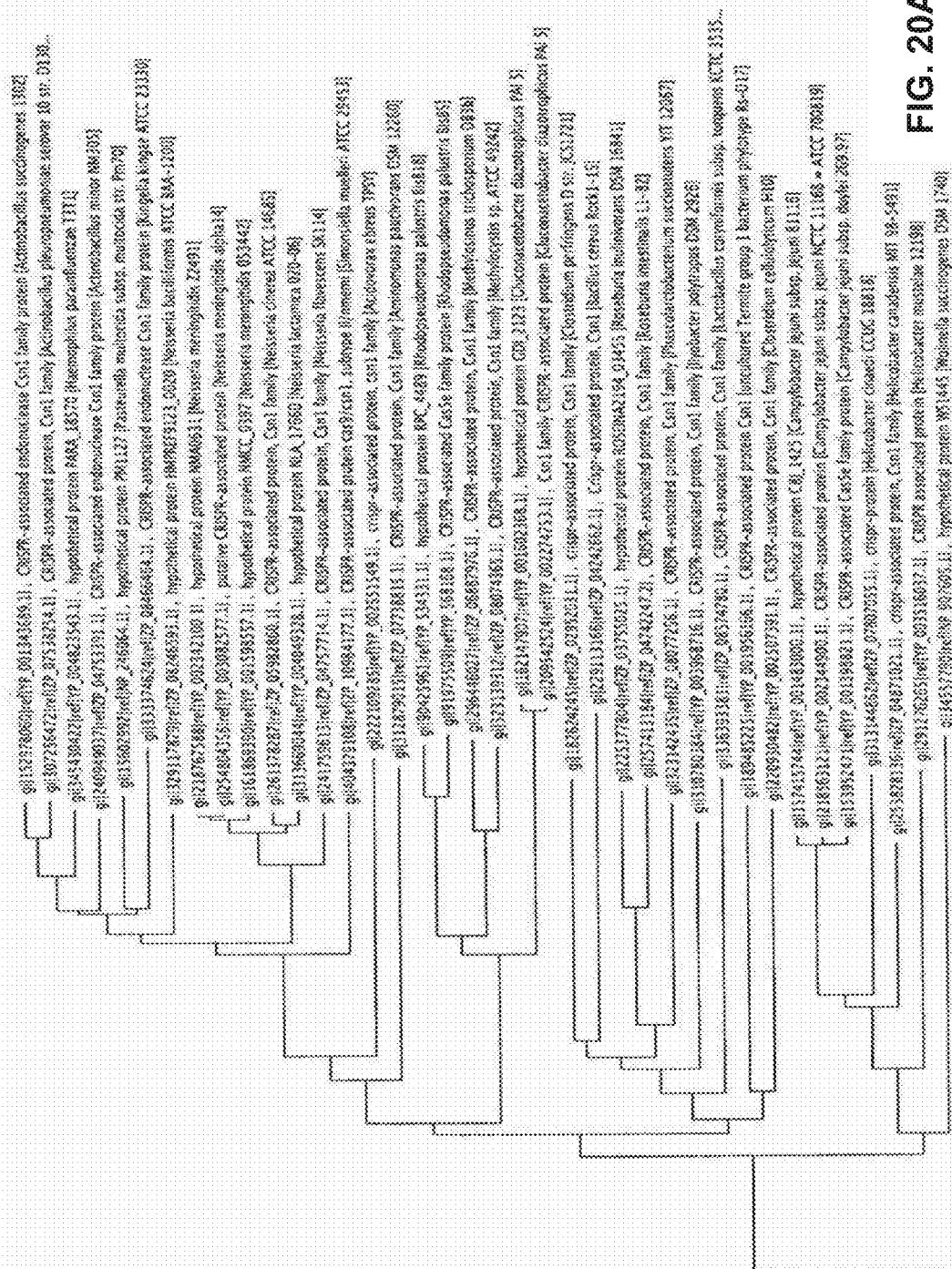
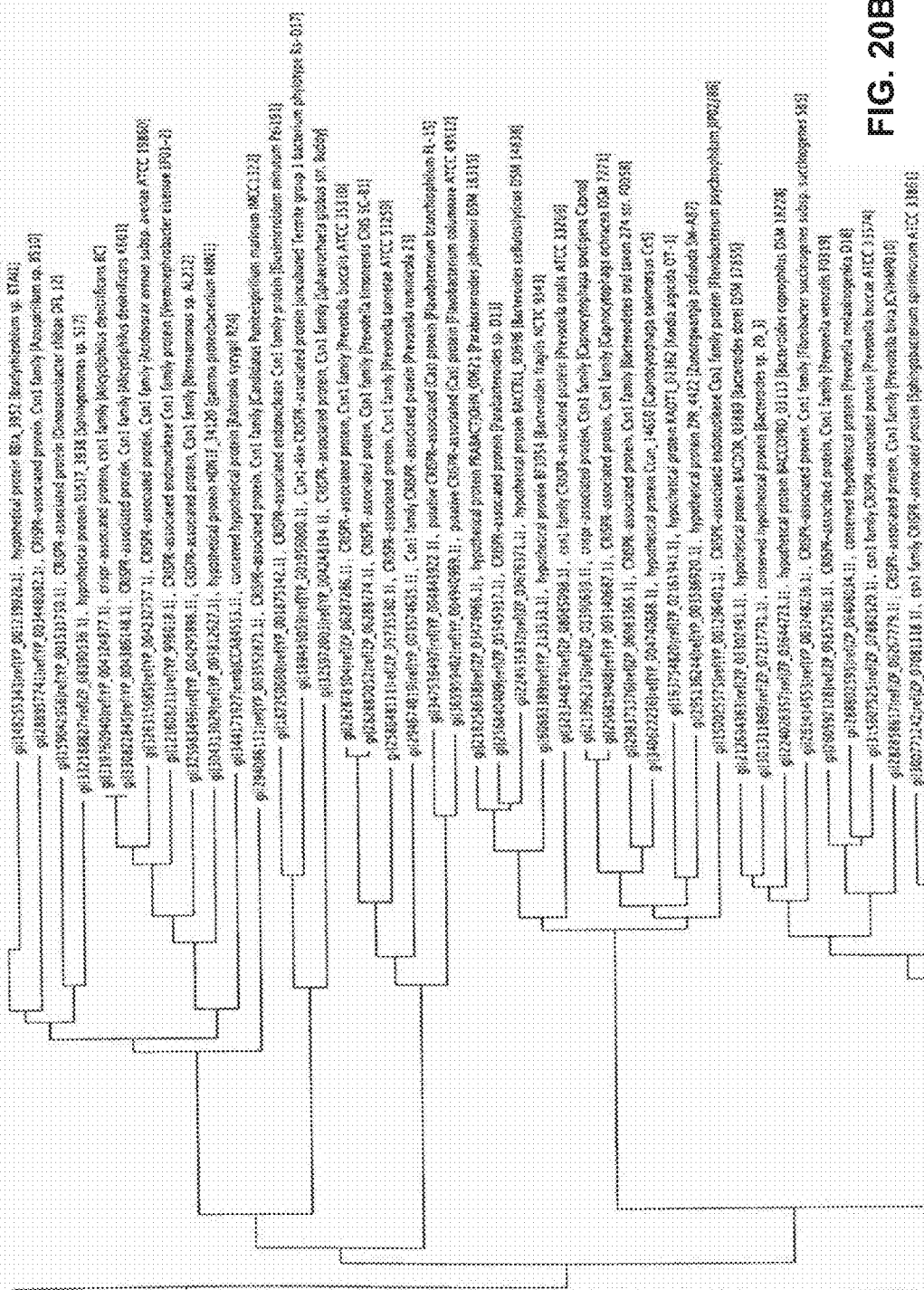


FIG. 19A









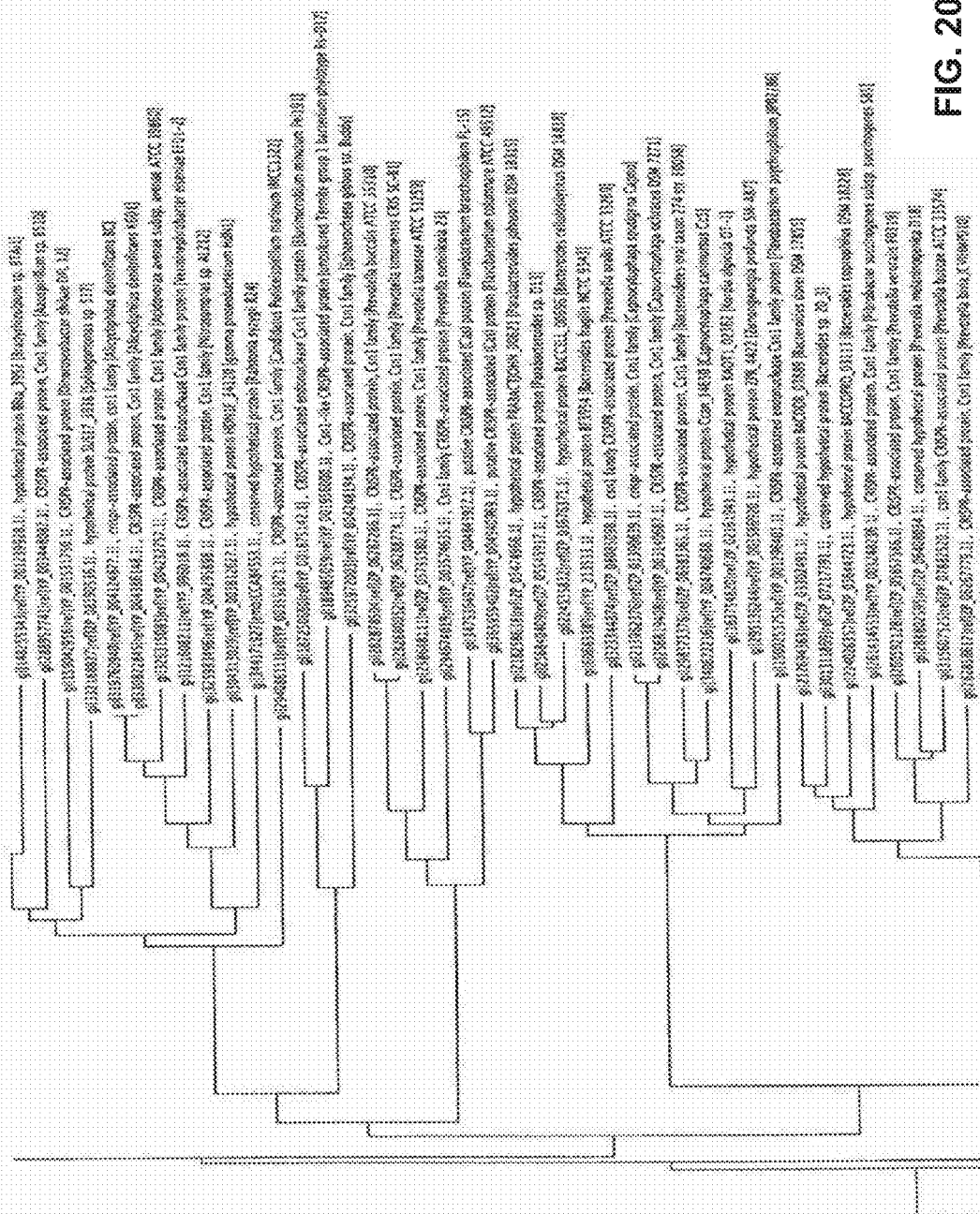
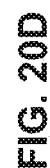
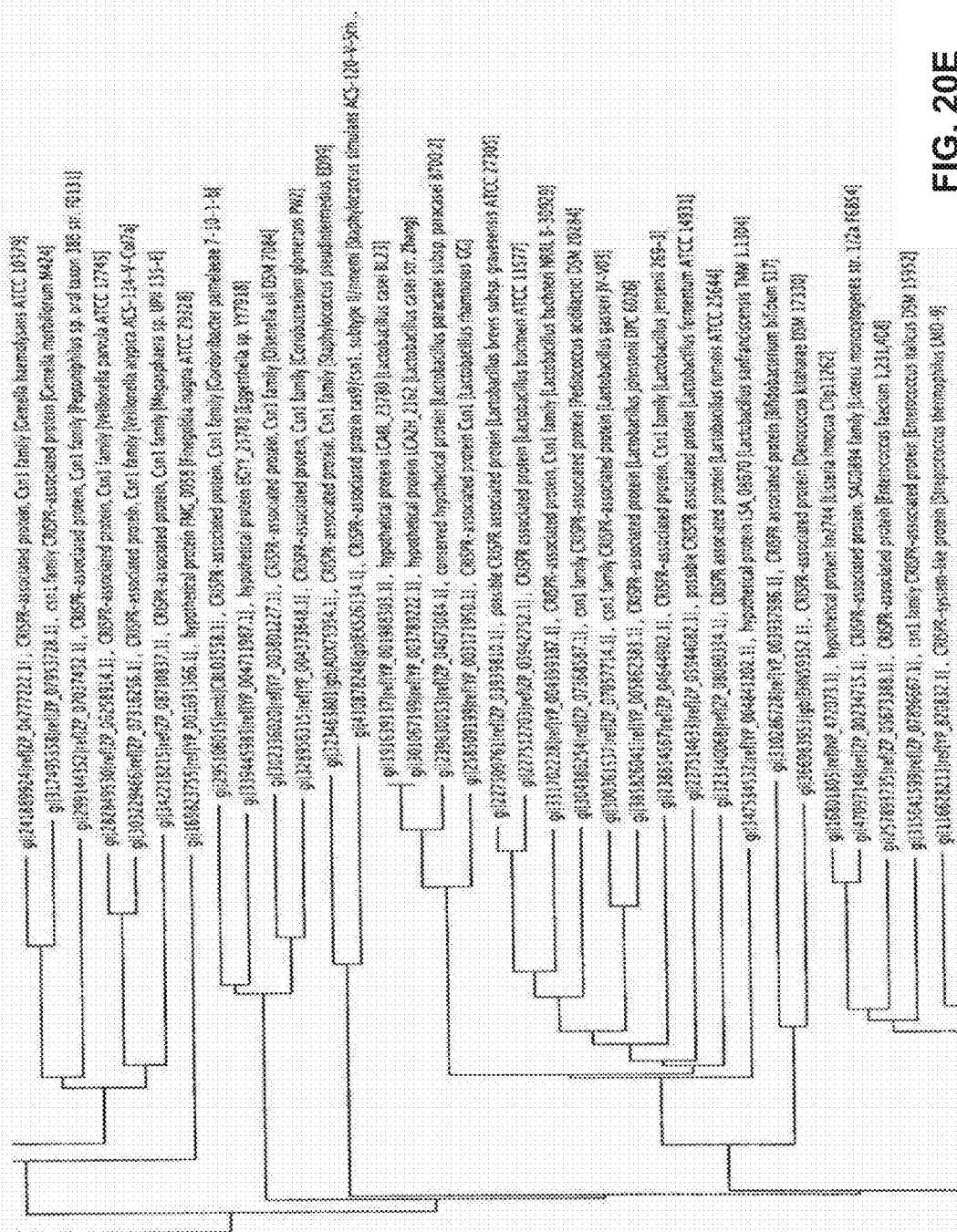


FIG. 20C





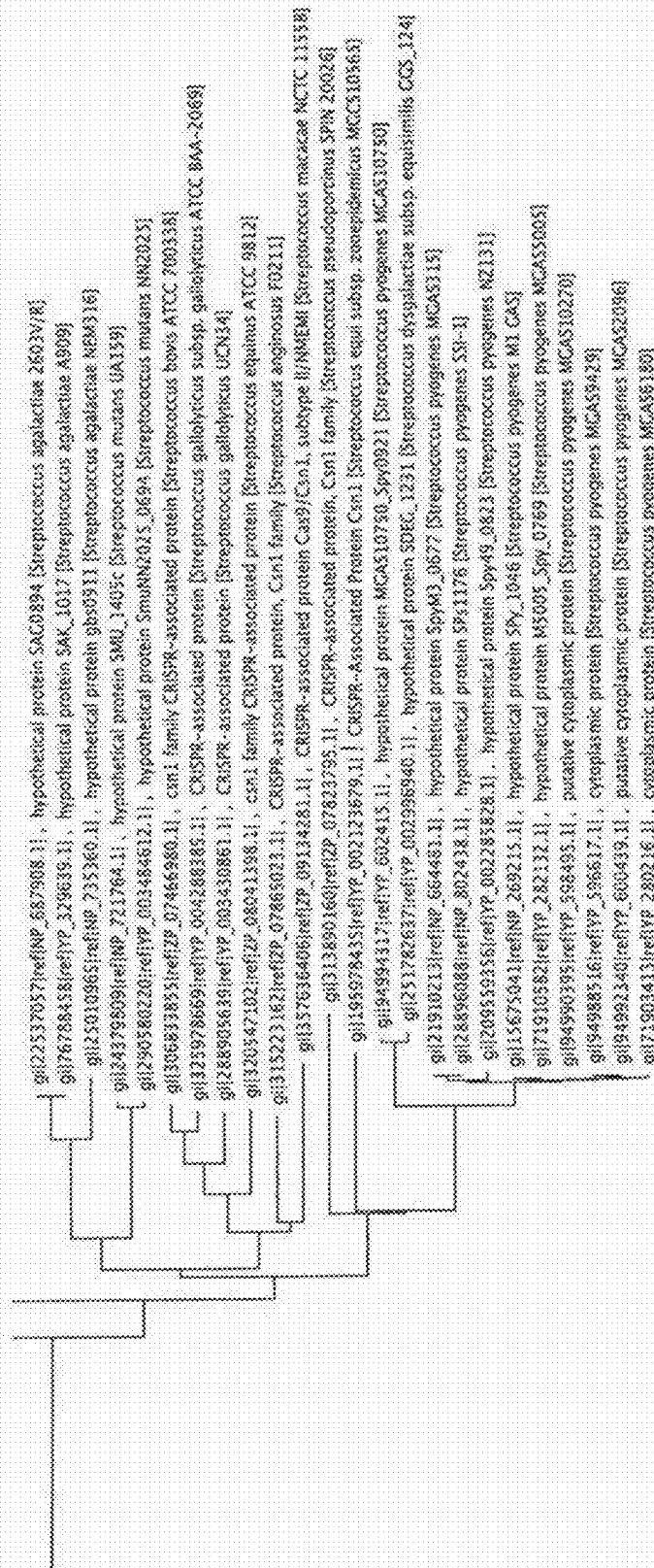


FIG. 20F

a

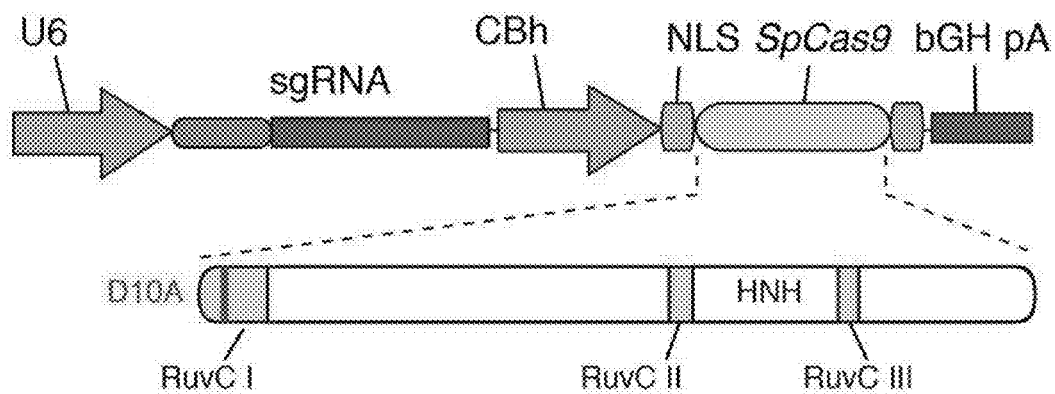


FIG. 21A

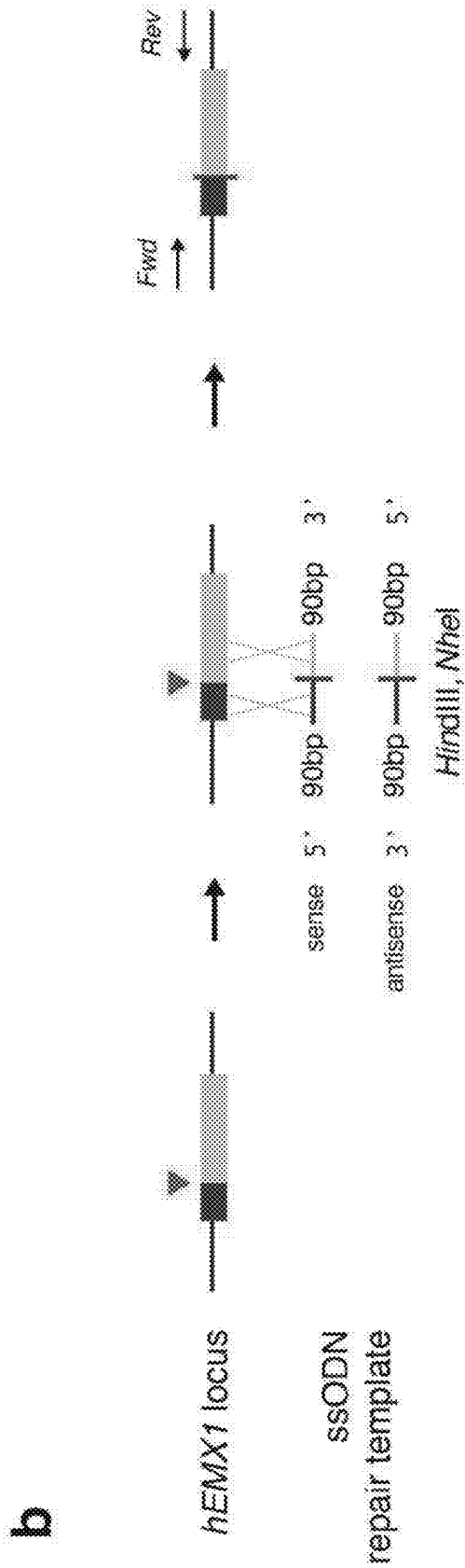


FIG. 21B

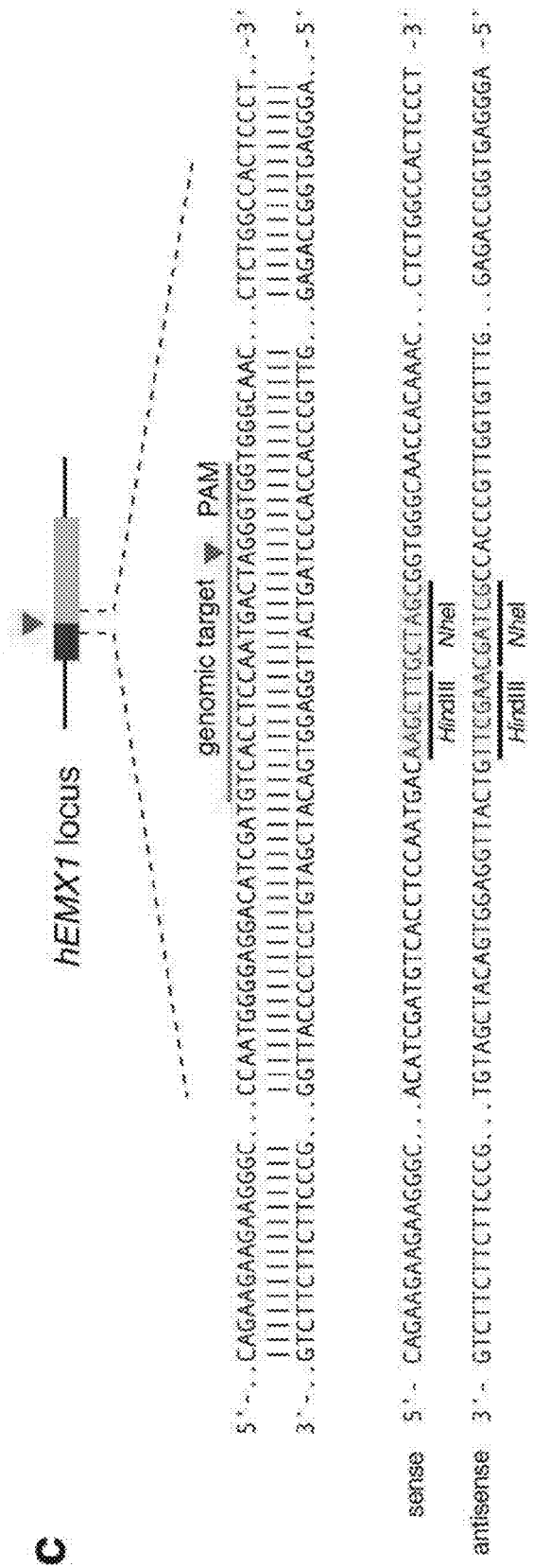


FIG. 21C

d

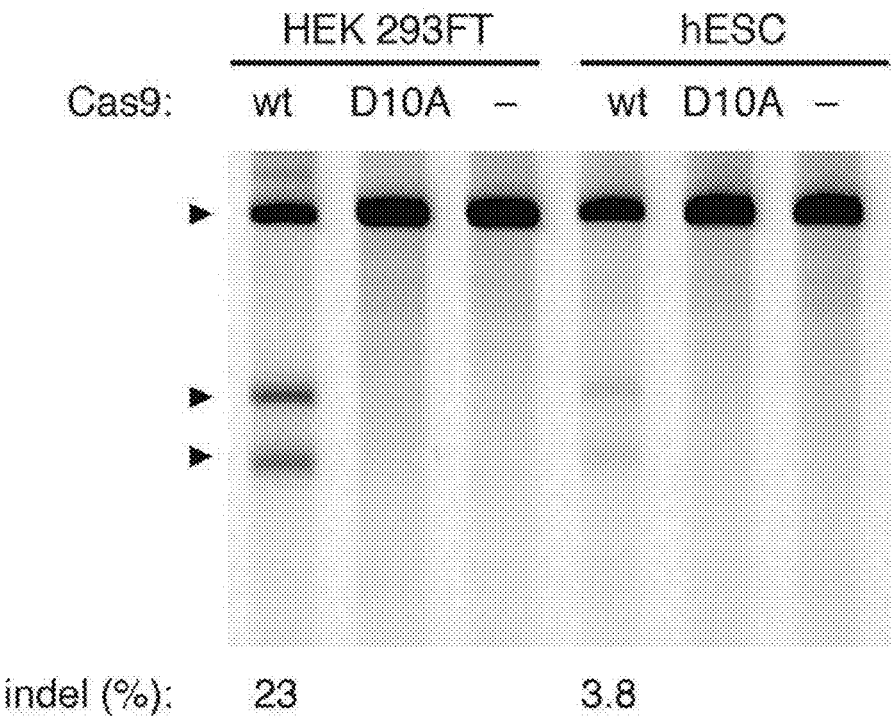
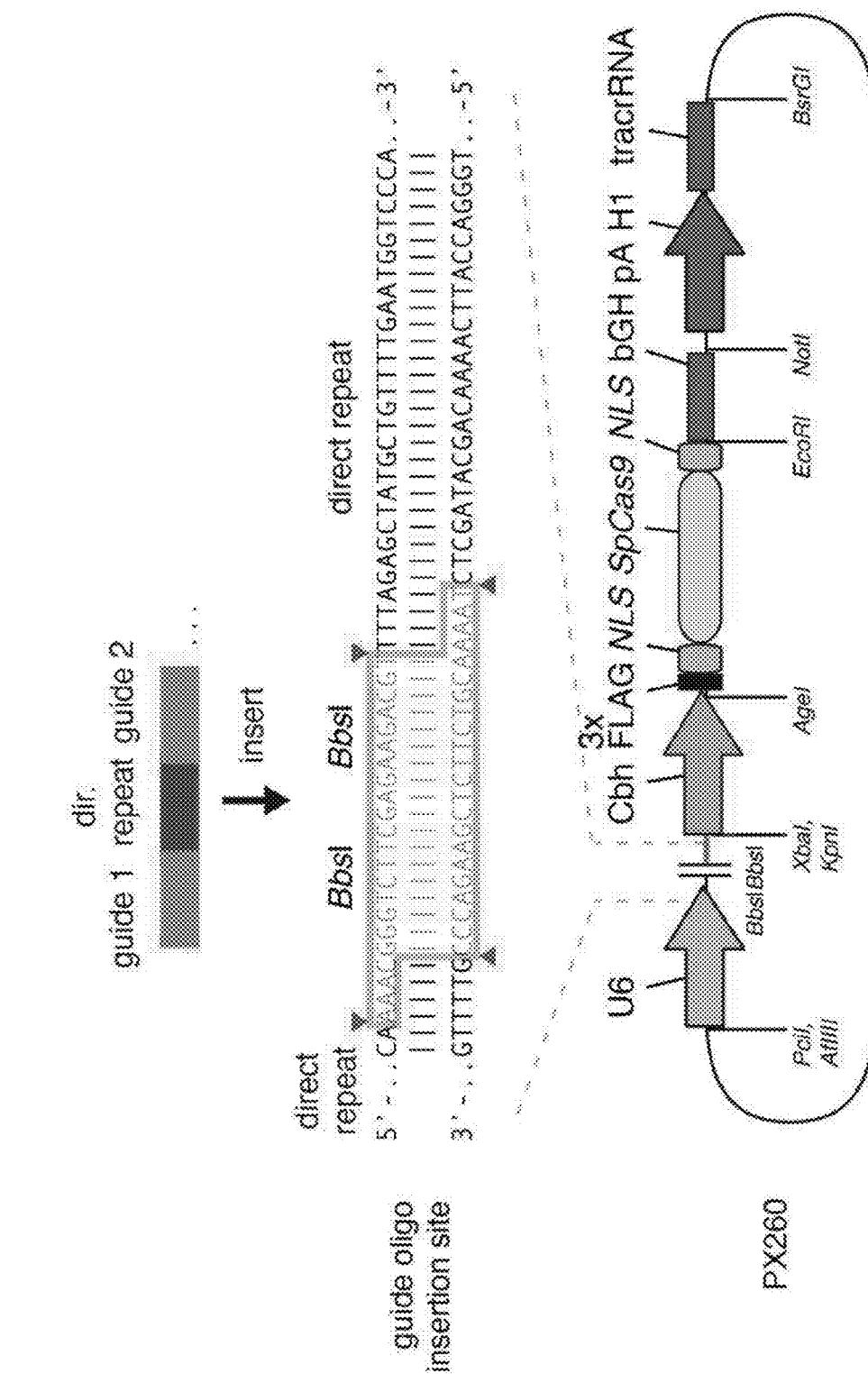


FIG. 21D



CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] This application claims priority to U.S. provisional patent application 61/842,322 having Broad reference BI-2011/008A, entitled CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS filed on Jul. 2, 2013. Priority is also claimed to U.S. provisional patent applications 61/736,527, 61/748,427, 61/791,409 and 61/835,931 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101, BI-2011/008/WSGR Docket No. 44063-701.102, Broad reference BI-2011/008/VP Docket No. 44790.02.2003 and BI-2011/008/VP Docket No. 44790.03.2003 respectively, all entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012, Jan. 2, 2013, Mar. 15, 2013 and Jun. 17, 2013, respectively.

[0002] The foregoing applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, and all documents cited or referenced herein ("herein cited documents"), and all documents cited or referenced in herein cited documents, together with any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0003] This invention was made with government support under NIH Pioneer Award DP1MH100706, awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

[0004] The present invention generally relates to systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that may use vector systems related to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof.

SEQUENCE LISTING

[0005] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Sep. 6, 2013, is named 44790.05.2003_SL.txt and is 56,781 bytes in size.

BACKGROUND OF THE INVENTION

[0006] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a

diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0007] There exists a pressing need for alternative and robust systems and techniques for sequence targeting with a wide array of applications. This invention addresses this need and provides related advantages. The CRISPR/Cas or the CRISPR-Cas system (both terms are used interchangeably throughout this application) does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target, in other words the Cas enzyme can be recruited to a specific DNA target using said short RNA molecule. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering and optimization of these genome engineering tools, which are aspects of the claimed invention.

[0008] In one aspect, the invention provides a method for altering or modifying expression of one or more gene products. The said method may comprise introducing into a eukaryotic cell containing and expressing DNA molecules encoding the one or more gene products an engineered, non-naturally occurring vector system comprising one or more vectors comprising: a) a first regulatory element operably linked to one or more Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) system guide RNAs that hybridize with target sequences in genomic loci of the DNA molecules encoding the one or more gene products, b) a second regulatory element operably linked to a Type-II Cas9 protein, wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products and the Cas9 protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas9 protein and the guide RNAs do not naturally occur together. The invention comprehends the expression of two or more gene products being altered and the vectors of the system further comprising one or more nuclear localization signal(s) (NLS (s)). The invention comprehends the guide RNAs comprising a guide sequence fused to a tracr sequence. The invention further comprehends the Cas9 protein being codon optimized for expression in the eukaryotic cell. In a preferred embodiment the eukaryotic cell is a mammalian cell or a human cell. In a further embodiment of the invention,

the expression of one or more of the gene products is decreased. In aspects of the invention cleaving the genomic loci of the DNA molecule encoding the gene product encompasses cleaving either one or both strands of the DNA duplex.

[0009] In one aspect, the invention provides an engineered, programmable, non-naturally occurring CRISPR-Cas system comprising a Cas9 protein and one or more guide RNAs that target the genomic loci of DNA molecules encoding one or more gene products in a eukaryotic cell and the Cas9 protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas9 protein and the guide RNAs do not naturally occur together. The invention comprehends the expression of two or more gene products being altered and the CRISPR-Cas system further comprising one or more NLS(s). The invention comprehends the guide RNAs comprising a guide sequence fused to a tracr sequence. The invention further comprehends the Cas9 protein being codon optimized for expression in the eukaryotic cell. In a preferred embodiment the eukaryotic cell is a mammalian cell or a human cell. In aspects of the invention cleaving the genomic loci of the DNA molecule encoding the gene product encompasses cleaving either one or both strands of the DNA duplex.

[0010] In another aspect, the invention provides an engineered, non-naturally occurring vector system comprising one or more vectors comprising a) a first regulatory element operably linked to one or more CRISPR-Cas system guide RNAs that hybridize with target sequences in genomic loci of DNA molecules encoding one or more gene products, b) a second regulatory element operably linked to a Type-II Cas9 protein, wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the genomic loci of the DNA molecules encoding the one or more gene products in a eukaryotic cell and the Cas9 protein cleaves the genomic loci of the DNA molecules encoding the one or more gene products, whereby expression of the one or more gene products is altered; and, wherein the Cas9 protein and the guide RNAs do not naturally occur together. The invention comprehends the expression of two or more gene products being altered and the vectors of the system further comprising one or more nuclear localization signal(s) (NLS(s)). The invention comprehends the guide RNAs comprising a guide sequence fused to a tracr sequence. The invention further comprehends the Cas9 protein being codon optimized for expression in the eukaryotic cell. In a preferred embodiment the eukaryotic cell is a mammalian cell or a human cell. In a further embodiment of the invention, the expression of one or more of the gene products is decreased. In aspects of the invention cleaving the genomic loci of the DNA molecule encoding the gene product encompasses cleaving either one or both strands of the DNA duplex.

[0011] In one aspect, the invention provides a vector system comprising one or more vectors. In some embodiments, the system comprises: (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a

CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence; wherein components (a) and (b) are located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system comprises the tracr sequence under the control of a third regulatory element, such as a polymerase III promoter. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. Determining optimal alignment is within the purview of one of skill in the art. For example, there are publically and commercially available alignment algorithms and programs such as, but not limited to, ClustalW, Smith-Waterman in matlab, Bowtie, Geneious, Biopython and SeqMan. In some embodiments, the CRISPR complex comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR complex in a detectable amount in the nucleus of a eukaryotic cell. Without wishing to be bound by theory, it is believed that a nuclear localization sequence is not necessary for CRISPR complex activity in eukaryotes, but that including such sequences enhances activity of the system, especially as to targeting nucleic acid molecules in the nucleus. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae*, *S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the first regulatory element is a polymerase II promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In general, and throughout this specification, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Vectors include, but are not limited to, nucleic acid molecules that are single-stranded, double-stranded, or partially double-stranded; nucleic acid molecules that comprise one or more free ends, no free ends (e.g. circular); nucleic acid molecules that comprise DNA, RNA, or both; and other varieties of polynucleotides known in the art. One type of vector is a "plasmid," which refers to a circular double stranded DNA loop into which additional DNA segments can be inserted, such as by standard molecular cloning techniques. Another type of vector is a viral vector, wherein virally-derived DNA or RNA sequences are present in the

vector for packaging into a virus (e.g. retroviruses, replication defective retroviruses, adenoviruses, replication defective adenoviruses, and adeno-associated viruses). Viral vectors also include polynucleotides carried by a virus for transfection into a host cell. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively-linked. Such vectors are referred to herein as “expression vectors.” Common expression vectors of utility in recombinant DNA techniques are often in the form of plasmids.

[0012] Recombinant expression vectors can comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory elements, which may be selected on the basis of the host cells to be used for expression, that is operatively-linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence of interest is linked to the regulatory element(s) in a manner that allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell).

[0013] The term “regulatory element” is intended to include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, *GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY* 185, Academic Press, San Diego, Calif. (1990). Regulatory elements include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). A tissue-specific promoter may direct expression primarily in a desired tissue of interest, such as muscle, neuron, bone, skin, blood, specific organs (e.g. liver, pancreas), or particular cell types (e.g. lymphocytes). Regulatory elements may also direct expression in a temporal-dependent manner, such as in a cell-cycle dependent or developmental stage-dependent manner, which may or may not also be tissue or cell-type specific. In some embodiments, a vector comprises one or more pol III promoter (e.g. 1, 2, 3, 4, 5, or more pol I promoters), one or more pol II promoters (e.g. 1, 2, 3, 4, 5, or more pol II promoters), one or more pol I promoters (e.g. 1, 2, 3, 4, 5, or more pol I promoters), or combinations thereof. Examples of pol III promoters include, but are not limited to, U6 and H1 promoters. Examples of pol II promoters include, but are not limited to, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al, *Cell*, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter. Also encompassed by the term “regulatory element” are enhancer elements, such as WPRE; CMV enhancers; the

R-U5' segment in LTR of HTLV-I (*Mol. Cell. Biol.*, Vol. 8(1), p. 466-472, 1988); SV40 enhancer; and the intron sequence between exons 2 and 3 of rabbit β -globin (*Proc. Natl. Acad. Sci. USA.*, Vol. 78(3), p. 1527-31, 1981). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression desired, etc. A vector can be introduced into host cells to thereby produce transcripts, proteins, or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (e.g., clustered regularly interspersed short palindromic repeats (CRISPR) transcripts, proteins, enzymes, mutant forms thereof, fusion proteins thereof, etc.).

[0014] Advantageous vectors include lentiviruses and adeno-associated viruses, and types of such vectors can also be selected for targeting particular types of cells.

[0015] In one aspect, the invention provides a eukaryotic host cell comprising (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the host cell comprises components (a) and (b). In some embodiments, component (a), component (b), or components (a) and (b) are stably integrated into a genome of the host eukaryotic cell. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the eukaryotic host cell further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length. In an aspect, the invention provides a non-human eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. In other

aspects, the invention provides a eukaryotic organism; preferably a multicellular eukaryotic organism, comprising a eukaryotic host cell according to any of the described embodiments. The organism in some embodiments of these aspects may be an animal; for example a mammal. Also, the organism may be an arthropod such as an insect. The organism also may be a plant. Further, the organism may be a fungus.

[0016] In one aspect, the invention provides a kit comprising one or more of the components described herein. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting one or more guide sequences upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. In some embodiments, the kit comprises components (a) and (b) located on the same or different vectors of the system. In some embodiments, component (a) further comprises the tracr sequence downstream of the tracr mate sequence under the control of the first regulatory element. In some embodiments, component (a) further comprises two or more guide sequences operably linked to the first regulatory element, wherein when expressed, each of the two or more guide sequences direct sequence specific binding of a CRISPR complex to a different target sequence in a eukaryotic cell. In some embodiments, the system further comprises a third regulatory element, such as a polymerase III promoter, operably linked to said tracr sequence. In some embodiments, the tracr sequence exhibits at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, the CRISPR enzyme comprises one or more nuclear localization sequences of sufficient strength to drive accumulation of said CRISPR enzyme in a detectable amount in the nucleus of a eukaryotic cell. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae*, *S. pyogenes* or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell. In some embodiments, the CRISPR enzyme directs cleavage of one or two strands at the location of the target sequence. In some embodiments, the CRISPR enzyme lacks DNA strand cleavage activity. In some embodiments, the first regulatory element is a polymerase III promoter. In some embodiments, the second regulatory element is a polymerase II promoter. In some embodiments, the guide sequence is at least 15, 16, 17, 18, 19, 20, 25 nucleotides, or between 10-30, or between 15-25, or between 15-20 nucleotides in length.

[0017] In one aspect, the invention provides a method of modifying a target polynucleotide in a eukaryotic cell. In

some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expressed from a gene comprising the target sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cell, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence. In some embodiments, said vectors are delivered to the eukaryotic cell in a subject. In some embodiments, said modifying takes place in said eukaryotic cell in a cell culture. In some embodiments, the method further comprises isolating said eukaryotic cell from a subject prior to said modifying. In some embodiments, the method further comprises returning said eukaryotic cell and/or cells derived therefrom to said subject.

[0018] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

[0019] In one aspect, the invention provides a method of generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors into a eukaryotic cell, wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a model

eukaryotic cell comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence.

[0020] In one aspect, the invention provides a method for developing a biologically active agent that modulates a cell signaling event associated with a disease gene. In some embodiments, a disease gene is any gene associated an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) contacting a test compound with a model cell of any one of the described embodiments; and (b) detecting a change in a readout that is indicative of a reduction or an augmentation of a cell signaling event associated with said mutation in said disease gene, thereby developing said biologically active agent that modulates said cell signaling event associated with said disease gene.

[0021] In one aspect, the invention provides a recombinant polynucleotide comprising a guide sequence upstream of a tracer mate sequence, wherein the guide sequence when expressed directs sequence-specific binding of a CRISPR complex to a corresponding target sequence present in a eukaryotic cell. In some embodiments, the target sequence is a viral sequence present in a eukaryotic cell. In some embodiments, the target sequence is a proto-oncogene or an oncogene.

[0022] In one aspect the invention provides for a method of selecting one or more cell(s) by introducing one or more mutations in a gene in the one or more cell (s), the method comprising: introducing one or more vectors into the cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracer mate sequence, a tracer sequence, and an editing template; wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage; allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected; allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracer mate sequence that is hybridized to the tracer sequence, wherein binding of the CRISPR complex to the target polynucleotide induces cell death, thereby allowing one or more cell(s) in which one or more mutations have been introduced to be selected. In a preferred embodiment, the CRISPR enzyme is Cas9. In another preferred embodiment of the invention the cell to be selected may be a eukaryotic cell. Aspects of the invention allow for selection of specific cells without requiring a selection marker or a two-step process that may include a counter-selection system. Accordingly, it is an object of the invention not to encompass within the invention any previously known product, process of making the product, or

method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[0023] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as “comprises”, “comprised”, “comprising” and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean “includes”, “included”, “including”, and the like; and that terms such as “consisting essentially of” and “consists essentially of” have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention. These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0025] FIG. 1 shows a schematic model of the CRISPR system. The Cas9 nuclease from *Streptococcus pyogenes* (yellow) is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence base-pairs with the DNA target (blue), directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM; magenta), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (red triangle).

[0026] FIG. 2A-F shows an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity. FIG. 2C discloses SEQ ID NOS 23-24, respectively, in order of appearance. FIG. 2E discloses SEQ ID NOS 25-27, respectively, in order of appearance. FIG. 2F discloses SEQ ID NOS 28-32, respectively, in order of appearance.

[0027] FIG. 3A-D shows results of an evaluation of SpCas9 specificity for an example target. FIG. 3A discloses SEQ ID NOS 33, 26 and 34-44, respectively, in order of appearance. FIG. 3C discloses SEQ ID NO: 33.

[0028] FIG. 4A-G shows an exemplary vector system and results for its use in directing homologous recombination in eukaryotic cells. FIG. 4E discloses SEQ ID NO: 45. FIG. 4F discloses SEQ ID NOS 46-47, respectively, in order of appearance. FIG. 4G discloses SEQ ID NOS 48-52, respectively, in order of appearance.

[0029] FIG. 5 provides a table of protospacer sequences (SEQ ID NOS 16, 15, 14, 53-58, 18, 17 and 59-63, respectively, in order of appearance) and summarizes modification

efficiency results for protospacer targets designed based on exemplary *S. pyogenes* and *S. thermophilus* CRISPR systems with corresponding PAMs against loci in human and mouse genomes. Cells were transfected with Cas9 and either pre-crRNA/tracrRNA or chimeric RNA, and analyzed 72 hours after transfection. Percent indels are calculated based on Surveyor assay results from indicated cell lines (N=3 for all protospacer targets, errors are S.E.M., N.D. indicates not detectable using the Surveyor assay, and N.T. indicates not tested in this study).

[0030] FIG. 6A-C shows a comparison of different tracrRNA transcripts for Cas9-mediated gene targeting. FIG. 6A discloses SEQ ID NOS 64-65, respectively, in order of appearance.

[0031] FIG. 7 shows a schematic of a surveyor nuclease assay for detection of double strand break-induced micro-insertions and -deletions.

[0032] FIG. 8A-B shows exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells. FIG. 8A discloses SEQ ID NOS 66-68, respectively, in order of appearance. FIG. 8B discloses SEQ ID NOS 69-71, respectively, in order of appearance.

[0033] FIG. 9A-C shows histograms of distances between adjacent *S. pyogenes* SF370 locus 1 PAM (NGG) (FIG. 9A) and *S. thermophilus* LMD9 locus 2 PAM (NNAGAAW) (FIG. 9B) in the human genome; and distances for each PAM by chromosome (Chr) (FIG. 9C).

[0034] FIG. 10A-D shows an exemplary CRISPR system, an example adaptation for expression in eukaryotic cells, and results of tests assessing CRISPR activity. FIG. 10B discloses SEQ ID NOS 72-73, respectively, in order of appearance. FIG. 10C discloses SEQ ID NO: 74.

[0035] FIG. 11A-C shows exemplary manipulations of a CRISPR system for targeting of genomic loci in mammalian cells. FIG. 11A discloses SEQ ID NO: 75. FIG. 11B discloses SEQ ID NOS 76-78, respectively, in order of appearance.

[0036] FIG. 12A-B shows the results of a Northern blot analysis of crRNA processing in mammalian cells. FIG. 12A discloses SEQ ID NO: 79.

[0037] FIG. 13A-B shows an exemplary selection of protospacers in the human PVALB and mouse Th loci. FIG. 13A discloses SEQ ID NO: 80. FIG. 13B discloses SEQ ID NO: 81.

[0038] FIG. 14 shows example protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human EMX1 locus. FIG. 14 discloses SEQ ID NO: 74.

[0039] FIG. 15 provides a table of sequences (SEQ ID NOS 82-93, respectively, in order of appearance) for primers and probes used for Surveyor, RFLP, genomic sequencing, and Northern blot assays.

[0040] FIG. 16A-C shows exemplary manipulation of a CRISPR system with chimeric RNAs and results of SURVEYOR assays for system activity in eukaryotic cells. FIG. 16A discloses SEQ ID NO: 94.

[0041] FIG. 17A-B shows a graphical representation of the results of SURVEYOR assays for CRISPR system activity in eukaryotic cells.

[0042] FIG. 18 shows an exemplary visualization of some *S. pyogenes* Cas9 target sites in the human genome using the UCSC genome browser. FIG. 18 discloses SEQ ID NOS 95-173, respectively, in order of appearance.

[0043] FIG. 19A-D shows a circular depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0044] FIG. 20A-F shows the linear depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[0045] FIG. 21A-D shows genome editing via homologous recombination. (a) Schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. Red arrow above indicates sgRNA cleavage site; PCR primers for genotyping (Tables J and K) are indicated as arrows in right panel. FIG. 21C discloses SEQ ID NOS 174-176, 174, 177 and 176, respectively, in order of appearance. (c) Sequence of region modified by HR. d, SURVEYOR assay for wild-type (wt) and nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

[0046] FIG. 22A-B shows single vector designs for SpCas9. FIG. 22A discloses SEQ ID NOS 178-180, respectively, in order of appearance. FIG. 22B discloses SEQ ID NO: 181.

[0047] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[0048] The terms “polynucleotide”, “nucleotide”, “nucleotide sequence”, “nucleic acid” and “oligonucleotide” are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0049] In aspects of the invention the terms “chimeric RNA”, “chimeric guide RNA”, “guide RNA”, “single guide RNA” and “synthetic guide RNA” are used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, the tracr sequence and the tracr mate sequence. The term “guide sequence” refers to the about 20 bp sequence within the guide RNA that specifies the target site and may be used interchangeably with the terms “guide” or “spacer”. The term “tracr mate sequence” may also be

used interchangeably with the term “direct repeat(s)”. An exemplary CRISPR-Cas system is illustrated in FIG. 1.

[0050] As used herein the term “wild type” is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms.

[0051] As used herein the term “variant” should be taken to mean the exhibition of qualities that have a pattern that deviates from what occurs in nature.

[0052] The terms “non-naturally occurring” or “engineered” are used interchangeably and indicate the involvement of the hand of man. The terms, when referring to nucleic acid molecules or polypeptides mean that the nucleic acid molecule or the polypeptide is at least substantially free from at least one other component with which they are naturally associated in nature and as found in nature.

[0053] “Complementarity” refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). “Perfectly complementary” means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. “Substantially complementary” as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

[0054] As used herein, “stringent conditions” for hybridization refer to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent, and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), *Laboratory Techniques In Biochemistry And Molecular Biology—Hybridization With Nucleic Acid Probes Part 1, Second Chapter “Overview of principles of hybridization and the strategy of nucleic acid probe assay”*, Elsevier, N.Y.

[0055] “Hybridization” refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogsteen binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PCR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the “complement” of the given sequence.

[0056] As used herein, “expression” refers to the process by which a polynucleotide is transcribed from a DNA

template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0057] The terms “polypeptide”, “peptide” and “protein” are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term “amino acid” includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0058] The terms “subject,” “individual,” and “patient” are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained in vivo or cultured in vitro are also encompassed.

[0059] The terms “therapeutic agent”, “therapeutic capable agent” or “treatment agent” are used interchangeably and refer to a molecule or compound that confers some beneficial effect upon administration to a subject. The beneficial effect includes enablement of diagnostic determinations; amelioration of a disease, symptom, disorder, or pathological condition; reducing or preventing the onset of a disease, symptom, disorder or condition; and generally counteracting a disease, symptom, disorder or pathological condition.

[0060] As used herein, “treatment” or “treating,” or “palliating” or “ameliorating” are used interchangeably. These terms refer to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not have yet been manifested.

[0061] The term “effective amount” or “therapeutically effective amount” refers to the amount of an agent that is sufficient to effect beneficial or desired results. The therapeutically effective amount may vary depending upon one or more of: the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The term also applies to a dose that will provide an image for detection by any one of the imaging methods described herein. The specific dose may vary depending on one or more of: the particular agent chosen, the dosing regimen to be followed, whether it is administered in combination with

other compounds, timing of administration, the tissue to be imaged, and the physical delivery system in which it is carried.

[0062] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd edition (1989); *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY* (F. M. Ausubel, et al. eds., (1987)); the series *METHODS IN ENZYMOLOGY* (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M. J. MacPherson, B. D. Hames and G. R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *ANTIBODIES, A LABORATORY MANUAL*, and *ANIMAL CELL CULTURE* (R. I. Freshney, ed. (1987)).

[0063] Several aspects of the invention relate to vector systems comprising one or more vectors, or vectors as such. Vectors can be designed for expression of CRISPR transcripts (e.g. nucleic acid transcripts, proteins, or enzymes) in prokaryotic or eukaryotic cells. For example, CRISPR transcripts can be expressed in bacterial cells such as *Escherichia coli*, insect cells (using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, *GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY* 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated in vitro, for example using T7 promoter regulatory sequences and T7 polymerase.

[0064] Vectors may be introduced and propagated in a prokaryote. In some embodiments, a prokaryote is used to amplify copies of a vector to be introduced into a eukaryotic cell or as an intermediate vector in the production of a vector to be introduced into a eukaryotic cell (e.g. amplifying a plasmid as part of a viral vector packaging system). In some embodiments, a prokaryote is used to amplify copies of a vector and express one or more nucleic acids, such as to provide a source of one or more proteins for delivery to a host cell or host organism. Expression of proteins in prokaryotes is most often carried out in *Escherichia coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, such as to the amino terminus of the recombinant protein. Such fusion vectors may serve one or more purposes, such as: (i) to increase expression of recombinant protein; (ii) to increase the solubility of the recombinant protein; and (iii) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Example fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson, 1988. *Gene* 67: 31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A. respectively, to the target recombinant protein.

[0065] Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY* 185, Academic Press, San Diego, Calif. (1990) 60-89).

[0066] In some embodiments, a vector is a yeast expression vector. Examples of vectors for expression in yeast *Saccharomyces cerevisiae* include pYepSec1 (Baldari, et al., 1987. *EMBO J.* 6: 229-234), pMFa (Kuijan and Herskowitz, 1982. *Cell* 30: 933-943), pJRY88 (Schultz et al., 1987. *Gene* 54: 113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (Invitrogen Corp, San Diego, Calif.).

[0067] In some embodiments, a vector drives protein expression in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., SF9 cells) include the pAc series (Smith, et al., 1983. *Mol. Cell. Biol.* 3: 2156-2165) and the pVL series (Lucklow and Summers, 1989. *Virology* 170: 31-39).

[0068] In some embodiments, a vector is capable of driving expression of one or more sequences in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, 1987. *Nature* 329: 840) and pMT2PC (Kaufman, et al., 1987. *EMBO J.* 6: 187-195). When used in mammalian cells, the expression vector's control functions are typically provided by one or more regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus, simian virus 40, and others disclosed herein and known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells see, e.g., Chapters 16 and 17 of Sambrook, et al., *MOLECULAR CLONING: A LABORATORY MANUAL*. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

[0069] In some embodiments, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, et al., 1987. *Genes Dev.* 1: 268-277), lymphoid-specific promoters (Calame and Eaton, 1988. *Adv. Immunol.* 43: 235-275), in particular promoters of T cell receptors (Winoto and Baltimore, 1989. *EMBO J.* 8: 729-733) and immunoglobulins (Baneiji, et al., 1983. *Cell* 33: 729-740; Queen and Baltimore, 1983. *Cell* 33: 741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle, 1989. *Proc. Natl. Acad. Sci. USA* 86: 5473-5477), pancreas-specific promoters (Edlund, et al., 1985. *Science* 230: 912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, e.g., the murine hox promoters (Kessel and Gruss, 1990. *Science* 249: 374-379) and the α -fetoprotein promoter (Campes and Tilghman, 1989. *Genes Dev.* 3: 537-546).

[0070] In some embodiments, a regulatory element is operably linked to one or more elements of a CRISPR system so as to drive expression of the one or more elements of the CRISPR system. In general, CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats), also known as SPIDRs (SPacer Interspersed Direct Repeats),

constitute a family of DNA loci that are usually specific to a particular bacterial species. The CRISPR locus comprises a distinct class of interspersed short sequence repeats (SSRs) that were recognized in *E. coli* (Ishino et al., J. Bacteriol., 169:5429-5433 [1987]; and Nakata et al., J. Bacteriol., 171:3553-3556 [1989]), and associated genes. Similar interspersed SSRs have been identified in *Haloferax mediterranei*, *Streptococcus pyogenes*, *Anabaena*, and *Mycobacterium tuberculosis* (See, Groenen et al., Mol. Microbiol., 10:1057-1065 [1993]; Hoe et al., Emerg. Infect. Dis., 5:254-263 [1999]; Masepohl et al., Biochim. Biophys. Acta 1307: 26-30 [1996]; and Mojica et al., Mol. Microbiol., 17:85-93 [1995]). The CRISPR loci typically differ from other SSRs by the structure of the repeats, which have been termed short regularly spaced repeats (SRSRs) (Janssen et al., OMICS J. Integ. Biol., 6:23-33 [2002]; and Mojica et al., Mol. Microbiol., 36:244-246 [2000]). In general, the repeats are short elements that occur in clusters that are regularly spaced by unique intervening sequences with a substantially constant length (Mojica et al., [2000], supra). Although the repeat sequences are highly conserved between strains, the number of interspersed repeats and the sequences of the spacer regions typically differ from strain to strain (van Embden et al., J. Bacteriol., 182:2393-2401 [2000]). CRISPR loci have been identified in more than 40 prokaryotes (See e.g., Jansen et al., Mol. Microbiol., 43:1565-1575 [2002]; and Mojica et al., [2005]) including, but not limited to *Aeropyrum*, *Pyrobaculum*, *Sulfolobus*, *Archaeoglobus*, *Halocarcula*, *Methanobacterium*, *Methanococcus*, *Methanosarcina*, *Methanopyrus*, *Pyrococcus*, *Picrophilus*, *Thermoplasma*, *Corynebacterium*, *Mycobacterium*, *Streptomyces*, *Aquifex*, *Porphyromonas*, *Chlorobium*, *Thermus*, *Bacillus*, *Listeria*, *Staphylococcus*, *Clostridium*, *Thermoanaerobacter*, *Mycoplasma*, *Fusobacterium*, *Azarcus*, *Chromobacterium*, *Neisseria*, *Nitrosomonas*, *Desulfovibrio*, *Geobacter*, *Mycrococcus*, *Campylobacter*, *Wolinella*, *Acinetobacter*, *Erwinia*, *Escherichia*, *Legionella*, *Methylococcus*, *Pasteurella*, *Photobacterium*, *Salmonella*, *Xanthomonas*, *Yersinia*, *Trponema*, and *Thermotoga*.

[0071] In general, “CRISPR system” refers collectively to transcripts and other elements involved in the expression of or directing the activity of CRISPR-associated (“Cas”) genes, including sequences encoding a Cas gene, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a “direct repeat” and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a “spacer” in the context of an endogenous CRISPR system), or other sequences and transcripts from a CRISPR locus. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system is derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes*. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, “target sequence” refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily

required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. A target sequence may comprise any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell. In some embodiments, the target sequence may be within an organelle of a eukaryotic cell, for example, mitochondrion or chloroplast. A sequence or template that may be used for recombination into the targeted locus comprising the target sequences is referred to as an “editing template” or “editing polynucleotide” or “editing sequence”. In aspects of the invention, an exogenous template polynucleotide may be referred to as an editing template. In an aspect of the invention the recombination is homologous recombination.

[0072] Typically, in the context of an endogenous CRISPR system, formation of a CRISPR complex (comprising a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of one or both strands in or near (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. Without wishing to be bound by theory, the tracr sequence, which may comprise or consist of all or a portion of a wild-type tracr sequence (e.g. about or more than about 20, 26, 32, 45, 48, 54, 63, 67, 85, or more nucleotides of a wild-type tracr sequence), may also form part of a CRISPR complex, such as by hybridization along at least a portion of the tracr sequence to all or a portion of a tracr mate sequence that is operably linked to the guide sequence. In some embodiments, the tracr sequence has sufficient complementarity to a tracr mate sequence to hybridize and participate in formation of a CRISPR complex. As with the target sequence, it is believed that complete complementarity is not needed, provided there is sufficient to be functional. In some embodiments, the tracr sequence has at least 50%, 60%, 70%, 80%, 90%, 95% or 99% of sequence complementarity along the length of the tracr mate sequence when optimally aligned. In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR system are introduced into a host cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target sites. For example, a Cas enzyme, a guide sequence linked to a tracr-mate sequence, and a tracr sequence could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements, may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to (“upstream” of) or 3' with respect to (“downstream” of) a second element. The coding sequence of one element may be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction. In some embodiments, a single promoter drives expression of a transcript encoding a CRISPR enzyme and one or more of the guide sequence, tracr mate sequence (optionally operably linked to the guide sequence), and a tracr sequence embedded within one or more intron sequences (e.g. each in a different intron, two or more in at least one intron, or all in a single intron). In some embodiments, the CRISPR

enzyme, guide sequence, tracr mate sequence, and tracr sequence are operably linked to and expressed from the same promoter. Single vector constructs for SpCas9 are illustrated in FIG. 22.

[0073] In some embodiments, a vector comprises one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a “cloning site”). In some embodiments, one or more insertion sites (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector comprises an insertion site upstream of a tracr mate sequence, and optionally downstream of a regulatory element operably linked to the tracr mate sequence, such that following insertion of a guide sequence into the insertion site and upon expression the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell. In some embodiments, a vector comprises two or more insertion sites, each insertion site being located between two tracr mate sequences so as to allow insertion of a guide sequence at each site. In such an arrangement, the two or more guide sequences may comprise two or more copies of a single guide sequence, two or more different guide sequences, or combinations of these. When multiple different guide sequences are used, a single expression construct may be used to target CRISPR activity to multiple different, corresponding target sequences within a cell. For example, a single vector may comprise about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more guide sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more such guide-sequence-containing vectors may be provided, and optionally delivered to a cell.

[0074] In some embodiments, a vector comprises a regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas10, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments the CRISPR enzyme is Cas9, and may be Cas9 from *S. pyogenes* or *S. pneumoniae*. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated to with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from *S.*

pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. In aspects of the invention, nickases may be used for genome editing via homologous recombination. For example, FIG. 21 shows genome editing via homologous recombination. FIG. 21 (a) shows the schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

[0075] In some embodiments, a Cas9 nickase may be used in combination with guide sequence(s), e.g., two guide sequences, which target respectively sense and antisense strands of the DNA target. This combination allows both strands to be nicked and used to induce NHEJ. Applicants have demonstrated (data not shown) the efficacy of two nickase targets (i.e., sgRNAs targeted at the same location but to different strands of DNA) in inducing mutagenic NHEJ. A single nickase (Cas9-D10A with a single sgRNA) is unable to induce NHEJ and create indels but Applicants have shown that double nickase (Cas9-D10A and two sgRNAs targeted to different strands at the same location) can do so in human embryonic stem cells (hESCs). The efficiency is about 50% of nuclease (i.e., regular Cas9 without D10 mutation) in hESCs.

[0076] As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a CRISPR enzyme is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form. Other mutations may be useful; where the Cas9 or other CRISPR enzyme is from a species other than *S. pyogenes*, mutations in corresponding amino acids may be made to achieve similar effects.

[0077] In some embodiments, an enzyme coding sequence encoding a CRISPR enzyme is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, mouse, rat, rabbit, dog, or non-human primate. In general, codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the

properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the “Codon Usage Database”, and these tables can be adapted in a number of ways. See Nakamura, Y., et al. “Codon usage tabulated from the international DNA sequence databases: status for the year 2000” Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, Pa.), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

[0078] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[0079] A guide sequence may be selected to target any target sequence. In some embodiments, the target sequence is a sequence within a genome of a cell. Exemplary target sequences include those that are unique in the target genome. For example, for the *S. pyogenes* Cas9, a unique

target sequence in a genome may include a Cas9 target site of the form MMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGG where NNNNNNNNNNNNNXGG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. For the *S. thermophilus* CRISPR1 Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMNNNNNNNNNNNNXXAGAAW (SEQ ID NO: 1) where NNNNNNNNNNNNNXXAGAAW (SEQ ID NO: 2) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. thermophilus* CRISPR1 Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXXAGAAW (SEQ ID NO: 3) where NNNNNNNNNNNNNXXAGAAW (SEQ ID NO: 4) (N is A, G, T, or C; X can be anything; and W is A or T) has a single occurrence in the genome. For the *S. pyogenes* Cas9, a unique target sequence in a genome may include a Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. A unique target sequence in a genome may include an *S. pyogenes* Cas9 target site of the form MMMMMMMMNNNNNNNNNNNNXGGXG where NNNNNNNNNNNNNXGGXG (N is A, G, T, or C; and X can be anything) has a single occurrence in the genome. In each of these sequences “M” may be A, G, T, or C, and need not be considered in identifying a sequence as unique.

[0080] In some embodiments, a guide sequence is selected to reduce the degree of secondary structure within the guide sequence. Secondary structure may be determined by any suitable polynucleotide folding algorithm. Some programs are based on calculating the minimal Gibbs free energy. An example of one such algorithm is mFold, as described by Zuker and Stiegler (Nucleic Acids Res. 9 (1981), 133-148). Another example folding algorithm is the online webserver RNAfold, developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A. R. Gruber et al., 2008, *Cell* 106(1): 23-24; and P A Carr and G M Church, 2009, *Nature Biotechnology* 27(12): 1151-62). Further algorithms may be found in U.S. application Ser. No. 61/836,080 (attorney docket 44790.11.2022; Broad Reference BI-2013/004A); incorporated herein by reference.

[0081] In general, a tracr mate sequence includes any sequence that has sufficient complementarity with a tracr sequence to promote one or more of: (1) excision of a guide sequence flanked by tracr mate sequences in a cell containing the corresponding tracr sequence; and (2) formation of a CRISPR complex at a target sequence, wherein the CRISPR complex comprises the tracr mate sequence hybridized to the tracr sequence. In general, degree of complementarity is with reference to the optimal alignment of the tracr mate sequence and tracr sequence, along the length of the shorter of the two sequences. Optimal alignment may be determined by any suitable alignment algorithm, and may further account for secondary structures, such as self-complementarity within either the tracr sequence or tracr mate sequence. In some embodiments, the degree of

complementarity between the tracr sequence and tracr mate sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%, 99%, or higher. Example illustrations of optimal alignment between a tracr sequence and a tracr mate sequence are provided in FIGS. 10B and 11B. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and tracr mate sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. Preferred loop forming sequences for use in hairpin structures are four nucleotides in length, and most preferably have the sequence GAAA. However, longer or shorter loop sequences may be used, as may alternative sequences. The sequences preferably include a nucleotide triplet (for example, AAA), and an additional nucleotide (for example C or G). Examples of loop forming sequences include CAAA and AAAG. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In some embodiments, the single transcript further includes a transcription termination sequence; preferably this is a polyT sequence, for example six T nucleotides. An example illustration of such a hairpin structure is provided in the lower portion of FIG. 11B, where the portion of the sequence 5' of the final "N" and upstream of the loop corresponds to the tracr mate sequence, and the portion of the sequence 3' of the loop corresponds to the tracr sequence. Further non-limiting examples of single polynucleotides comprising a guide sequence, a tracr mate sequence, and a tracr sequence are as follows (listed 5' to 3'), where "N" represents a base of a guide sequence, the first block of lower case letters represent the tracr mate sequence, and the second block of lower case letters represent the tracr sequence, and the final poly-T sequence represents the transcription terminator: (1) NNNNNNNNNNNNNNNNNNNNNgttttgtagctctcaagatttGAAAtaatcttcagaaagctacaaagataaggctt catgcgaaatcaacaccctgtcattttatggcagggtgttttcgtatttaaTTTTTT (SEQ ID NO: 5); (2) NNNNNNNNNNNNNNNNNNNNNgttttgtagctctcaGAAAtgcagaaagctacaaagataaggcttcagtcgcaaatca acaccctgtcattttatggcagggtgttttcgtatttaaTTTTTT (SEQ ID NO: 6); (3) NNNNNNNNNNNNNNNNNNNNNgttttgtagctctcaGAAAtgcagaaagctacaaagataaggcttcagtcgcaaatca acaccctgtcattttatggcagggtgtTTTTTT (SEQ ID NO: 7); (4) NNNNNNNNNNNNNNNNNNNNNgttttagagctaGAAAtagcaagttaaataaggctagtcggttatcaacttgaaaa agtggcaccgagtcggtgcTTTTTT (SEQ ID NO: 8); (5) NNNNNNNNNNNNNNNNNNNNNgttttagagctaGAAATAGcaagttaaataaggctagtcggttatcaacttgaaa aaagtgtTTTTTT (SEQ ID NO: 9); and (6) NNNNNNNNNNNNNNNNNNNNNgttttagagctagAAATAGcaagttaaataaggctagtcggttatcaTTTTTT (SEQ ID NO: 10). In some embodiments, sequences (1) to (3) are used in combination with Cas9 from *S. thermophilus* CRISPR1. In some embodiments, sequences (4) to (6) are used in combination with Cas9 from *S. pyogenes*. In some embodiments, the tracr sequence is a separate transcript from a transcript comprising the tracr mate sequence (such as illustrated in the top portion of FIG. 11B).

[0082] In some embodiments, the CRISPR enzyme is part of a fusion protein comprising one or more heterologous protein domains (e.g. about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more domains in addition to the CRISPR enzyme). A CRISPR enzyme fusion protein may comprise any additional protein sequence, and optionally a linker sequence between any two domains. Examples of protein domains that may be fused to a CRISPR enzyme include, without limitation, epitope tags, reporter gene sequences, and protein domains having one or more of the following activities: methylase activity, demethylase activity, transcription activation activity, transcription repression activity, transcription release factor activity, histone modification activity, RNA cleavage activity and nucleic acid binding activity. Non-limiting examples of epitope tags include histidine (His) tags, V5 tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Examples of reporter genes include, but are not limited to, glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and auto-fluorescent proteins including blue fluorescent protein (BFP). A CRISPR enzyme may be fused to a gene sequence encoding a protein or a fragment of a protein that bind DNA molecules or bind other cellular molecules, including but not limited to maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DBD) fusions, GAL4A DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions. Additional domains that may form part of a fusion protein comprising a CRISPR enzyme are described in US20110059502, incorporated herein by reference. In some embodiments, a tagged CRISPR enzyme is used to identify the location of a target sequence.

[0083] In an aspect of the invention, a reporter gene which includes but is not limited to glutathione-S-transferase (GST), horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT) beta-galactosidase, beta-glucuronidase, luciferase, green fluorescent protein (GFP), HcRed, DsRed, cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), and autofluorescent proteins including blue fluorescent protein (BFP), may be introduced into a cell to encode a gene product which serves as a marker by which to measure the alteration or modification of expression of the gene product. In a further embodiment of the invention, the DNA molecule encoding the gene product may be introduced into the cell via a vector. In a preferred embodiment of the invention the gene product is luciferase. In a further embodiment of the invention the expression of the gene product is decreased.

[0084] In some aspects, the invention provides methods comprising delivering one or more polynucleotides, such as or one or more vectors as described herein, one or more transcripts thereof, and/or one or more proteins transcribed therefrom, to a host cell. In some aspects, the invention further provides cells produced by such methods, and organisms (such as animals, plants, or fungi) comprising or produced from such cells. In some embodiments, a CRISPR enzyme in combination with (and optionally complexed with) a guide sequence is delivered to a cell. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids in mammalian cells or target tissues. Such methods can be used to administer nucleic acids

encoding components of a CRISPR system to cells in culture, or in a host organism. Non-viral vector delivery systems include DNA plasmids, RNA (e.g. a transcript of a vector described herein), naked nucleic acid, and nucleic acid complexed with a delivery vehicle, such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10): 1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada et al., in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu et al., *Gene Therapy* 1:13-26 (1994).

[0085] Methods of non-viral delivery of nucleic acids include lipofection, nucleofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in e.g., U.S. Pat. Nos. 5,049,386, 4,946,787; and 4,897,355 and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424; WO 91/16024. Delivery can be to cells (e.g. in vitro or ex vivo administration) or target tissues (e.g. in vivo administration).

[0086] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (see, e.g., Crystal, *Science* 270:404-410 (1995); Blaese et al., *Cancer Gene Ther.* 2:291-297 (1995); Behr et al., *Bioconjugate Chem.* 5:382-389 (1994); Remy et al., *Bioconjugate Chem.* 5:647-654 (1994); Gao et al., *Gene Therapy* 2:710-722 (1995); Ahmad et al., *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0087] The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (in vivo) or they can be used to treat cells in vitro, and the modified cells may optionally be administered to patients (ex vivo). Conventional viral based systems could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0088] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are

comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., *J. Virol.* 66:2731-2739 (1992); Johann et al., *J. Virol.* 66:1635-1640 (1992); Sommerfelt et al., *Virology* 176:58-59 (1990); Wilson et al., *J. Virol.* 63:2374-2378 (1989); Miller et al., *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700). In applications where transient expression is preferred, adenoviral based systems may be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors may also be used to transduce cells with target nucleic acids, e.g., in the in vitro production of nucleic acids and peptides, and for in vivo and ex vivo gene therapy procedures (see, e.g., West et al., *Virology* 160:38-47 (1987); U.S. Pat. No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin et al., *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, et al., *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski et al., *J. Virol.* 63:03822-3828 (1989).

[0089] Packaging cells are typically used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and ψ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producing a cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the polynucleotide(s) to be expressed. The missing viral functions are typically supplied in trans by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely rep and cap, but lacking ITR sequences. The cell line may also be infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV. Additional methods for the delivery of nucleic acids to cells are known to those skilled in the art. See, for example, US20030087817, incorporated herein by reference.

[0090] In some embodiments, a host cell is transiently or non-transiently transfected with one or more vectors described herein. In some embodiments, a cell is transfected as it naturally occurs in a subject. In some embodiments, a

cell that is transfected is taken from a subject. In some embodiments, the cell is derived from cells taken from a subject, such as a cell line. A wide variety of cell lines for tissue culture are known in the art. Examples of cell lines include, but are not limited to, C8161, CCRF-CEM, MOLT, mIMCD-3, NHDF, HeLa-S3, Huh1, Huh4, Huh7, HUVEC, HASMC, HEK293, HEK293T, MiaPaCell, Panc1, PC-3, TF1, CTLL-2, C1R, Rat6, CV1, RPTE, A10, T24, J82, A375, ARH-77, Calu1, SW480, SW620, SKOV3, SK-UT, CaCo2, P388D1, SEM-K2, WEHI-231, HB56, TIB55, Jurkat, J45.01, LRMB, Bcl-1, BC-3, IC21, DLD2, Raw264.7, NRK, NRK-52E, MRC5, MEF, Hep G2, HeLa B, HeLa T4, COS, COS-1, COS-6, COS-M6A, BS-C-1 monkey kidney epithelial, BALB/3T3 mouse embryo fibroblast, 3T3 Swiss, 3T3-L1, 132-d5 human fetal fibroblasts; 10.1 mouse fibroblasts, 293-T, 3T3, 721, 9L, A2780, A2780ADR, A2780cis, A172, A20, A253, A431, A-549, ALC, B16, B35, BCP-1 cells, BEAS-2B, bEnd.3, BHK-21, BR 293, BxPC3, C3H-10T1/2, C6/36, Cal-27, CHO, CHO-7, CHO-IR, CHO-K1, CHO-K2, CHO-T, CHO Dhfr $-/-$, COR-L23, COR-L23/CPR, COR-L235010, COR-L23/R23, COS-7, COV-434, CML T1, CMT, CT26, D17, DH82, DU145, DuCaP, EL4, EM2, EM3, EMT6/AR1, EMT6/AR10.0, FM3, H1299, H69, HB54, HB55, HCA2, HEK-293, HeLa, Hepa1c1c7, HL-60, HMEC, HT-29, Jurkat, JY cells, K562 cells, Ku812, KCL22, KG1, KYO1, LNCap, Ma-Mel 1-48, MC-38, MCF-7, MCF-10A, MDA-MB-231, MDA-MB-468, MDA-MB-435, MDCK II, MDCK II, MOR/0.2R, MONO-MAC 6, MTD-1A, MyEnd, NCI-H69/CPR, NCI-H69/LX10, NCI-H69/LX20, NCI-H69/LX4, NIH-3T3, NALM-1, NW-145, OPCN/OPCT cell lines, Peer, PNT-1A/PNT 2, RenCa, RIN-5F, RMA/RMAS, Saos-2 cells, Sf-9, SkBr3, T2, T-47D, T84, THP1 cell line, U373, U87, U937, VCaP, Vero cells, WM39, WT-49, X63, YAC-1, YAR, and transgenic varieties thereof. Cell lines are available from a variety of sources known to those with skill in the art (see, e.g., the American Type Culture Collection (ATCC) (Manassas, Va.)). In some embodiments, a cell transfected with one or more vectors described herein is used to establish a new cell line comprising one or more vector-derived sequences. In some embodiments, a cell transiently transfected with the components of a CRISPR system as described herein (such as by transient transfection of one or more vectors, or transfection with RNA), and modified through the activity of a CRISPR complex, is used to establish a new cell line comprising cells containing the modification but lacking any other exogenous sequence. In some embodiments, cells transiently or non-transiently transfected with one or more vectors described herein, or cell lines derived from such cells are used in assessing one or more test compounds.

[0091] In some embodiments, one or more vectors described herein are used to produce a non-human transgenic animal or transgenic plant. In some embodiments, the transgenic animal is a mammal, such as a mouse, rat, or rabbit. In certain embodiments, the organism or subject is a plant. In certain embodiments, the organism or subject or plant is algae. Methods for producing transgenic plants and animals are known in the art, and generally begin with a method of cell transfection, such as described herein. Transgenic animals are also provided, as are transgenic plants, especially crops and algae. The transgenic animal or plant may be useful in applications outside of providing a disease model. These may include food or feed production through expression of, for instance, higher protein, carbohydrate,

nutrient or vitamins levels than would normally be seen in the wildtype. In this regard, transgenic plants, especially pulses and tubers, and animals, especially mammals such as livestock (cows, sheep, goats and pigs), but also poultry and edible insects, are preferred.

[0092] Transgenic algae or other plants such as rape may be particularly useful in the production of vegetable oils or biofuels such as alcohols (especially methanol and ethanol), for instance. These may be engineered to express or over-express high levels of oil or alcohols for use in the oil or biofuel industries.

[0093] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell, which may be in vivo, ex vivo or in vitro. In some embodiments, the method comprises sampling a cell or population of cells from a human or non-human animal or plant (including micro-algae), and modifying the cell or cells. Culturing may occur at any stage ex vivo. The cell or cells may even be re-introduced into the non-human animal or plant (including micro-algae).

[0094] In one aspect, the invention provides for methods of modifying a target polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracer sequence which in turn hybridizes to a tracer sequence.

[0095] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracer sequence which in turn hybridizes to a tracer sequence.

[0096] With recent advances in crop genomics, the ability to use CRISPR-Cas systems to perform efficient and cost effective gene editing and manipulation will allow the rapid selection and comparison of single and multiplexed genetic manipulations to transform such genomes for improved production and enhanced traits. In this regard reference is made to US patents and publications: U.S. Pat. No. 6,603, 061—*Agrobacterium*-Mediated Plant Transformation Method; U.S. Pat. No. 7,868,149—Plant Genome Sequences and Uses Thereof and US 2009/0100536—Transgenic Plants with Enhanced Agronomic Traits, all the contents and disclosure of each of which are herein incorporated by reference in their entirety. In the practice of the invention, the contents and disclosure of Morrell et al “Crop genomics: advances and applications” Nat Rev Genet. 2011 Dec. 29; 13(2):85-96 are also herein incorporated by reference in their entirety.

[0097] In plants, pathogens are often host-specific. For example, *Fusarium oxysporum* f. sp. *lycopersici* causes tomato wilt but attacks only tomato, and *F. oxysporum* f. *dianthii* *Puccinia graminis* f. sp. *tritici* attacks only wheat. Plants have existing and induced defenses to resist most pathogens. Mutations and recombination events across plant

generations lead to genetic variability that gives rise to susceptibility, especially as pathogens reproduce with more frequency than plants. In plants there can be non-host resistance, e.g., the host and pathogen are incompatible. There can also be Horizontal Resistance, e.g., partial resistance against all races of a pathogen, typically controlled by many genes and Vertical Resistance, e.g., complete resistance to some races of a pathogen but not to other races, typically controlled by a few genes. In a Gene-for-Gene level, plants and pathogens evolve together, and the genetic changes in one balance changes in other. Accordingly, using Natural Variability, breeders combine most useful genes for Yield, Quality, Uniformity, Hardiness, Resistance. The sources of resistance genes include native or foreign Varieties, Heirloom Varieties, Wild Plant Relatives, and Induced Mutations, e.g., treating plant material with mutagenic agents. Using the present invention, plant breeders are provided with a new tool to induce mutations. Accordingly, one skilled in the art can analyze the genome of sources of resistance genes, and in Varieties having desired characteristics or traits employ the present invention to induce the rise of resistance genes, with more precision than previous mutagenic agents and hence accelerate and improve plant breeding programs.

[0098] In one aspect, the invention provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, the kit comprises a vector system and instructions for using the kit. In some embodiments, the vector system comprises (a) a first regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence, wherein when expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a eukaryotic cell, wherein the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence; and/or (b) a second regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence. Elements may be provided individually or in combinations, and may be provided in any suitable container, such as a vial, a bottle, or a tube. In some embodiments, the kit includes instructions in one or more languages, for example in more than one language.

[0099] In some embodiments, a kit comprises one or more reagents for use in a process utilizing one or more of the elements described herein. Reagents may be provided in any suitable container. For example, a kit may provide one or more reaction or storage buffers. Reagents may be provided in a form that is usable in a particular assay, or in a form that requires addition of one or more other components before use (e.g. in concentrate or lyophilized form). A buffer can be any buffer, including but not limited to a sodium carbonate buffer, a sodium bicarbonate buffer, a borate buffer, a Tris buffer, a MOPS buffer, a HEPES buffer, and combinations thereof. In some embodiments, the buffer is alkaline. In some embodiments, the buffer has a pH from about 7 to about 10. In some embodiments, the kit comprises one or more oligonucleotides corresponding to a guide sequence for insertion into a vector so as to operably link the guide

sequence and a regulatory element. In some embodiments, the kit comprises a homologous recombination template polynucleotide.

[0100] In one aspect, the invention provides methods for using one or more elements of a CRISPR system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utility including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene therapy, drug screening, disease diagnosis, and prognosis. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0101] The target polynucleotide of a CRISPR complex can be any polynucleotide endogenous or exogenous to the eukaryotic cell. For example, the target polynucleotide can be a polynucleotide residing in the nucleus of the eukaryotic cell. The target polynucleotide can be a sequence coding a gene product (e.g., a protein) or a non-coding sequence (e.g., a regulatory polynucleotide or a junk DNA). Without wishing to be bound by theory, it is believed that the target sequence should be associated with a PAM (protospacer adjacent motif); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence). Examples of PAM sequences are given in the examples section below, and the skilled person will be able to identify further PAM sequences for use with a given CRISPR enzyme.

[0102] The target polynucleotide of a CRISPR complex may include a number of disease-associated genes and polynucleotides as well as signaling biochemical pathway-associated genes and polynucleotides as listed in U.S. provisional patent applications 61/736,527 and 61/748,427 having Broad reference BI-2011/008/WSGR Docket No. 44063-701.101 and BI-2011/008/WSGR Docket No. 44063-701.102 respectively, both entitled SYSTEMS METHODS AND COMPOSITIONS FOR SEQUENCE MANIPULATION filed on Dec. 12, 2012 and Jan. 2, 2013, respectively, the contents of all of which are herein incorporated by reference in their entirety.

[0103] Examples of target polynucleotides include a sequence associated with a signaling biochemical pathway, e.g., a signaling biochemical pathway-associated gene or polynucleotide. Examples of target polynucleotides include a disease associated gene or polynucleotide. A “disease-associated” gene or polynucleotide refers to any gene or polynucleotide which is yielding transcription or translation products at an abnormal level or in an abnormal form in cells derived from a disease-affected tissues compared with tissues or cells of a non disease control. It may be a gene that becomes expressed at an abnormally high level; it may be a gene that becomes expressed at an abnormally low level, where the altered expression correlates with the occurrence and/or progression of the disease. A disease-associated gene also refers to a gene possessing mutation(s) or genetic variation that is directly responsible or is in linkage disequi-

librium with a gene(s) that is responsible for the etiology of a disease. The transcribed or translated products may be known or unknown, and may be at a normal or abnormal level.

[0104] Examples of disease-associated genes and polynucleotides are available from McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web.

[0105] Examples of disease-associated genes and polynucleotides are listed in Tables A and B. Disease specific information is available from McKusick-Nathans Institute

of Genetic Medicine, Johns Hopkins University (Baltimore, Md.) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, Md.), available on the World Wide Web. Examples of signaling biochemical pathway-associated genes and polynucleotides are listed in Table C.

[0106] Mutations in these genes and pathways can result in production of improper proteins or proteins in improper amounts which affect function. Further examples of genes, diseases and proteins are hereby incorporated by reference from US Provisional application. Such genes, proteins and pathways may be the target polynucleotide of a CRISPR complex.

TABLE A

| DISEASE/DISORDERS | GENE(S) |
|----------------------------------|---|
| Neoplasia | PTEN; ATM; ATR; EGFR; ERBB2; ERBB3; ERBB4; Notch1; Notch2; Notch3; Notch4; AKT; AKT2; AKT3; HIF; HIF1a; HIF3a; Met; HRG; Bcl2; PPAR alpha; PPAR gamma; WTI (Wilms Tumor); FGF Receptor Family members (5 members: 1, 2, 3, 4, 5); CDKN2a; APC; RB (retinoblastoma); MEN1; VHL; BRCA1; BRCA2; AR (Androgen Receptor); TSG101; IGF; IGF Receptor; Igf1 (4 variants); Igf2 (3 variants); Igf 1 Receptor; Igf 2 Receptor; Bax; Bcl2; caspases family (9 members: 1, 2, 3, 4, 6, 7, 8, 9, 12); Kras; Ape |
| Age-related Macular Degeneration | Aber; Ccl2; Cc2; cp (ceruloplasmin); Timp3; eathepsinD; Vldlr; Ccr2 |
| Schizophrenia | Neuregulin1 (Nrg1); Erb4 (receptor for Neuregulin); Complexin1 (Cplx1); Tph1 Tryptophan hydroxylase; Tph2 Tryptophan hydroxylase 2; Neurexin 1; GSK3; GSK3a; GSK3b |
| Disorders | 5-HTT (Slc6a4); COMT; DRD (Drd1a); SLC6A3; DAOA; DTNBP1; Dao (Dao1) |
| Trinucleotide Repeat Disorders | HTT (Huntington's Dx); SBMA/SMAX1/AR (Kennedy's Dx); FXN/X25 (Friedrich's Ataxia); ATX3 (Machado-Joseph's Dx); ATXN1 and ATXN2 (spinocerebellar ataxias); DMPK (myotonic dystrophy); Atrophin-1 and Atn1 (DRPLA Dx); CBP (Creb-BP-global instability); VLDLR (Alzheimer's); Atxn7; Atxn10 |
| Fragile X Syndrome | FMR2; FXR1; FXR2; mGLUR5 |
| Secretase Related Disorders | APH-1 (alpha and beta); Presenilin (Psen1); nicastrin Ncstn); PEN-2 |
| Others | Nos1; Parp1; Nat1; Nat2 |
| Prion-related disorders | Prp |
| ALS | SOD1; ALS2; STEX; FUS; TARDBP; VEGF (VEGF-a; VEGF-b; VEGF-c) |
| Drug addiction | Prkce (alcohol); Drd2; Drd4; ABAT (alcohol); GRIA2; Grm5; Grin1; Htr1b; Grin2a; Drd3; Pdyn; Gria1 (alcohol) |
| Autism | Mecp2; BZRAP1; MDGA2; Sema5A; Neurexin 1; Fragile X (FMR2 (AFF2); FXR1; FXR2; Mglur5) |
| Alzheimer's Disease | E1; CHIP; UCH; UBB; Tau; LRP; PICALM; Clusterin; PS1; SORL1; CR1; Vldlr; Uba1; Uba3; CHIP28 (Aqp1, Aquaporin 1); Uchl1; Uchl3; APP |
| Inflammation | IL-10; IL-1 (IL-1a; IL-1b); IL-13; IL-17 (IL-17a (CTLA8); IL-17b; IL-17c; IL-17d; IL-17f); IL-23; Cx3cr1; ptpn22; TNFa; NOD2/CARD15 for IBD; IL-6; IL-12 (IL-12a; IL-12b); CTLA4; Cx3cl1 |
| Parkinson's Disease | x-Synuclein; DJ-1; LRRK2; Parkin; PINK1 |

TABLE B

| | |
|--|--|
| Blood and coagulation diseases and disorders | Anemia (CDAN1, CDA1, RPS19, DBA, PKLR, PK1, NT5C3, UMPH1, PSN1, RHAG, RH50A, NRAMP2, SPTB, ALAS2, ANH1, ASB, ABCB7, ABC7, ASAT); Bare lymphocyte syndrome (TAPBP, TPSN, TAP2, ABCB3, PSF2, RING11, MHC2TA, C2TA, RFX5, RFXAP, RFX5) Bleeding disorders (TBXA2R, P2RX1, P2X1); Factor H and factor H-like 1 (HF1, CFH, HUS); Factor V and factor VIII (MCFD2); Factor VII deficiency (F7); Factor X deficiency (F10); Factor XI deficiency (F11); Factor XII deficiency (F12, HAF); Factor XIIIa deficiency (F13A1, F13A); Factor XIIIb deficiency (F13B); Fanconi anemia (FANCA, FACA, FA1, FA, FAA, FAAP95, FAAP90, FLJ34064, |
|--|--|

TABLE B-continued

| | |
|---|--|
| | FANCB, FANCC, FACC, BRCA2, FANCD1, FANCD2, FANCD, FACD, FAD, FANCE, FACE, FANCF, XRCC9, FANCG, BRIP1, BACH1, FANCI, PHF9, FANCL, FANCM, KIAA1596); Hemophagocytic lymphohistiocytosis disorders (PRF1, HPLH2, UNC13D, MUNC13-4, HPLH3, HLH3, FHL3); Hemophilia A (F8, F8C, HEMA); Hemophilia B (F9, HEMB), Hemorrhagic disorders (PI, ATT, F5); Leukocyte deficiencies and disorders (ITGB2, CD18, LCAMB, LAD, EIF2B1, EIF2BA, EIF2B2, EIF2B3, EIF2B5, LVWM, CACH, CLE, EIF2B4); Sickle cell anemia (HBB); Thalassemia (HBA2, HBB, HBD, LCRB, HBA1). |
| Cell dysregulation and oncology diseases and disorders | B-cell non-Hodgkin lymphoma (BCL7A, BCL7); Leukemia (TAL1, TCL5, SCL, TAL2, FLT3, NBS1, NBS, ZNFN1A1, IK1, LYF1, HOXD4, HOX4B, BCR, CML, PHL, ALL, ARNT, KRAS2, RASK2, GMPS, AF10, ARHGEF12, LARG, KIAA0382, CALM, CLTH, CEBPA, CEBP, CHIC2, BTL, FLT3, KIT, PBT, LPP, NPM1, NUP214, D9S46E, CAN, CAIN, RUNX1, CBFA2, AML1, WHSC1L1, NSD3, FLT3; AF1Q, NPM1, NUMA1, ZNF145, PLZF, PML, MYL, STAT5B, AF10, CALM, CLTH, ARL11, ARLTS1, P2RX7, P2X7, BCR, CML, PHL, ALL, GRAF, NF1, VRNF, WSS, NFNS, PTPN11, PTP2C, SHP2, NS1, BCL2, CCND1, PRAD1, BCL1, TCRA, GATA1, GF1, ERYF1, NFE1, ABL1, NQO1, DIA4, NMOR1, NUP214, D9S46E, CAN, CAIN). AIDS (KIR3DL1, NKAT3, NKB1, AMB11, KIR3DS1, IFNG, CXCL12, SDF1); Autoimmune lymphoproliferative syndrome (TNFRSF6, APT1, FAS, CD95, ALPS1A); Combined immunodeficiency, (IL2RG, SCIDX1, SCIDX, IMD4); HIV-1 (CCL5, SCYA5, D17S136E, TCD228), HIV susceptibility or infection (IL10, CSIF, CMKBR2, CCR2, CMKBR5, CCKCR5 (CCU)); Immunodeficiencies (CD3E, CD3G, AICDA, AID, HIGM2, TNFRSF5, CD40, UNG, DGU, HIGM4, TNFRSF5, CD40LG, HIGM1, IGM, FOXP3, IPEX, AIID, XPID, PIDX, TNFRSF14B, TACD); Inflammation (IL-10, IL-1 (IL-1a, IL-1b), IL-13, IL-17 (IL-17a (CTLA8), IL-17b, IL-17c, IL-17d, IL-17f), IL-23, Cx3cr1, ptpn22, TNFa, NOD2/CARD15 for IBD, IL-6, IL-12 (IL-12a, IL-12b), CTLA4, Cx3cl1); Severe combined immunodeficiencies (SCIDs)(JAK3, JAK1, DCLRE1C, ARTEMIS, SCIDA, RAG1, RAG2, ADA, PTPRC, CD45, LCA, IL7R, CD3D, T3D, IL2RG, SCIDM, SCIDX, IMD4). |
| Inflammation and immune related diseases and disorders | |
| Metabolic, liver, kidney and protein diseases and disorders | Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPR1, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2, ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKU1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63). |
| Muscular/Skeletal diseases and disorders | Becker muscular dystrophy (DMD, BMD, MYF6), Duchenne Muscular Dystrophy (DMD, BMD); Emery-Dreifuss muscular dystrophy (LMNA, LMN1, EMD2, FPLD, CMD1A, HGPS, LGMD1B, LMNA, LMN1, EMD2, FPLD, CMD1A); Facioscapulohumeral muscular dystrophy (FSHMa1A, FSHD1A); Muscular dystrophy (FKRP, MDC1C, LGMD2I, LAMA2, LAMM, LARGE, KIAA0609, MDC1D, FCMD, TTID, MYOT, CAPN3, CANP3, DYSF, LGMD2B, SGCG, LGMD2C, DMDA1, SCG3, SGCA, ADL, DAG2, LGMD2D, DMDA2, SGCB, LGMD2E, SGCD, SGD, LGMD2F, CMD1L, TCAP, LGMD2G, CMD1N, TRIM32, HT2A, LGMD2H, FKRP, MDC1C, LGMD2I, TTN, CMD1G, TMD, LGMD2J, POMT1, CAV3, LGMD1C, SEPNI, SELN, RSMN1, PLEC1, PLTN, EBS1); Osteopetrosis (LRP5, BMND1, LRP7, LR3, OPG, VBCH2, CLCN7, CLC7, OPTA2, OSTM1, GL, TCIRG1, TIRC7, OC116, OPTB1); Muscular atrophy (VAPB, VAPC, ALS8, SMN1, SMA1, SMA2, SMA3, SMA4, BSCL2, SPG17, GARS, SMAD1, CMT2D, HEXB, IGHMBP2, SMUBP2, CATF1, SMARD1), |
| Neurological and neuronal diseases and disorders | ALS (SOD1, ALS2, STEX, FUS, TARDBP, VEGF (VEGF-a, VEGF-b, VEGF-c); Alzheimer disease (APP, AAA, CVAP, AD1, APOE, AD2, PSEN2, AD4, STM2, APBB2, FE65L1, NOS3, PLA2, URK, ACE, DCP1, ACE1, MPO, PACIP1, PAXIP1L, PTP1, A2M, BLMH, BMH, PSEN1, AD3); Autism (Mecp2, BZRAP1, MDGA2, Sema5A, Neurexin1 GLO1, MECP2, RTT, PPMX, MRX16, MRX79, NLGN3, NLGN4, KIAA1260, AUTSX2); Fragile X Syndrome (FMR2, FXR1, FXR2, mGLUR5); Huntington's disease and disease like disorders (HD, IT15, PRNP, PRIP, JPH3, JP3, HDL2, TBP, SCA17); Parkinson disease (NR4A2, NURR1, NOT, TINUR, SNCAIP, TBP, SCA17, SNCA, NACP, PARK1, PARK4, DJ1, PARK7, LRRK2, PARK8, PINK1, PARK6, UCHL1, PARK5, SNCA, NACP, PARK1, PARK4, PRKN, |

TABLE B-continued

| | |
|--|---|
| | PARK2, PDI, DBH, NDUFV2); Rett syndrome (MECP2, RTT, PPMX, MRX16, MRX79, CDKL5, STK9, MECP2, RTT, PPMX, MRX16, MRX79, x-Synuclein, DJ-1); Schizophrenia (Neuregulin1 (Nrg1), Erb4 (receptor for Neuregulin), Complexin1 (Cplx1), Tph1 Tryptophan hydroxylase, Tph2, Tryptophan hydroxylase 2, Neurexin 1, GSK3, GSK3a, GSK3b, 5-HTT (Slc6a4), COMT, DRD (Drd1a), SLC6A3, DAOA, DTNBP1, Dao (Dao1)); Secretase Related Disorders (APH-1 (alpha and beta), Presenitin (Psen1), nicastrin, (Ncstn), PEN-2, Nos1, Parp1, Nat1, Nat2); Trinucleotide Repeat Disorders (HTT (Huntington's Dx), SBMA/SMAX1/AR (Kennedy's Dx), FXN/X25 (Friedrich's Ataxia), ATX3 (Machado-Joseph's Dx), ATXN1 and ATXN2 (spinocerebellar ataxias), DMPK (myotonic dystrophy), Atrophin-1 and Atm1 (DRPLA Dx), CBP (Creb-BP-global instability), VLDLR (Alzheimer's), Atxn7, Atxn.10). |
| Occular diseases and disorders | Age-related macular degeneration (Aber, Ccl2, Cc2, cp (ceruloplasmin), Timp3, cathepsinD, Vldlr, Ccr2); Cataract (CRYAA, CRYA1, CRYBB2, CRYB2, PITX3, BFSP2, CP49, CP47, CRYAA, CRYA1, PAX6, AN2, MGDA, CRYBA1, CRYB1, CRYGC, CRYG3, CCL, LIM2, MP19, CRYGD, CRYG4, BFSP2, CP49, CP47, HSF4, CTM, HST4, CTM, MIP, AQP0, CRYAB, CRYA2, CTPP2, CRYBB1, CRYGD, CRYG4, CRYBB2, CRYB2, CRYGC, CRYG3, CCL, CRYAA, CRYA1, GJA8, CX50, CAE1, GJA3, CX46, CZP3, CAE3, CCM1, CAM, KRIT1); Corneal clouding and dystrophy (APOA1, TGFBI, CSD2, CDGG1, CSD, BIGH3, CDG2, TACSTD2, TROP2, M1S1, VSX1, RINX, PPCD, PPD, KTCN, COL8A2, FECD, PPCD2, PIP5K3, CFD); Cornea plana congenital (KERA, CNA2); Glaucoma (MYOC, TIGR, GLC1A, JOAG, GPOA, OPTN, GLC1E, FIP2, HYPL, NRP, CYP1B1, GLC3A, OPA1, NTG, NPG, CYP1B1, GLC3A); Leber congenital amaurosis (CRB1, RP12, CRX, CORD2, CRD, RPGRIP1, LCA6, CORD9, RPE65, RP20, AIPL1, LCA4, GUCY2D, GUC2D, LCA1, CORD6, RDH12, LCA3); Macular dystrophy (ELOVL4, ADMD, STGD2, STGD3, RDS, RP7, PRPH2, PRPH, AVMD, AOFMD, VMD2). |
| Epilepsy, myoclonic Lafora type, 254780 | EPM2A, MELF, EPM2 |
| Epilepsy, myoclonic, Lafora type, 254780 | NHLRC1, EPM2A, EPM2B |
| Duchenne muscular dystrophy, 310200 (3) | DMD, BMD |
| AIDS, delayed/rapid progression to (3) | KIR3DL1, NKAT3, NKB1, AMB11 |
| AIDS, rapid progression to, 609423 (3) | KIR3DS1 |
| AIDS, resistance to (3) | IFNG |
| Alpha 1-Antitrypsin Deficiency | CXCL12, SDF1 |
| | SERPINA1 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1]; SERPINA2 [serpin peptidase inhibitor, clade A alpha-1 antiproteinase, antitrypsin), member 2]; SERPINA3 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3]; SERPINA5 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5]; SERPINA6 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6]; SERPINA7 [serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7];" AND "SERPINA6 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6) |

TABLE C

| CELLULAR FUNCTION | GENES |
|--------------------|---|
| PI3K/AKT Signaling | PRKCE; ITGAM; ITGA5; IRAK1; PRKAA2; EIF2AK2; PTEN; EIF4E; PRKCZ; GRK6; MAPK1; TSC1; PLK1; AKT2; IKKBK; PIK3CA; CDK8; CDKN1B; NFKB2; BCL2; PIK3CB; PPP2R1A; MAPK8; BCL2L1; MAPK3; TSC2; ITGA1; KRAS; EIF4EBP1; RELA; PRKCD; NOS3; PRKAA1; MAPK9; CDK2; PPP2CA; PIM1; ITGB7; YWHAZ; ILK; TP53; RAF1; IKBK; RELB; DYRK1A; CDKN1A; ITGB1; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; CHUK; PDPK1; PPP2R5C; CTNNB1; MAP2K1; NFKB1; PAK3; ITGB3; CCND1; GSK3A; FRAP1; SFN; ITGA2; TTK; CSNK1A1; BRAF; GSK3B; AKT3; FOXO1; SGK; HSP90AA1; RPS6KB1 |
| ERK/MAPK Signaling | PRKCE; ITGAM; ITGA5; HSPB1; IRAK1; PRKAA2; EIF2AK2; RAC1; RAP1A; TLN1; EIF4E; ELK1; GRK6; |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|--------------------------------------|--|
| Glucocorticoid Receptor Signaling | <p>MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; CREB1; PRKC1; PTK2; FOS; RPS6KA4; PIK3CB; PPP2R1A; PIK3C3; MAPK8; MAPK3; ITGA1; ETS1; KRAS; MYCN; EIF4EBP1; PPARG; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PPP2CA; PIM1; PIK3C2A; ITGB7; YWHAZ; PPP1CC; KSR1; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; PIK3R1; STAT3; PPP2R5C; MAP2K1; PAK3; ITGB3; ESR1; ITGA2; MYC; TTK; CSNK1A1; CRKL; BRAF; ATF4; PRKCA; SRF; STAT1; SGK RAC1; TAF4B; EP300; SMAD2; TRAF6; PCAF; ELK1; MAPK1; SMAD3; AKT2; IKKBK; NCOR2; UBE2I; PIK3CA; CREB1; FOS; HSPA5; NFKB2; BCL2; MAP3K14; STAT5B; PIK3CB; PIK3C3; MAPK8; BCL2L1; MAPK3; TSC22D3; MAPK10; NRIP1; KRAS; MAPK13; RELA; STAT5A; MAPK9; NOS2A; PBX1; NR3C1; PIK3C2A; CDKN1C; TRAF2; SERPINE1; NCOA3; MAPK14; TNF; RAF1; IKBK; MAP3K7; CREBBP; CDKN1A; MAP2K2 ; JAK1; IL8; NCOA2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; TGBR1; ESR1; SMAD4; CEBPB; JUN; AR; AKT3; CCL2; MMP1; STAT1; IL6; HSP90AA1</p> |
| Axonal Guidance Signaling | <p>PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; ADAM12; IGF1; RAC1; RAP1A; EIF4E; PRKCZ; NRP1; NTRK2; ARGF7; SMO; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; AKT2; PIK3CA; ERBB2; PRKCI; PTK2; CFL1; GNAQ; PIK3CB; CXCL12; PIK3C3; WNT11; PRKD1; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA; PRKCD; PIK3C2A; ITGB7; GLI2; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; ADAM17; AKT1; PIK3R1; GLI1; WNT5A; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; CRKL; RND1; GSK3B; AKT3; PRKCA</p> |
| Ephrin Receptor Signaling | <p>PRKCE; ITGAM; ROCK1; ITGA5; CXCR4; IRAK1; PRKAA2; EIF2AK2; RAC1; RAP1A; GRK6; ROCK2; MAPK1; PGF; RAC2; PTPN11; GNAS; PLK1; AKT2; DOK1; CDK8; CREB1; PTK2; CFL1; GNAQ; MAP3K14; CXCL12; MAPK8; GNB2L1; ABL1; MAPK3; ITGA1; KRAS; RHOA; PRKCD; PRKAA1; MAPK9; SRC; CDK2; PIM1; ITGB7; PXN; RAF1; FYN; DYRK1A; ITGB1; MAP2K2; PAK4; AKT1; JAK2 ; STAT3; ADAM10; MAP2K1; PAK3; ITGB3; CDC42; VEGFA; ITGA2; EPHA8; TTK; CSNK1A1; CRKL; BRAF; PTPN13; ATF4; AKT3; SGK</p> |
| Actin Cytoskeleton Signaling | <p>ACTN4; PRKCE; ITGAM; ROCK1; ITGA5; IRAK1; PRKAA2; EIF2AK2; RAC1; INS; ARHGEF7; GRK6; ROCK2; MAPK1; RAC2; PLK1; AKT2; PIK3CA; CDK8; PTK2; CFL1; PIK3CB; MYH9; DIAPH1; PIK3C3; MAPK8; F2R; MAPK3; SLC9A1; ITGA1; KRAS; RHOA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; ITGB7; PPP1CC; PXN; VIL2; RAF1; GSN; DYRK1A; ITGB1; MAP2K2; PAK4; PIP5K1A; PIK3R1; MAP2K1; PAK3; ITGB3; CDC42; APC; ITGA2; TTK; CSNK1A1; CRKL; BRAF; VAV3; SGK</p> |
| Huntington's Disease Signaling | <p>PRKCE; IGF1; EP300; RCOR1; PRKCZ; HDAC4; TGM2; MAPK1; CAPNS1; AKT2; EGFR; NCOR2; SP1; CAPN2; PIK3CA; HDAC5; CREB1; PRKCI; HSPA5; REST; GNAQ; PIK3CB; PIK3C3; MAPK8; IGF1R; PRKD1; GNB2L1; BCL2L1; CAPN1; IMAPK3; CASP8; HDAC2; HDAC7A; PRKCD; HDAC11; MAPK9; HDAC9; PIK3C2A; HDAC3; TP53; CASP9; CREBBP; AKT1; PIK3R1; PDPK1; CASP1; APAF1; FRAP1; CASP2; JUN; BAX; ATF4; AKT3; PRKCA; CLTC; SGK; HDAC6; CASP3</p> |
| Apoptosis Signaling | <p>PRKCE; ROCK1; BID; IRAK1; PRKAA2; EIF2AK2; BAK1; BIRC4; GRK6; MAPK1; CAPNS1; PLK1; AKT2; IKKBK; CAPN2; CDK8; FAS; NFKB2; BCL2; MAP3K14; MAPK8; BCL2L1; CAPN1; MAPK3; CASP8; KRAS; RELA; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; TP53; TNF; RAF1; IKBK; RELB; CASP9; DYRK1A; MAP2K2; CHUK; APAF1; MAP2K1; NFKB1; PAK3; LMNA; CASP2; BIRC2; TTK; CSNK1A1; BRAF; BAX; PRKCA; SGK; CASP3; BIRC3; PARP1</p> |
| B Cell Receptor Signaling | <p>RAC1; PTEN; LYN; ELK1; MAPK1; RAC2; PTPN11; AKT2; IKKBK; PIK3CA; CREB1; SYK; NFKB2; CAMK2A; MAP3K14; PIK3CB; PIK3C3; MAPK8; BCL2L1; ABL1;</p> |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|---|--|
| Leukocyte Extravasation Signaling | MAPK3; ETS1; KRAS; MAPK13; RELA; PTPN6; MAPK9; EGR1; PIK3C2A; BTK; MAPK14; RAF1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; PIK3R1; CHUK; MAP2K1; NFKB1; CDC42; GSK3A; FRAP1; BCL6; BCL10; JUN; GSK3B; ATF4; AKT3; VAV3; RPS6KB1 |
| Integrin Signaling | ACTN4; CD44; PRKCE; ITGAM; ROCK1; CXCR4; CYBA; RAC1; RAP1A; PRKCZ; ROCK2; RAC2; PTPN11; MMP14; PIK3CA; PRKCI; PTK2; PIK3CB; CXCL12; PIK3C3; MAPK8; PRKBI; ABL1; MAPK10; CYBB; MAPK13; RHOA; PRKCD; MAPK9; SRC; PIK3C2A; BTK; MAPK14; NOX1; PXN; VIL2; VASP; ITGB1; MAP2K2; CTNND1; PIK3R1; CTNNB1; CLDN1; CDC42; F11R; ITK; CRKL; VAV3; CTTN; PRKCA; MMP1; MMP9 |
| Acute Phase Response Signaling | ACTN4; ITGAM; ROCK1; ITGA5; RAC1; PTEN; RAP1A; TLN1; ARHGEF7; MAPK1; RAC2; CAPNS1; AKT2; CAPN2; PIK3CA; PTK2; PIK3CB; PIK3C3; MAPK8; CAV1; CAPN1; ABL1; MAPK3; ITGA1; KRAS; RHOA; SRC; PIK3C2A; ITGB7; PPP1CC; ILK; PXN; VASP; RAF1; FYN; ITGB1; MAP2K2; PAK4; AKT1; PIK3R1; TNK2; MAP2K1; PAK3; ITGB3; CDC42; RND3; ITGA2; CRKL; BRAF; GSK3B; AKT3 |
| PTEN Signaling | IRAK1; SOD2; MYD88; TRAF6; ELK1; MAPK1; PTPN11; AKT2; IKBKB; PIK3CA; FOS; NFKB2; MAP3K14; PIK3CB; MAPK8; RIPK1; MAPK3; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; FTL; NR3C1; TRAF2; SERPINE1; MAPK14; TNF; RAF1; PDK1; IKBKG; RELB; MAP3K7; MAP2K2; AKT1; JAK2; PIK3R1; CHUK; STAT3; MAP2K1; NFKB1; FRAP1; CEBPB; JUN; AKT3; IL2R1; IL6 |
| p53 Signaling | ITGAM; ITGA5; RAC1; PTEN; PRKCZ; BCL2L1; MAPK1; RAC2; AKT2; EGFR; IKBKB; CBL; PIK3CA; CDKN1B; PTK2; NFKB2; BCL2; PIK3CB; BCL2L1; MAPK3; ITGA1; KRAS; ITGB7; ILK; PDGFRB; INSR; RAF1; IKBKG; CASP9; CDKN1A; ITGB1; MAP2K2; AKT1; PIK3R1; CHUK; PDGFRA; PDPK1; MAP2K1; NFKB1; ITGB3; CDC42; CCND1; GSK3A; ITGA2; GSK3B; AKT3; FOXO1; CASP3; RPS6KB1 |
| Aryl Hydrocarbon Receptor Signaling | PTEN; EP300; BBC3; PCAF; FASN; BRCA1; GADD45A; BIRC5; AKT2; PIK3CA; CHEK1; TP53INP1; BCL2; PIK3CB; PIK3C3; MAPK8; THBS1; ATR; BCL2L1; E2F1; PMAIP1; CHEK2; TNFRSF10B; TP73; RB1; HDAC9; CDK2; PIK3C2A; MAPK14; TP53; LRDD; CDKN1A; HIPK2; AKT1; PIK3R1; RRM2B; APAF1; CTNNB1; SIRT1; CCND1; PRKDC; ATM; SFN; CDKN2A; JUN; SNAI2; GSK3B; BAX; AKT3 |
| Xenobiotic Metabolism Signaling | HSPR1; EP300; FASN; TGM2; RXRA; MAPK1; NQO1; NCOR2; SP1; ARNT; CDKN1B; FOS; CHEK1; SMARCA4; NFKB2; MAPK8; ALDH1A1; ATR; E2F1; MAPK3; NRIP1; CHEK2; RELA; TP73; GSTP1; RB1; SRC; CDK2; AHR; NFE2L2; NCOA3; TP53; TNF; CDKN1A; NCOA2; APAF1; NFKB1; CCND1; ATM; ESR1; CDKN2A; MYC; JUN; ESR2; BAX; IL6; CYP1B1; HSP90AA1 |
| SAPK/JNK Signaling | PRKCE; EP300; PRKCZ; RXRA; MAPK1; NQO1; NCOR2; PIK3CA; ARNT; PRKCI; NFKB2; CAMK2A; PIK3CB; PPP2R1A; PIK3C3; MAPK8; PRKD1; ALDH1A1; MAPK3; NRIP1; KRAS; MAPK13; PRKCD; GSTP1; MAPK9; NOS2A; ABCB1; AHR; PPP2CA; FTL; NFE2L2; PIK3C2A; PPARGC1A; MAPK14; TNF; RAF1; CREBBP; MAP2K2; PIK3R1; PPP2R5C; MAP2K1; NFKB1; KEAP1; PRKCA; EIF2AK3; IL6; CYP1B1; HSP90AA1 |
| PPAr/RXR Signaling | PRKCE; IRAK1; PRKAA2; EIF2AK2; RAC1; ELK1; GRK6; MAPK1; GADD45A; RAC2; PLK1; AKT2; PIK3CA; FADD; CDK8; PIK3CB; PIK3C3; MAPK8; RIPK1; GNB2L1; IRS1; MAPK3; MAPK10; DAXX; KRAS; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; TRAF2; TP53; LCK; MAP3K7; DYRK1A; MAP2K2; PIK3R1; MAP2K1; PAK3; CDC42; JUN; TTK; CSNK1A1 ; CRKL; BRAF; SGK |
| | PRKAA2; EP300; INS; SMAD2; TRAF6; PPARA; FASN; RXRA; MAPK1; SMAD3; GNAS; IKBKB; NCOR2; ABCA1; GNAQ; NFKB2; MAP3K14; STAT5B; MAPK8; IRS1; MAPK3; KRAS; RELA; PRKAA1; PPARGC1A; |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|---|---|
| NF-KB Signaling | NCOA3; MAPK14; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; JAK2; CHUK; MAP2K1; NFKB1; TGFBF1; SMAD4; JUN; IL1R1; PRKCA; IL6; HSP90AA1; ADIPOQ |
| Neuregulin Signaling | IRAK1; EIF2AK2; EP300; INS; MYD88; PRKCZ; TRAF6; TNK1; AKT2; EGFR; IKBKB; PIK3CA; BTRC; NFKB2; MAP3K14; PIK3CB; PIK3C3; MAPK8; RIPK1; HDAC2; KRAS; RELA; PIK3C2A; TRAF2; TLR4; PDGFRB; TNF; INSR; LCK; IKBKG; RELB; MAP3K7; CREBBP; AKT1; PIK3R1; CHUK; PDGFRA; NFKB1; TLR2; BCL10; GSK3B; AKT3; TNFAIP3; IL1R1 |
| Wnt & Beta catenin Signaling | ERBB4; PRKCE; ITGAM; ITGA5; PTEN; PRKCZ; ELK1; MAPK1; PTPN11; AKT2; EGFR; ERBB2; PRKCI; CDKN1B; STAT5B; PRKD1; MAPK3; ITGA1; KRAS; PRKCD; STAT5A; SRC; ITGB7; RAF1; ITGB1; MAP2K2; ADAM17; AKT1; PIK3R1; PDPK1; MAP2K1; ITGB3; EREG; FRAP1; PSEN1; ITGA2; MYC; NRG1; CRKL; AKT3; PRKCA; HSP90AA1; RPS6KB1 |
| Insulin Receptor Signaling | CD44; EP300; LRP6; DVL3; CSNK1E; GJA1; SMO; AKT2; PIN1; CDH1; BTRC; GNAQ; MARK2; PPP2R1A; WNT11; SRC; DKK1; PPP2CA; SOX6; SFRP2; ILK; LEF1; SOX9; TP53; MAP3K7; CREBBP; TCF7L2; AKT1; PPP2R5C; WNT5A; LRP5; CTNBN1; TGFBF1; CCND1; GSK3A; DVL1; APC; CDKN2A; MYC; CSNK1A1; GSK3B; AKT3; SOX2 |
| IL-6 Signaling | PTEN; INS; EIF4E; PTPN1; PRKCZ; MAPK1; TSC1; PTPN11; AKT2; CBL; PIK3CA; PRKCI; PIK3CB; PIK3C3; MAPK8; IRS1; MAPK3; TSC2; KRAS; EIF4EBP1; SLC2A4; PIK3C2A; PPP1CC; INSR; RAF1; FYN; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; PDPK1; MAP2K1; GSK3A; FRAP1; CRKL; GSK3B; AKT3; FOXO1; SGK; RPS6KB1 |
| Hepatic Cholestasis | HSPB1; TRAF6; MAPKAPK2; ELK1; MAPK1; PTPN11; IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK3; MAPK10; IL6ST; KRAS; MAPK13; IL6R; RELA; SOCS1; MAPK9; ABCB1; TRAF2; MAPK14; TNF; RAF1; IKBKG; RELB; MAP3K7; MAP2K2; IL8; JAK2; CHUK; STAT3; MAP2K1; NFKB1; CEBPB; JUN; IL1R1; SRF; IL6 |
| IGF-1 Signaling | PRKCE; IRAK1; INS; MYD88; PRKCZ; TRAF6; PPARA; RXRA; IKBKB; PRKCI; NFKB2; MAP3K14; MAPK8; PRKD1; MAPK10; RELA; PRKCD; MAPK9; ABCB1; TRAF2; TLR4; TNF; INSR; IKBKG; RELB; MAP3K7; IL8; CHUK; NR1H2; TJP2; NFKB1; ESR1; SREBF1; FGFR4; JUN; IL1R1; PRKCA; IL6 |
| NRF2-mediated Oxidative Stress Response | IGF1; PRKCZ; ELK1; MAPK1; PTPN11; NEDD4; AKT2; PIK3CA; PRKCI; PTK2; FOS; PIK3CB; PIK3C3; MAPK8; IGF1R; IRS1; MAPK3; IGFBP7; KRAS; PIK3C2A; YWHAZ; PXN; RAF1; CASP9; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; IGFBP2; SFN; JUN; CYR61; AKT3; FOXO1; SRF; CTGF; RPS6KB1 |
| Hepatic Fibrosis/Hepatic Stellate Cell Activation | PRKCE; EP300; SOD2; PRKCZ; MAPK1; SQSTM1; NQO1; PIK3CA; PRKCI; FOS; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; KRAS; PRKCD; GSTP1; MAPK9; FTL; NFE2L2; PIK3C2A; MAPK14; RAF1; MAP3K7; CREBBP; MAP2K2; AKT1; PIK3R1; MAP2K1; PP1B; JUN; KEAP1; GSK3B; ATF4; PRKCA; EIF2AK3; HSP90AA1 |
| PPAR Signaling | EDN1; IGF1; KDR; FLT1; SMAD2; FGFR1; MET; PGF; SMAD3; EGFR; FAS; CSF1; NFKB2; BCL2; MYH9; IGF1R; IL6R; RELA; TLR4; PDGFRB; TNF; RELB; IL8; PDGFRA; NFKB1; TGFBF1; SMAD4; VEGFA; BAX; IL1R1; CCL2; HGF; MMP1; STAT1; IL6; CTGF; MMP9 |
| Fc Epsilon RI Signaling | EP300; INS; TRAF6; PPARA; RXRA; MAPK1; IKBKB; NCOR2; FOS; NFKB2; MAP3K14; STAT5B; MAPK3; NRIP1; KRAS; PPARG; RELA; STAT5A; TRAF2; PPARGC1A; PDGFRB; TNF; INSR; RAF1; IKBKG; RELB; MAP3K7; CREBBP; MAP2K2; CHUK; PDGFRA; MAP2K1; NFKB1; JUN; IL1R1; HSP90AA1 |
| | PRKCE; RAC1; PRKCZ; LYN; MAPK1; RAC2; PTPN11; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; MAPK8; PRKD1; MAPK3; MAPK10; KRAS; MAPK13; PRKCD; MAPK9; PIK3C2A; BTK; MAPK14; TNF; RAF1; FYN; MAP2K2; AKT 1; PIK3R11; PDPK1; MAP2K1; AKT3; VAV3; PRKCA |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|--|--|
| G-Protein Coupled Receptor Signaling | PRKCE; RAPIA; RGS16; MAPK1; GNAS; AKT2; IKBKB; PIK3CA; CREB1; GNAQ; NFKB2; CAMK2A; PIK3CB; PIK3C3; MAPK3; KRAS; RELA; SRC; PIK3C2A; RAF1; IKBK; RELB; FYN; MAP2K2; AKT1; PIK3R1; CHUK; PDPK1; STAT3; MAP2K1; NFKB1; BRAF; ATF4; AKT3; PRKCA |
| Inositol Phosphate Metabolism | PRKCE; IRAK1; PRKAA2; EIF2AK2; PTEN; GRK6; MAPK1; PLK1; AKT2; PIK3CA; CDK8; PIK3CB; PIK3C3; MAPK8; MAPK3; PRKCD; PRKAA1; MAPK9; CDK2; PIM1; PIK3C2A; DYRK1A; MAP2K2; PIP5K1A; PIK3R1; MAP2K1; PAK3; ATM; TTK; CSNK1A1; BRAF; SGK |
| PDGF Signaling | EIF2AK2; ELK1; ABL2; MAPK1; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; CAV1; ABL1; MAPK3; KRAS; SRC; PIK3C2A; PDGFRB; RAF1; MAP2K2; JAK1; JAK2; PIK3R1; PDGFRA; STAT3; SPHK1; MAP2K1; MYC; JUN; CRKL; PRKCA; SRF; STAT1; SPHK2 |
| VEGF Signaling | ACTN4; ROCK1; KDR; FLT1; ROCK2; MAPK1; PGF; AKT2; PIK3CA; ARNT; PTK2; BCL2; PIK3CB; PIK3C3; BCL2L1; MAPK3; KRAS; HIF1A; NOS3; PIK3C2A; PXN; RAF1; MAP2K2; ELAVL1; AKT1; PIK3R1; MAP2K1; SFN; VEGFA; AKT3; FOXO1; PRKCA |
| Natural Killer Cell Signaling | PRKCE; RAC1; PRKCZ; MAPK1; RAC2; PTPN11; KIR2DL3; AKT2; PIK3CA; SYK; PRKCI; PIK3CB; PIK3C3; PRKD1; MAPK3; KRAS; PRKCD; PTPN6; PIK3C2A; LCK; RAF1; FYN; MAP2K2; PAK4; AKT1; PIK3R1; MAP2K1; PAK3; AKT3; VAV3; PRKCA |
| Cell Cycle: G1/S Checkpoint Regulation | HDAC4; SMAD3; SUV39H1; HDAC5; CDKN1B; BTRC; ATR; ABL1; E2F1; HDAC2; HDAC7A; RB1; HDAC11; HDAC9; CDK2; E2F2; HDACS; TP53; CDKN1A; CCND1; E2F4; ATM; RBL2; SMAD4; CDKN2A; MYC; NRG1; GSK3B; RBL1; HDAC6 |
| T Cell Receptor Signaling | RAC1; ELK1; MAPK1; IKBK13; CBL; PIK3CA; FOS; NFKB2; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; RELA; PIK3C2A; BTK; LCK; RAF1; IKBKG; RELB; FYN; MAP2K2; PIK3R1; CHUK; MAP2K1; NFKB1; ITK; BCL10 JUN; VAV3 |
| Death Receptor Signaling | CRADD; HSPB1; BID; BIRC4; TBK1; IKBKB; FADD; FAS; NFKB2; BCL2; MAP3K14; MAPK8; RIPK1; CASP8; DAXX; TNFRSF10B; RELA; TRAF2; TNF; IKBKG; RELB; CASP9; CHUK; APAF1; NFKB1; CASP2; BIRC2; CASP3; BIRC3 |
| FGF Signaling | RAC1; FGFR1; MET; MAPKAPK2; MAPK1; PTPN11; AKT2; PIK3CA; CREB1; PIK3CB; PIK3C3; MAPK8; MAPK3; MAPK13; PTPN6; PIK3C2A; MAPK14; RAF1; AKT1; PIK3R1; STAT3; MAP2K1; .FGFR4; CRKL; ATF; AKT3; PRKCA; HGF |
| GM-CSF Signaling | LYN; ELK1; MAPK1; PTPN11; AKT2; PIK3CA; CAMK2A; STAT5B; PIK3CB; PIK3C3; GNB2L1; BCL2L1; MAPK3; ETS1; KRAS; RUNX1; PIM1; PIK3C2A; RAF1; MAP2K2; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; CCND1; AKT3; STAT1 |
| Amyotrophic Lateral Sclerosis Signaling | BID; IGF1; RAC1; BIRC4; PGF; CAPNS1; CAPN2; PIK3CA; BCL2; PIK3CB; PIK3C3; BCL2L1; CAPN1; PIK3C2A; TP53; CASP9; PIK3R1; RAB5A; CASP1; APAF1; VEGFA; BIRC2; BAX; AKT3; CASP3; BIRC3 |
| JAK/Stat Signaling | PTPN1; MAPK1; PTPN11; AKT2; PIK3CA; STAT5B; PIK3CB; PIK3C3; MAPK3; KRAS; SOCS1; STAT5A; PTPN6; PIK3C2A; RAF1; CDKN1A; MAP2K2; JAK1; AKT1; JAK2; PIK3R1; STAT3; MAP2K1; FRAP1; AKT3; STAT1 |
| Nicotinate and Nicotinamide Metabolism | PRKCE; IRAK1; PRKAA2; EIF2AK2; GRK6; MAPK1; PLK1; AKT2; CDK8; MAPK8; MAPK3; PRKCD; PRKAA1; PBEF1; MAPK9; CDK2; PIM1; DYRK1A; MAP2K2; MAP2K1; PAK3; NT5E; TTK; CSNK1A1; BRAF; SGK |
| Chernokine Signaling | CXCR4; ROCK2; MAPK1; PTK2; FOS; CFL1; GNAQ; CAMK2A; CXCL12; MAPK8; MAPK3; KRAS; MAPK13; RHOA; CCR3; SRC; PPP1CC; MAPK14; NOX1; RAF1; MAP2K2; MAP2K1; JUN; CCL2; PRKCA |
| IL-2 Signaling | ELK1; MAPK1; PTPN11; AKT2; PIK3CA; SYK; FOS; STAT5B; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; SOCS1; STAT5A; PIK3C2A; LCK; RAF1; MAP2K2; JAK1; AKT1; PIK3R1; MAP2K1; JUN; AKT3 |
| Synaptic Long Term Depression | PRKCE; IGF1; PRKCZ; PRDX6; LYN; MAPK1; GNAS; PRKCI; GNAQ; PPP2R1A; IGF1R; PRKD1; MAPK3; |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|---|--|
| Estrogen Receptor Signaling | KRAS; GRN; PRKCD; NOS3; NOS2A; PPP2CA; YWHAZ; RAF1; MAP2K2; PPP2R5C; MAP2K1; PRKCA TAF4B; EP300; CARM1; PCAF; MAPK1; NCOR2; SMARCA4; MAPK3; NR1P1; KRAS; SRC; NR3C1; HDAC3; PPARGC1A; RBM9; NCOA3; RAF1; CREBBP; MAP2K2; NCOA2; MAP2K1; PRKDC; ESR1; ESR2 |
| Protein Ubiquitination Pathway | TRAF6; SMURF1; BIRC4; BRCA1; UCHL1; NEDD4; CBL; UBE2I; BTRC; HSPA5; USP7; USP10; FBXW7; USP9X; STUB1; USP22; B2M; BIRC2; PARK2; USP8; USP1; VHL; HSP90AA1; BIRC3 |
| IL-10 Signaling | TRAF6; CCR1; ELK1; IKBKB; SP1; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; MAPK14; TNF; IKBKG; RELB; MAP3K7; JAK1; CHUK; STAT3; NFKB1; JUN; IL1R1; IL6 |
| VDR/RXR Activation | PRKCE; EP300; PRKCD; RXRA; GADD45A; HES1; NCOR2; SP1; PRKCI; CDKN1B; PRKD; PRKCD; RUNX2; KLF4; YY1; NCOA3; CDKN1A; NCOA2; SPP1; LRP5; CEBPB; FOXO1; PRKCA |
| TGF-beta Signaling | EP300; SMAD2; SMURF1; MAPK1; SMAD3; SMAD1; FOS; MAPK8; MAPK3; KRAS; MAPK9; RUNX2; SERPINE1; RAF1; MAP3K7; CREBBP; MAP2K2; MAP2K1; TGFBR1; SMAD4; JUN; SMAD5 |
| Toll-like Receptor Signaling | IRAK1; EIF2AK2; MYD88; TRAF6; PPARA; ELK1; IKBKB; FOS; NFKB2; MAP3K14; MAPK8; MAPK13; RELA; TLR4; MAPK14; IKBKG; RELB; MAP3K7; CHUK; NFKB1; TLR2; JUN |
| p38 MAPK Signaling | HSPB1; IRAK1; TRAF6; MAPKAPK2; ELK1; FADD; FAS; CREB1; DDIT3; RPS6KA4; DAXX; MAPK13; TRAF2; MAPK14; TNF; MAP3K7; TGFBR1; MYC; ATF4; IL1R1; SRF; STAT1 |
| Neurotrophin/TRK Signaling | NTRK2; MAPK1; PTPN11; PIK3CA; CREB1; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; KRAS; PIK3C2A; RAF1; MAP2K2; AKT1; PIK3R1; PDPK1; MAP2K1; CDC42; JUN; ATF4 |
| FXR/RXR Activation | INS; PPARA; FASN; RXRA; AKT2; SDC1; MAPK8; APOB; MAPK10; PPARG; MTPP; MAPK9; PPARGC1A; TNF; CREBBP; AKT1; SREBF1; FGFR4; AKT3; FOXO1 |
| Synaptic Long Term Potentiation | PRKCE; RAP1A; EP300; PRKCD; MAPK1; CREB1; PRKCI; GNAQ; CAMK2A; PRKD1; MAPK3; KRAS; PRKCD; PPP1CC; RAF1; CREBBP; MAP2K2; MAP2K1; ATF4; PRKCA |
| Calcium Signaling | RAP1A; EP300; HDAC4; MAPK1; HDAC5; CREB1; CAMK2A; MYH9; MAPK3; HDAC2; HDAC7A; HDAC11; HDAC9; HDAC3; CREBBP; CALR; CAMKK2; ATF4; HDAC6 |
| EGF Signaling | ELK1; MAPK1; EGFR; PIK3CA; FOS; PIK3CB; PIK3C3; MAPK8; MAPK3; PIK3C2A; RAF1; JAK1; PIK3R1; STAT3; MAP2K1; JUN; PRKCA; SRF; STAT1 |
| Hypoxia Signaling in the Cardiovascular System | EDN1; PTEN; EP300; NQO1; UBE21; CREB1; ARNT; HIF1A; SLC2A4; NOS3; TP53; LDHA; AKT1; ATM; VEGFA; JUN; ATF4; VHL; HSP90AA1 |
| LPS/IL-1 Mediated Inhibition of RXR Function | IRAK1; MYD88; TRAF6; PPARA; RXRA; ABCA1; MAPK8; ALDH1A1; GSTP1; MAPK9; ABCB1; TRAF2; TLR4; TNF; MAP3K7; NR1H2; SREBF1; JUN; IL1R1 |
| LXR/RXR Activation | FASN; RXRA; NCOR2; ABCA1; NFKB2; IRF3; RELA; NOS2A; TLR4; TNF; RELB; LDLR; NR1H2; NFKB1; SREBF1; IL1R1; CCL2; IL6; MMP9 |
| Amyloid Processing | PRKCE; CSNK1E; MAPK1; CAPNS1; AKT2; CAPN2; CAPN1; MAPK3; MAPK13; MAPT; MAPK14; AKT1; PSEN1; CSNK1A1; GSK3B; AKT3; APP |
| IL-4 Signaling | AKT2; PIK3CA; PIK3CE3; PIK3C3; IRS1; KRAS; SOCS1; PTPN6; NR3C1; PIK3C2A; JAK1; AKT1; JAK2; PIK3R1; FRAP1; AKT3; RPS6KB1 |
| Cell Cycle: G2/M DNA Damage Checkpoint Regulation | EP300; PCAF; BRCA1; GADD45A; PLK1; BTRC; CHEK1; ATR; CHEK2; YWHAZ; TP53; CDKN1A; PRKDC; ATM; SFN; CDKN2A |
| Nitric Oxide Signaling in the Cardiovascular System | KDR; FLT1; PGF; AKT2; PIK3CA; PIK3CB; PIK3C3; CAV1; PRKCD; NOS3; PIK3C2A; AKT1; PIK3R1; VEGFA; AKT3; HSP90AA1 |
| Purine Metabolism | NME2; SMARCA4; MYH9; RRM2; ADAR; EIF2AK4; PKM2; ENTPD1; RAD51; RRM2B; TJP2; RAD51C; NT5E; POLD1; NME1 |
| cAMP-mediated Signaling | RAP1A; MAPK1; GNAS; CREB1; CAMK2A; MAPK3; SRC; RAF1; MAP2K2; STAT3; MAP2K1; BRAF; ATF4 |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|---|--|
| Mitochondrial Dysfunction | SOD2; MAPK8; CASP8; MAPK10; MAPK9; CASP9; PARK7; PSEN1; PARK2; APP; CASP3 |
| Notch Signaling | HES1; JAG1; NUMB; NOTCH4; ADAM17; NOTCH2; PSEN1; NOTCH3; NOTCH 1; DLL4 |
| Endoplasmic Reticulum Stress Pathway | HSPA5; MAPK8; XBP1; TRAF2; ATF6; CASP9; ATF4; EIF2AK3; CASP3 |
| Pyrimidine Metabolism | NME2; AICDA; RRM2; EIF2AK4; ENTPD1; RRM2B; NT5E; POLD1; NME1 |
| Parkinson's Signaling | UCHL1; MAPK8; MAPK13; MAPK14; CASP9; PARK7; PARK2; CASP3 |
| Cardiac & Beta Adrenergic Signaling | GNAS; GNAQ; PPP2R1A; GNB2L1; PPP2CA; PPP1CC; PPP2R5C |
| Glycolysis/Gluconeogenesis | HK2; GCK; GPI; ALDH1A1; PKM2; LDHA; HK1 |
| Interferon Signaling | IRF1; SOCS1; JAK1; JAK2; IFITM1; STAT1; IFIT3 |
| Sonic Hedgehog Signaling | ARRB2; SMO; GLI2; DYRK1A; GLI1; GSK3B; DYRK1B |
| Glycerophospholipid Metabolism | PLD1; GRN; GPAM; YWHAZ; SPHK1; SPHK2 |
| Phospholipid Degradation | PRDX6; PLD1; GRN; YWHAZ; SPHK1; SPHK2 |
| Tryptophan Metabolism | SIAH2; PRMT5; NEDD4; ALDH1A1; CYP1B1; SIAH1 |
| Lysine Degradation | SUV39H1; EHMT2; NSD1; SETD7; PPP2R5C |
| Nucleotide Excision Repair Pathway | ERCC5; ERCC4; XPA; XPC; ERCC1 |
| Starch and Sucrose Metabolism | UCHL1; HK2; GCK; GPI; HK1 |
| Aminosugars Metabolism | NQO1; HK2; GCK; HK1 |
| Arachidonic Acid Metabolism | PRDX6; GRN; YWHAZ; CYP1B1 |
| Circadian Rhythm Signaling | CSNK1E; CREB1; ATF4; NR1D1 |
| Coagulation System | BDKRB1; F2R; SERPINE1; F3 |
| Dopamine Receptor Signaling | PPP2R1A; PPP2CA; PPP1CC; PPP2R5C |
| Glutathione Metabolism | IDH2; GSTP1; ANPEP; IDH1 |
| Glycerolipid Metabolism | ALDH1A1; GPAM; SPHK1; SPHK2 |
| Linoleic Acid Metabolism | PRDX6; GRN; YWHAZ; CYP1B1 |
| Methionine Metabolism | DNMT1; DNMT3B; AHCY; DNMT3A |
| Pyruvate Metabolism | GLO1; ALDH1A1; PKM2; LDHA |
| Arginine and Proline Metabolism | ALDH1A1; NOS3; NOS2A |
| Eicosanoid Signaling | PRDX6; GRN; YWHAZ |
| Fructose and Mannose Metabolism | HK2; GCK; HK1 |
| Galactose Metabolism | HK2; GCK; HK1 |
| Stilbene, Coumarin and Lignin Biosynthesis | PRDX6; PRDX1; TYR |
| Antigen Presentation Pathway | CALR; B2M |
| Biosynthesis of Steroids | NQO1; DHCR7 |
| Butanoate Metabolism | ALDH1A1; NLGN1 |
| Citrate Cycle | IDH2; IDH1 |
| Fatty Acid Metabolism | ALDH1A1; CYP1B1 |
| Glycerophospholipid Metabolism | PRDX6; CHKA |
| Histidine Metabolism | PRMT5; ALDH1A1 |
| Inositol Metabolism | ERO1L; APEX1 |
| Metabolism of Xenobiotics | GSTP1; CYP1B1 |
| by Cytochrome p450 | |
| Methane Metabolism | PRDX6; PRDX1 |
| Phenylalanine Metabolism | PRDX6; PRDX1 |
| Propanoate Metabolism | ALDH1A1; LDHA |
| Selenoamino Acid Metabolism | PRMT5; AHCY |
| Sphingolipid Metabolism. | SPHK1; SPHK2 |
| Aminophosphonate Metabolism | PRMT5 |
| Androgen and Estrogen Metabolism | PRMT5 |

TABLE C-continued

| CELLULAR FUNCTION | GENES |
|---|---|
| Ascorbate and Aldarate Metabolism | ALDH1A1 |
| Bile Acid Biosynthesis | ALDH1A1 |
| Cysteine Metabolism | LDHA |
| Fatty Acid Biosynthesis | FASN |
| Glutamate Receptor Signaling | GNB2L1 |
| NRF2-mediated Oxidative Stress Response | PRDX1 |
| Pentose Phosphate Pathway | GPI |
| Pentose and Glucuronate interconversions | UCHL1 |
| Retinol Metabolism | ALDH1A1 |
| Riboflavin Metabolism | TYR |
| Tyrosine Metabolism | PRMT5, TYR |
| Ubiquinone Biosynthesis | PRMT5 |
| Valine, Leucine and Isoleucine Degradation | ALDH1A1 |
| Glycine, Serine and Threonine Metabolism | CHKA |
| Lysine Degradation | ALDH1A1 |
| Pain/Taste | TRPM5; TRPA1 |
| Pain | TRPM7; TRPC5; TRPC6; TRPC1; Cnr1; cnr2; Grk2; Trpa1; Pomc; Cgrp; Crf; Pka; Era; Nr2b; TRPM5; Prkaca; Prkacb; Prkar1a; Prkar2a |
| Mitochondrial Function | AIF; CytC; SMAC (Diablo); Aifm-1; Aifm-2 |
| Developmental | BMP-4; Chordin (Chrd); Noggin (Nog); WNT (WM2; Wnt2b; Wnt3a; Wnt4; Wnt5a; Wnt6; Wnt7b; Wnt8b; Wnt9a; Wnt9b; Wnt10a; Wnt10b; Wnt16); beta-catenin; Dkk-1; Frizzled related proteins; Otx-2; Gbx2; FGF-8; Reelin; Dab1; unc-86 (Pou4f1 or Bm3a); Numb; Reln |
| Neurology | |

[0107] Embodiments of the invention also relate to methods and compositions related to knocking out genes, amplifying genes and repairing particular mutations associated with DNA repeat instability and neurological disorders (Robert D. Wells, Tetsuo Ashizawa, Genetic Instabilities and Neurological Diseases, Second Edition, Academic Press, Oct. 13, 2011—Medical). Specific aspects of tandem repeat sequences have been found to be responsible for more than twenty human diseases (New insights into repeat instability: role of RNA.DNA hybrids. McIvor E I, Polak U, Napierala M. RNA Biol. 2010 September-October; 7(5):551-8). The CRISPR-Cas system may be harnessed to correct these defects of genomic instability.

[0108] A further aspect of the invention relates to utilizing the CRISPR-Cas system for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease. Lafora disease is an autosomal recessive condition which is characterized by progressive myoclonus epilepsy which may start as epileptic seizures in adolescence. A few cases of the disease may be caused by mutations in genes yet to be identified. The disease causes seizures, muscle spasms, difficulty walking, dementia, and eventually death. There is currently no therapy that has proven effective against disease progression. Other genetic abnormalities associated with epilepsy may also be targeted by the CRISPR-Cas system and the underlying genetics is further described in Genetics of Epilepsy and Genetic Epilepsies, edited by Giuliano Avanzini, Jeffrey L. Noebels, Mariani Foundation Paediatric Neurology; 20; 2009).

[0109] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[0110] Several further aspects of the invention relate to correcting defects associated with a wide range of genetic diseases which are further described on the website of the National Institutes of Health under the topic subsection Genetic Disorders (website at health.nih.gov/topic/GeneticDisorders). The genetic brain diseases may include but are not limited to Adrenoleukodystrophy, Agenesis of the Corpus Callosum, Aicardi Syndrome, Alpers' Disease, Alzheimer's Disease, Barth Syndrome, Batten Disease, CADASIL, Cerebellar Degeneration, Fabry's Disease, Gerstmann-Straussler-Scheinker Disease, Huntington's Disease and other Triplet Repeat Disorders, Leigh's Disease, Lesch-Nyhan Syndrome, Menkes Disease, Mitochondrial Myopathies and NINDS Colpocephaly. These diseases are further described on the website of the National Institutes of Health under the subsection Genetic Brain Disorders.

[0111] In some embodiments, the condition may be neoplasia. In some embodiments, where the condition is neoplasia, the genes to be targeted are any of those listed in Table A (in this case PTEN as well as so forth). In some embodiments, the condition may be Age-related Macular Degeneration. In some embodiments, the condition may be a Schizophrenic Disorder. In some embodiments, the condition may be a Trinucleotide Repeat Disorder. In some embodiments, the condition may be Fragile X Syndrome. In some embodiments, the condition may be a Secretase

Related Disorder. In some embodiments, the condition may be a Prion—related disorder. In some embodiments, the condition may be ALS. In some embodiments, the condition may be a drug addiction. In some embodiments, the condition may be Autism. In some embodiments, the condition may be Alzheimer's Disease. In some embodiments, the condition may be inflammation. In some embodiments, the condition may be Parkinson's Disease.

[0112] Examples of proteins associated with Parkinson's disease include but are not limited to α -synuclein, DJ-1, LRRK2, PINK1, Parkin, UCHL1, Synphilin-1, and NURR1.

[0113] Examples of addiction-related proteins may include ABAT for example.

[0114] Examples of inflammation-related proteins may include the monocyte chemoattractant protein-1 (MCP1) encoded by the Ccr2 gene, the C—C chemokine receptor type 5 (CCR5) encoded by the Ccr5 gene, the IgG receptor IIB (FCGR2b, also termed CD32) encoded by the Fcgr2b gene, or the Fe epsilon R1g (FCER1g) protein encoded by the Fcer1g gene, for example.

[0115] Examples of cardiovascular diseases associated proteins may include IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin 12 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), or CTSK (cathepsin K), for example.

[0116] Examples of Alzheimer's disease associated proteins may include the very low density lipoprotein receptor protein (VLDLR) encoded by the VLDLR gene, the ubiquitin-like modifier activating enzyme 1 (UBA1) encoded by the UBA1 gene, or the NEDD8-activating enzyme E1 catalytic subunit protein (UBE1C) encoded by the UBA3 gene, for example.

[0117] Examples of proteins associated Autism Spectrum Disorder may include the benzodiazepine receptor (peripheral) associated protein 1 (BZRAP1) encoded by the BZRAP1 gene, the AF4/FMR2 family member 2 protein (AFF2) encoded by the AFF2 gene (also termed MFR2), the fragile X mental retardation autosomal homolog 1 protein (FXR1) encoded by the FXR1 gene, or the fragile X mental retardation autosomal homolog 2 protein (FXR2) encoded by the FXR2 gene, for example.

[0118] Examples of proteins associated Macular Degeneration may include the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, or the chemokine (C—C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, for example.

[0119] Examples of proteins associated Schizophrenia may include NRG1, ErbB4, CPLX1, TPH1, TPH2, NRXN1, GSK3A, BDNF, DISC1, GSK3B, and combinations thereof.

[0120] Examples of proteins involved in tumor suppression may include ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3 related), EGFR (epidermal growth factor receptor), ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2), ERBB3 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 3), ERBB4 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 4), Notch 1, Notch 2, Notch 3, or Notch 4, for example.

[0121] Examples of proteins associated with a secretase disorder may include PSENEN (presenilin enhancer 2 homolog (*C. elegans*)), CTSB (cathepsin B), PSEN1 (presenilin 1), APP (amyloid beta (A4) precursor protein),

APH1B (anterior pharynx defective 1 homolog B (*C. elegans*)), PSEN2 (presenilin 2 (Alzheimer disease 4)), or BACE1 (beta-site APP-cleaving enzyme 1), for example.

[0122] Examples of proteins associated with Amyotrophic Lateral Sclerosis may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0123] Examples of proteins associated with prion diseases may include SOD1 (superoxide dismutase 1), ALS2 (amyotrophic lateral sclerosis 2), FUS (fused in sarcoma), TARDBP (TAR DNA binding protein), VAGFA (vascular endothelial growth factor A), VAGFB (vascular endothelial growth factor B), and VAGFC (vascular endothelial growth factor C), and any combination thereof.

[0124] Examples of proteins related to neurodegenerative conditions in prion disorders may include A2M (Alpha-2-Macroglobulin), AATF (Apoptosis antagonizing transcription factor), ACPP (Acid phosphatase prostate), ACTA2 (Actin alpha 2 smooth muscle aorta), ADAM22 (ADAM metalloproteinase domain), ADORA3 (Adenosine A3 receptor), or ADRA1D (Alpha-1D adrenergic receptor for Alpha-1D adrenoreceptor), for example.

[0125] Examples of proteins associated with Immunodeficiency may include A2M [alpha-2-macroglobulin]; AANAT [arylalkylamine N-acetyltransferase]; ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1]; ABCA2 [ATP-binding cassette, sub-family A (ABC1), member 2]; or ABCA3 [ATP-binding cassette, sub-family A (ABC1), member 3]; for example.

[0126] Examples of proteins associated with Trinucleotide Repeat Disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), or DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), for example.

[0127] Examples of proteins associated with Neurotransmission Disorders include SST (somatostatin), NOS1 (nitric oxide synthase 1 (neuronal)), ADRA2A (adrenergic, alpha-2A-, receptor), ADRA2C (adrenergic, alpha-2C-, receptor), TACR1 (tachykinin receptor 1), or HTR2c (5-hydroxytryptamine (serotonin) receptor 2C), for example.

[0128] Examples of neurodevelopmental-associated sequences include A2BP1 [ataxin 2-binding protein 1], AADAT [amino acid transferase], AANAT [arylalkylamine N-acetyltransferase], ABAT [4-aminobutyrate aminotransferase], ABCA1 [ATP-binding cassette, sub-family A (ABC1), member 1], or ABCA13 [ATP-binding cassette, sub-family A (ABC1), member 13], for example.

[0129] Further examples of preferred conditions treatable with the present system include may be selected from: Aicardi-Goutières Syndrome; Alexander Disease; Allan-Herndon-Dudley Syndrome; POLG-Related Disorders; Alpha-Mannosidosis (Type II and III); Alström Syndrome; Angelman Syndrome; Ataxia-Telangiectasia; Neuronal Ceroid-Lipofuscinoses; Beta-Thalassemia; Bilateral Optic Atrophy and (Infantile) Optic Atrophy Type 1; Retinoblastoma (bilateral); Canavan Disease; Cerebrooculofacioskeletal Syndrome 1 [COFS1]; Cerebrotendinous Xanthomatosis; Cornelia de Lange Syndrome; MAPT-Related Disorders; Genetic Prion Diseases; Dravet Syndrome; Early-Onset Familial Alzheimer Disease; Friedreich Ataxia [FRDA]; Fryns Syndrome; Fucosidosis; Fukuyama Con-

genital Muscular Dystrophy; Galactosialidosis; Gaucher Disease; Organic Acidemias; Hemophagocytic Lymphohistiocytosis; Hutchinson-Gilford Progeria Syndrome; Mucopolidosis II; Infantile Free Sialic Acid Storage Disease; PLA2G6-Associated Neurodegeneration; Jervell and Lange-Nielsen Syndrome; Junctional Epidermolysis Bullosa; Huntington Disease; Krabbe Disease (Infantile); Mitochondrial DNA-Associated Leigh Syndrome and NARP; Lesch-Nyhan Syndrome; LIS1-Associated Lissencephaly; Lowe Syndrome; Maple Syrup Urine Disease; MECP2 Duplication Syndrome; ATP7A-Related Copper Transport Disorders; LAMA2-Related Muscular Dystrophy; Arylsulfatase A Deficiency; Mucopolysaccharidosis Types I, II or III; Peroxisome Biogenesis Disorders, Zellweger Syndrome Spectrum; Neurodegeneration with Brain Iron Accumulation Disorders; Acid Sphingomyelinase Deficiency; Niemann-Pick Disease Type C; Glycine Encephalopathy; ARX-Related Disorders; Urea Cycle Disorders; COL1A1/2-Related Osteogenesis Imperfecta; Mitochondrial DNA Deletion Syndromes; PLP1-Related Disorders; Perry Syndrome; Phelan-McDermid Syndrome; Glycogen Storage Disease Type II (Pompe Disease) (Infantile); MAPT-Related Disorders; MECP2-Related Disorders; Rhizomelic Chondrodysplasia Punctata Type 1; Roberts Syndrome; Sandhoff Disease; Schindler Disease—Type 1; Adenosine Deaminase Deficiency; Smith-Lemli-Opitz Syndrome; Spinal Muscular Atrophy, Infantile-Onset Spinocerebellar Ataxia; Hexosaminidase A Deficiency; Thanatophoric Dysplasia Type 1; Collagen Type VI-Related Disorders; Usher Syndrome Type I; Congenital Muscular Dystrophy; Wolf-Hirschhorn Syndrome; Lysosomal Acid Lipase Deficiency; and Xeroderma Pigmentosum.

[0130] As will be apparent, it is envisaged that the present system can be used to target any polynucleotide sequence of interest. Some examples of conditions or diseases that might be usefully treated using the present system are included in the Tables above and examples of genes currently associated with those conditions are also provided there. However, the genes exemplified are not exhaustive.

EXAMPLES

[0131] The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1: CRISPR Complex Activity in the Nucleus of a Eukaryotic Cell

[0132] An example type II CRISPR system is the type II CRISPR locus from *Streptococcus pyogenes* SF370, which contains a cluster of four genes Cas9, Cas1, Cas2, and Csn1, as well as two non-coding RNA elements, tracrRNA and a characteristic array of repetitive sequences (direct repeats) interspaced by short stretches of non-repetitive sequences (spacers, about 30 bp each). In this system, targeted DNA double-strand break (DSB) is generated in four sequential steps (FIG. 2A). First, two non-coding RNAs, the pre-crRNA array and tracrRNA, are transcribed from the

CRISPR locus. Second, tracrRNA hybridizes to the direct repeats of pre-crRNA, which is then processed into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the DNA target consisting of the protospacer and the corresponding PAM via heteroduplex formation between the spacer region of the crRNA and the protospacer DNA. Finally, Cas9 mediates cleavage of target DNA upstream of PAM to create a DSB within the protospacer (FIG. 2A). This example describes an example process for adapting this RNA-programmable nuclease system to direct CRISPR complex activity in the nuclei of eukaryotic cells.

[0133] Cell Culture and Transfection

[0134] Human embryonic kidney (HEK) cell line HEK 293FT (Life Technologies) was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37° C. with 5% CO₂ incubation. Mouse neuro2A (N2A) cell line (ATCC) was maintained with DMEM supplemented with 5% fetal bovine serum (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37° C. with 5% CO₂.

[0135] HEK 293FT or N2A cells were seeded into 24-well plates (Corning) one day prior to transfection at a density of 200,000 cells per well. Cells were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's recommended protocol. For each well of a 24-well plate a total of 800 ng of plasmids were used.

[0136] Surveyor Assay and Sequencing Analysis for Genome Modification

[0137] HEK 293FT or N2A cells were transfected with plasmid DNA as described above. After transfection, the cells were incubated at 37° C. for 72 hours before genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA extraction kit (Epicentre) following the manufacturer's protocol. Briefly, cells were resuspended in QuickExtract solution and incubated at 65° C. for 15 minutes and 98° C. for 10 minutes. Extracted genomic DNA was immediately processed or stored at -20° C.

[0138] The genomic region surrounding a CRISPR target site for each gene was PCR amplified, and products were purified using QiaQuick Spin Column (Qiagen) following manufacturer's protocol. A total of 400 ng of the purified PCR products were mixed with 2 µl 10× Taq polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 20 µl, and subjected to a re-annealing process to enable heteroduplex formation: 95° C. for 10 min, 95° C. to 85° C. ramping at -2° C./s, 85° C. to 25° C. at -0.25° C./s, and 25° C. hold for 1 minute. After re-annealing, products were treated with Surveyor nuclease and Surveyor enhancer S (Transgenomics) following the manufacturer's recommended protocol, and analyzed on 4-20% Novex TBE polyacrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technologies) for 30 minutes and imaged with a Gel Doc gel imaging system (Bio-rad). Quantification was based on relative band intensities, as a measure of the fraction of cleaved DNA. FIG. 7 provides a schematic illustration of this Surveyor assay.

[0139] Restriction fragment length polymorphism assay for detection of homologous recombination.

[0140] HEK 293FT and N2A cells were transfected with plasmid DNA, and incubated at 37° C. for 72 hours before genomic DNA extraction as described above. The target

genomic region was PCR amplified using primers outside the homology arms of the homologous recombination (HR) template. PCR products were separated on a 1% agarose gel and extracted with MinElute GelExtraction Kit (Qiagen). Purified products were digested with HindIII (Fermentas) and analyzed on a 6% Novex TBE poly-acrylamide gel (Life Technologies).

[0141] RNA Secondary Structure Prediction and Analysis

[0142] RNA secondary structure prediction was performed using the online webserver RNAfold developed at Institute for Theoretical Chemistry at the University of Vienna, using the centroid structure prediction algorithm (see e.g. A. R. Gruber et al., 2008, Cell 106(1): 23-24; and P A Carr and G M Church, 2009, Nature Biotechnology 27(12): 1151-62).

[0143] RNA Purification

[0144] HEK 293FT cells were maintained and transfected as stated above. Cells were harvested by trypsinization followed by washing in phosphate buffered saline (PBS). Total cell RNA was extracted with TRI reagent (Sigma) following manufacturer's protocol. Extracted total RNA was quantified using Nanodrop (Thermo Scientific) and normalized to same concentration.

[0145] Northern Blot Analysis of crRNA and tracrRNA Expression in Mammalian Cells

[0146] RNAs were mixed with equal volumes of 2× loading buffer (Ambion), heated to 95° C. for 5 min, chilled on ice for 1 min, and then loaded onto 8% denaturing polyacrylamide gels (SequaGel, National Diagnostics) after pre-running the gel for at least 30 minutes. The samples were electrophoresed for 1.5 hours at 40 W limit. Afterwards, the RNA was transferred to Hybond N+ membrane (GE Healthcare) at 300 mA in a semi-dry transfer apparatus (Bio-rad) at room temperature for 1.5 hours. The RNA was crosslinked to the membrane using autocrosslink button on Stratagene UV Crosslinker the Stratalinker (Stratagene). The membrane was pre-hybridized in ULTRAhyb-Oligo Hybridization Buffer (Ambion) for 30 min with rotation at 42° C., and probes were then added and hybridized overnight. Probes were ordered from IDT and labeled with [γ -³²P] ATP (Perkin Elmer) with T4 polynucleotide kinase (New England Biolabs). The membrane was washed once with pre-warmed (42° C.) 2×SSC, 0.5% SDS for 1 min followed by two 30 minute washes at 42° C. The membrane was exposed to a phosphor screen for one hour or overnight at room temperature and then scanned with a phosphorimager (Typhoon).

[0147] Bacterial CRISPR System Construction and Evaluation

[0148] CRISPR locus elements, including tracrRNA, Cas9, and leader were PCR amplified from *Streptococcus pyogenes* SF370 genomic DNA with flanking homology arms for Gibson Assembly. Two BsaI type IIS sites were introduced in between two direct repeats to facilitate easy insertion of spacers (FIG. 8). PCR products were cloned into EcoRV-digested pACYC184 downstream of the tet promoter using Gibson Assembly Master Mix (NEB). Other endogenous CRISPR system elements were omitted, with the exception of the last 50 bp of Csn2. Oligos (Integrated DNA Technology) encoding spacers with complimentary overhangs were cloned into the BsaI-digested vector pDC000 (NEB) and then ligated with T7 ligase (Enzymatics) to generate pCRISPR plasmids. Challenge plasmids containing spacers with PAM

[0149] expression in mammalian cells (expression constructs illustrated in FIG. 6A, with functionality as determined by results of the Surveyor assay shown in FIG. 6B). Transcription start sites are marked as +1, and transcription terminator and the sequence probed by northern blot are also indicated. Expression of processed tracrRNA was also confirmed by Northern blot. FIG. 6C shows results of a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying long or short tracrRNA, as well as SpCas9 and DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III, respectively. U6 indicate loading control blotted with a probe targeting human U6 snRNA. Transfection of the short tracrRNA expression construct led to abundant levels of the processed form of tracrRNA (~75 bp). Very low amounts of long tracrRNA are detected on the Northern blot.

[0150] To promote precise transcriptional initiation, the RNA polymerase III-based U6 promoter was selected to drive the expression of tracrRNA (FIG. 2C). Similarly, a U6 promoter-based construct was developed to express a pre-crRNA array consisting of a single spacer flanked by two direct repeats (DRs, also encompassed by the term "tracr-mate sequences"; FIG. 2C). The initial spacer was designed to target a 33-base-pair (bp) target site (30-bp protospacer plus a 3-bp CRISPR motif (PAM) sequence satisfying the NGG recognition motif of Cas9) in the human EMX1 locus (FIG. 2C), a key gene in the development of the cerebral cortex.

[0151] To test whether heterologous expression of the CRISPR system (SpCas9, SpRNase III, tracrRNA, and pre-crRNA) in mammalian cells can achieve targeted cleavage of mammalian chromosomes, HEK 293FT cells were transfected with combinations of CRISPR components. Since DSBs in mammalian nuclei are partially repaired by the non-homologous end joining (NHEJ) pathway, which leads to the formation of indels, the Surveyor assay was used to detect potential cleavage activity at the target EMX1 locus (FIG. 7) (see e.g. Guschin et al., 2010, Methods Mol Biol 649: 247). Co-transfection of all four CRISPR components was able to induce up to 5.0% cleavage in the protospacer (see FIG. 2D). Co-transfection of all CRISPR components minus SpRNase III also induced up to 4.7% indel in the protospacer, suggesting that there may be endogenous mammalian RNases that are capable of assisting with crRNA maturation, such as for example the related Dicer and Drosha enzymes. Removing any of the remaining three components abolished the genome cleavage activity of the CRISPR system (FIG. 2D). Sanger sequencing of amplicons containing the target locus verified the cleavage activity: in 43 sequenced clones, 5 mutated alleles (11.6%) were found. Similar experiments using a variety of guide sequences produced indel percentages as high as 29% (see FIGS. 3-6, 10, and 11). These results define a three-component system for efficient CRISPR-mediated genome modification in mammalian cells. To optimize the cleavage efficiency, Applicants also tested whether different isoforms of tracrRNA affected the cleavage efficiency and found that, in this example system, only the short (89-bp) transcript form was able to mediate cleavage of the human EMX1 genomic locus (FIG. 6B).

[0152] FIG. 12 provides an additional Northern blot analysis of crRNA processing in mammalian cells. FIG. 12A illustrates a schematic showing the expression vector for a

single spacer flanked by two direct repeats (DR-EMX1(1)-DR). The 30 bp spacer targeting the human EMX1 locus protospacer 1 (see FIG. 6) and the direct repeat sequences are shown in the sequence beneath FIG. 12A. The line indicates the region whose reverse-complement sequence was used to generate Northern blot probes for EMX1(1) crRNA detection. FIG. 12B shows a Northern blot analysis of total RNA extracted from 293FT cells transfected with U6 expression constructs carrying DR-EMX1(1)-DR. Left and right panels are from 293FT cells transfected without or with SpRNase III respectively. DR-EMX1(1)-DR was processed into mature crRNAs only in the presence of SpCas9 and short tracrRNA and was not dependent on the presence of SpRNase III. The mature crRNA detected from transfected 293FT total RNA is ~33 bp and is shorter than the 39-42 bp mature crRNA from *S. pyogenes*. These results demonstrate that a CRISPR system can be transplanted into eukaryotic cells and reprogrammed to facilitate cleavage of endogenous mammalian target polynucleotides.

[0153] FIG. 2 illustrates the bacterial CRISPR system described in this example. FIG. 2A illustrates a schematic showing the CRISPR locus 1 from *Streptococcus pyogenes* SF370 and a proposed mechanism of CRISPR-mediated DNA cleavage by this system. Mature crRNA processed from the direct repeat-spacer array directs Cas9 to genomic targets consisting of complementary protospacers and a protospacer-adjacent motif (PAM). Upon target-spacer base pairing, Cas9 mediates a double-strand break in the target DNA. FIG. 2B illustrates engineering of *S. pyogenes* Cas9 (SpCas9) and RNase III (SpRNase III) with nuclear localization signals (NLSs) to enable import into the mammalian nucleus. FIG. 2C illustrates mammalian expression of SpCas9 and SpRNase III driven by the constitutive EF1a promoter and tracrRNA and pre-crRNA array (DR-Spacer-DR) driven by the RNA Pol3 promoter U6 to promote precise transcription initiation and termination. A protospacer from the human EMX1 locus with a satisfactory PAM sequence is used as the spacer in the pre-crRNA array. FIG. 2D illustrates surveyor nuclease assay for SpCas9-mediated minor insertions and deletions. SpCas9 was expressed with and without SpRNase III, tracrRNA, and a pre-crRNA array carrying the EMX1-target spacer. FIG. 2E illustrates a schematic representation of base pairing between target locus and EMX1-targeting crRNA, as well as an example chromatogram showing a micro deletion adjacent to the SpCas9 cleavage site. FIG. 2F illustrates mutated alleles identified from sequencing analysis of 43 clonal amplicons showing a variety of micro insertions and deletions. Dashes indicate deleted bases, and non-aligned or mismatched bases indicate insertions or mutations. Scale bar=10 μ m.

[0154] To further simplify the three-component system, a chimeric crRNA-tracrRNA hybrid design was adapted, where a mature crRNA (comprising a guide sequence) may be fused to a partial tracrRNA via a stem-loop to mimic the natural crRNA:tracrRNA duplex. To increase co-delivery efficiency, a bicistronic expression vector was created to drive co-expression of a chimeric RNA and SpCas9 in transfected cells. In parallel, the bicistronic vectors were used to express a pre-crRNA (DR-guide sequence-DR) with SpCas9, to induce processing into crRNA with a separately expressed tracrRNA (compare FIG. 11B top and bottom). FIG. 8 provides schematic illustrations of bicistronic expression vectors for pre-crRNA array (FIG. 8A) or chimeric crRNA (represented by the short line downstream of the

guide sequence insertion site and upstream of the EF1a promoter in FIG. 8B) with hSpCas9, showing location of various elements and the point of guide sequence insertion. The expanded sequence around the location of the guide sequence insertion site in FIG. 8B also shows a partial DR sequence (GTTTTAGAGCTA) (SEQ ID NO: 11) and a partial tracrRNA sequence (TAGCAAGT-TAAAATAAGGCTAGTCCGTTTTT) (SEQ ID NO: 12). Guide sequences can be inserted between BbsI sites using annealed oligonucleotides. Sequence design for the oligonucleotides are shown below the schematic illustrations in FIG. 8, with appropriate ligation adapters indicated. WPRE represents the Woodchuck hepatitis virus post-transcriptional regulatory element. The efficiency of chimeric RNA-mediated cleavage was tested by targeting the same EMX1 locus described above. Using both Surveyor assay and Sanger sequencing of amplicons, Applicants confirmed that the chimeric RNA design facilitates cleavage of human EMX1 locus with approximately a 4.7% modification rate (FIG. 3).

[0155] Generalizability of CRISPR-mediated cleavage in eukaryotic cells was tested by targeting additional genomic loci in both human and mouse cells by designing chimeric RNA targeting multiple sites in the human EMX1 and PVALB, as well as the mouse Th loci. FIG. 13 illustrates the selection of some additional targeted protospacers in human PVALB (FIG. 13A) and mouse Th (FIG. 13B) loci. Schematics of the gene loci and the location of three protospacers within the last exon of each are provided. The underlined sequences include 30 bp of protospacer sequence and 3 bp at the 3' end corresponding to the PAM sequences. Protospacers on the sense and anti-sense strands are indicated above and below the DNA sequences, respectively. A modification rate of 6.3% and 0.75% was achieved for the human PV4LB and mouse Th loci respectively, demonstrating the broad applicability of the CRISPR system in modifying different loci across multiple organisms (FIG. 5). While cleavage was only detected with one out of three spacers for each locus using the chimeric constructs, all target sequences were cleaved with efficiency of indel production reaching 27% when using the co-expressed pre-crRNA arrangement (FIGS. 6 and 13).

[0156] FIG. 11 provides a further illustration that SpCas9 can be reprogrammed to target multiple genomic loci in mammalian cells. FIG. 11A provides a schematic of the human EMX1 locus showing the location of five protospacers, indicated by the underlined sequences. FIG. 11B provides a schematic of the pre-crRNA/tracrRNA complex showing hybridization between the direct repeat region of the pre-crRNA and tracrRNA (top), and a schematic of a chimeric RNA design comprising a 20 bp guide sequence, and tracr mate and tracr sequences consisting of partial direct repeat and tracrRNA sequences hybridized in a hairpin structure (bottom). Results of a Surveyor assay comparing the efficacy of Cas9-mediated cleavage at five protospacers in the human EMX1 locus is illustrated in FIG. 11C. Each protospacer is targeted using either processed pre-crRNA/tracrRNA complex (crRNA) or chimeric RNA (chiRNA).

[0157] Since the secondary structure of RNA can be crucial for intermolecular interactions, a structure prediction algorithm based on minimum free energy and Boltzmann-weighted structure ensemble was used to compare the putative secondary structure of all guide sequences used in the

genome targeting experiment (see e.g. Gruber et al., 2008, *Nucleic Acids Research*, 36: W70). Analysis revealed that in most cases, the effective guide sequences in the chimeric crRNA context were substantially free of secondary structure motifs, whereas the ineffective guide sequences were more likely to form internal secondary structures that could prevent base pairing with the target protospacer DNA. It is thus possible that variability in the spacer secondary structure might impact the efficiency of CRISPR-mediated interference when using a chimeric crRNA.

[0158] Further vector designs for SpCas9 are shown in FIG. 22, which illustrates single expression vectors incorporating a U6 promoter linked to an insertion site for a guide oligo, and a Cbh promoter linked to SpCas9 coding sequence. The vector shown in FIG. 22b includes a tracrRNA coding sequence linked to an H1 promoter.

[0159] In the bacterial assay, all spacers facilitated efficient CRISPR interference (FIG. 3C). These results suggest that there may be additional factors affecting the efficiency of CRISPR activity in mammalian cells.

[0160] To investigate the specificity of CRISPR-mediated cleavage, the effect of single-nucleotide mutations in the guide sequence on protospacer cleavage in the mammalian genome was analyzed using a series of EMX1-targeting chimeric crRNAs with single point mutations (FIG. 3A). FIG. 3B illustrates results of a Surveyor nuclease assay comparing the cleavage efficiency of Cas9 when paired with different mutant chimeric RNAs. Single-base mismatch up to 12-bp 5' of the PAM substantially abrogated genomic cleavage by SpCas9, whereas spacers with mutations at farther upstream positions retained activity against the original protospacer target (FIG. 3B). In addition to the PAM, SpCas9 has single-base specificity within the last 12-bp of the spacer. Furthermore, CRISPR is able to mediate genomic cleavage as efficiently as a pair of TALE nucleases (TALEN) targeting the same EMX1 protospacer. FIG. 3C provides a schematic showing the design of TALENs targeting EMX1, and FIG. 3D shows a Surveyor gel comparing the efficiency of TALEN and Cas9 (n=3).

[0161] Having established a set of components for achieving CRISPR-mediated gene editing in mammalian cells through the error-prone NHEJ mechanism, the ability of CRISPR to stimulate homologous recombination (HR), a high fidelity gene repair pathway for making precise edits in the genome, was tested. The wild type SpCas9 is able to mediate site-specific DSBs, which can be repaired through both NHEJ and HR. In addition, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of SpCas9 was engineered to convert the nuclease into a nickase (SpCas9n; illustrated in FIG. 4A) (see e.g. Sapranasaks et al., 2011, *Nucleic Acids Research*, 39: 9275; Gasiunas et al., 2012, *Proc. Natl. Acad. Sci. USA*, 109: E2579), such that nicked genomic DNA undergoes the high-fidelity homology-directed repair (HDR). Surveyor assay confirmed that SpCas9n does not generate indels at the EMX1 protospacer target. As illustrated in FIG. 4B, co-expression of EMX1-targeting chimeric crRNA with SpCas9 produced indels in the target site, whereas co-expression with SpCas9n did not (n=3). Moreover, sequencing of 327 amplicons did not detect any indels induced by SpCas9n. The same locus was selected to test CRISPR-mediated HR by co-transfecting HEK 293FT cells with the chimeric RNA targeting EMX1, hSpCas9 or hSpCas9n, as well as a HR template to introduce a pair of restriction sites

(HindIII and NheI) near the protospacer. FIG. 4C provides a schematic illustration of the HR strategy, with relative locations of recombination points and primer annealing sequences (arrows). SpCas9 and SpCas9n indeed catalyzed integration of the HR template into the EMX1 locus. PCR amplification of the target region followed by restriction digest with HindIII revealed cleavage products corresponding to expected fragment sizes (arrows in restriction fragment length polymorphism gel analysis shown in FIG. 4D), with SpCas9 and SpCas9n mediating similar levels of HR efficiencies. Applicants further verified HR using Sanger sequencing of genomic amplicons (FIG. 4E). These results demonstrate the utility of CRISPR for facilitating targeted gene insertion in the mammalian genome. Given the 14-bp (12-bp from the spacer and 2-bp from the PAM) target specificity of the wild type SpCas9, the availability of a nickase can significantly reduce the likelihood of off-target modifications, since single strand breaks are not substrates for the error-prone NHEJ pathway.

[0162] Expression constructs mimicking the natural architecture of CRISPR loci with arrayed spacers (FIG. 2A) were constructed to test the possibility of multiplexed sequence targeting. Using a single CRISPR array encoding a pair of EMX1- and PVALB-targeting spacers, efficient cleavage at both loci was detected (FIG. 4F, showing both a schematic design of the crRNA array and a Surveyor blot showing efficient mediation of cleavage). Targeted deletion of larger genomic regions through concurrent DSBs using spacers against two targets within EMX1 spaced by 119 bp was also tested, and a 1.6% deletion efficacy (3 out of 182 amplicons; FIG. 4G) was detected. This demonstrates that the CRISPR system can mediate multiplexed editing within a single genome.

Example 2: CRISPR System Modifications and Alternatives

[0163] The ability to use RNA to program sequence-specific DNA cleavage defines a new class of genome engineering tools for a variety of research and industrial applications. Several aspects of the CRISPR system can be further improved to increase the efficiency and versatility of CRISPR targeting. Optimal Cas9 activity may depend on the availability of free Mg²⁺ at levels higher than that present in the mammalian nucleus (see e.g. Jinek et al., 2012, *Science*, 337:816), and the preference for an NGG motif immediately downstream of the protospacer restricts the ability to target on average every 12-bp in the human genome (FIG. 9, evaluating both plus and minus strands of human chromosomal sequences). Some of these constraints can be overcome by exploring the diversity of CRISPR loci across the microbial metagenome (see e.g. Makarova et al., 2011, *Nat Rev Microbiol*, 9:467). Other CRISPR loci may be transplanted into the mammalian cellular milieu by a process similar to that described in Example 1. For example, FIG. 10 illustrates adaptation of the Type II CRISPR system from CRISPR 1 of *Streptococcus thermophilus* LMD-9 for heterologous expression in mammalian cells to achieve CRISPR-mediated genome editing. FIG. 10A provides a Schematic illustration of CRISPR 1 from *S. thermophilus* LMD-9. FIG. 10B illustrates the design of an expression system for the *S. thermophilus* CRISPR system. Human codon-optimized hStCas9 is expressed using a constitutive EF1 α promoter. Mature versions of tracrRNA and crRNA are expressed using the U6 promoter to promote precise

transcription initiation. Sequences from the mature crRNA and tracrRNA are illustrated. A single base indicated by the lower case “a” in the crRNA sequence is used to remove the polyU sequence, which serves as a RNA polIII transcriptional terminator. FIG. 10C provides a schematic showing guide sequences targeting the human EMX1 locus. FIG. 10D shows the results of hStCas9-mediated cleavage in the target locus using the Surveyor assay. RNA guide spacers 1 and 2 induced 14% and 6.4%, respectively. Statistical analysis of cleavage activity across biological replica at these two protospacer sites is also provided in FIG. 5. FIG. 14 provides a schematic of additional protospacer and corresponding PAM sequence targets of the *S. thermophilus* CRISPR system in the human EMX1 locus. Two protospacer sequences are highlighted and their corresponding PAM sequences satisfying NNAGAAW motif are indicated by underlining 3' with respect to the corresponding highlighted sequence. Both protospacers target the anti-sense strand.

Example 3: Sample Target Sequence Selection Algorithm

[0164] A software program is designed to identify candidate CRISPR target sequences on both strands of an input DNA sequence based on desired guide sequence length and a CRISPR motif sequence (PAM) for a specified CRISPR enzyme. For example, target sites for Cas9 from *S. pyogenes*, with PAM sequences NGG, may be identified by searching for 5'-N_x-NGG-3' both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR1, with PAM sequence NNAGAAW, may be identified by searching for 5'-N_x-NNAGAAW-3' both on the input sequence and on the reverse-complement of the input. Likewise, target sites for Cas9 of *S. thermophilus* CRISPR3, with PAM sequence NGGNG, may be identified by searching for 5'-N_x-NGGNG-3' both on the input sequence and on the reverse-complement of the input. The value “x” in N, may be fixed by the program or specified by the user, such as 20.

[0165] Since multiple occurrences in the genome of the DNA target site may lead to nonspecific genome editing, after identifying all potential sites, the program filters out sequences based on the number of times they appear in the relevant reference genome. For those CRISPR enzymes for which sequence specificity is determined by a ‘seed’ sequence, such as the 11-12 bp 5' from the PAM sequence, including the PAM sequence itself, the filtering step may be based on the seed sequence. Thus, to avoid editing at additional genomic loci, results are filtered based on the number of occurrences of the seed:PAM sequence in the

relevant genome. The user may be allowed to choose the length of the seed sequence. The user may also be allowed to specify the number of occurrences of the seed:PAM sequence in a genome for purposes of passing the filter. The default is to screen for unique sequences. Filtration level is altered by changing both the length of the seed sequence and the number of occurrences of the sequence in the genome. The program may in addition or alternatively provide the sequence of a guide sequence complementary to the reported target sequence(s) by providing the reverse complement of the identified target sequence(s). An example visualization of some target sites in the human genome is provided in FIG. 18.

[0166] Further details of methods and algorithms to optimize sequence selection can be found in U.S. application Ser. No. 61/064,798 (Attorney docket 44790.11.2022; Broad Reference BI-2012/084); incorporated herein by reference.

Example 4: Evaluation of Multiple Chimeric crRNA-tracrRNA Hybrids

[0167] This example describes results obtained for chimeric RNAs (chiRNAs; comprising a guide sequence, a tracr mate sequence, and a tracr sequence in a single transcript) having tracr sequences that incorporate different lengths of wild-type tracrRNA sequence. FIG. 16a illustrates a schematic of a bicistronic expression vector for chimeric RNA and Cas9. Cas9 is driven by the CBh promoter and the chimeric RNA is driven by a U6 promoter. The chimeric guide RNA consists of a 20 bp guide sequence (Ns) joined to the tracr sequence (running from the first “U” of the lower strand to the end of the transcript), which is truncated at various positions as indicated. The guide and tracr sequences are separated by the tracr-mate sequence GUUUUA-GAGCUA (SEQ ID NO: 13) followed by the loop sequence GAAA. Results of SURVEYOR assays for Cas9-mediated indels at the human EMX1 and PVALB loci are illustrated in FIGS. 16b and 16c, respectively. Arrows indicate the expected SURVEYOR fragments. ChiRNAs are indicated by their “+n” designation, and crRNA refers to a hybrid RNA where guide and tracr sequences are expressed as separate transcripts. Quantification of these results, performed in triplicate, are illustrated by histogram in FIGS. 17a and 17b, corresponding to FIGS. 16b and 16c, respectively (“N.D.” indicates no indels detected). Protospacer IDs and their corresponding genomic target, protospacer sequence, PAM sequence, and strand location are provided in Table D. Guide sequences were designed to be complementary to the entire protospacer sequence in the case of separate transcripts in the hybrid system, or only to the underlined portion in the case of chimeric RNAs.

TABLE D

| proto-spacer ID | genomic target | protospacer sequence (5' to 3') | PAM | SEQ ID NO: | strand |
|-----------------|----------------|---------------------------------|-----|------------|--------|
| 1 | EMX1 | GGACATCGATGTCACCTCCAATGACTAGGG | TGG | 14 | + |
| 2 | EMX1 | CATTGGAGGTGACATCGATGTCCTCCCAT | TGG | 15 | - |
| 3 | EMX1 | GGAAGGGCCTGAGTCCGAGCAGAAGAAGAA | GGG | 16 | + |
| 4 | PVALB | GGTGGCGAGAGGGCCGAGATTGGGTGTTT | AGG | 17 | + |
| 5 | PVALB | ATGcAGGAGGGTGGCGAGAGGGCCGAGAT | TGG | 18 | + |

[0168] Further details to optimize guide sequences can be found in U.S. application Ser. No. 61/836,127 (Attorney docket 44790.08.2022; Broad Reference B1-2013/004G); incorporated herein by reference.

[0169] Initially, three sites within the EMX1 locus in human HEK 293FT cells were targeted. Genome modification efficiency of each chiRNA was assessed using the SURVEYOR nuclease assay, which detects mutations resulting from DNA double-strand breaks (DSBs) and their subsequent repair by the non-homologous end joining (NHEJ) DNA damage repair pathway. Constructs designated chiRNA(+n) indicate that up to the +n nucleotide of wild-type tracrRNA is included in the chimeric RNA construct, with values of 48, 54, 67, and 85 used for n. Chimeric RNAs containing longer fragments of wild-type tracrRNA (chiRNA(+67) and chiRNA(+85)) mediated DNA cleavage at all three EMX1 target sites, with chiRNA(+85) in particular demonstrating significantly higher levels of DNA cleavage than the corresponding crRNA/tracrRNA hybrids that expressed guide and tracr sequences in separate transcripts (FIGS. 16b and 17a). Two sites in the PVALB locus that yielded no detectable cleavage using the hybrid system (guide sequence and tracr sequence expressed as separate transcripts) were also targeted using chiRNAs. chiRNA(+67) and chiRNA(+85) were able to mediate significant cleavage at the two PVALB protospacers (FIGS. 16c and 17b).

[0170] For all five targets in the EMX1 and PVALB loci, a consistent increase in genome modification efficiency with increasing tracr sequence length was observed. Without wishing to be bound by any theory, the secondary structure formed by the 3' end of the tracrRNA may play a role in enhancing the rate of CRISPR complex formation.

Example 5: Cas9 Diversity

[0171] The CRISPR-Cas system is an adaptive immune mechanism against invading exogenous DNA employed by diverse species across bacteria and archaea. The type II CRISPR-Cas9 system consists of a set of genes encoding proteins responsible for the “acquisition” of foreign DNA into the CRISPR locus, as well as a set of genes encoding the “execution” of the DNA cleavage mechanism; these include the DNA nuclease (Cas9), a non-coding transactivating crRNA (tracrRNA), and an array of foreign DNA-derived spacers flanked by direct repeats (crRNAs). Upon maturation by Cas9, the tracrRNA and crRNA duplex guide the Cas9 nuclease to a target DNA sequence specified by the spacer guide sequences, and mediates double-stranded breaks in the DNA near a short sequence motif in the target DNA that is required for cleavage and specific to each CRISPR-Cas system. The type II CRISPR-Cas systems are found throughout the bacterial kingdom and highly diverse in Cas9 protein sequence and size, tracrRNA and crRNA direct repeat sequence, genome organization of these elements, and the motif requirement for target cleavage. One species may have multiple distinct CRISPR-Cas systems.

[0172] Applicants evaluated 207 putative Cas9s from bacterial species identified based on sequence homology to known Cas9s and structures orthologous to known subdomains, including the HNH endonuclease domain and the RuvC endonuclease domains [information from the Eugene Koonin and Kira Makarova]. Phylogenetic analysis based on the protein sequence conservation of this set revealed five families of Cas9s, including three groups of large Cas9s

(~1400 amino acids) and two of small Cas9s (~1100 amino acids) (see FIGS. 19 and 20A-F).

[0173] Further details of Cas9s and mutations of the Cas9 enzyme to convert into a nickase or DNA binding protein and use of same with altered functionality can be found in U.S. application Ser. Nos. 61/836,101 and 61/835,936 (Attorney docket 44790.09.2022 and 4790.07.2022 and Broad Reference B1-2013/004E and B1-2013/004F respectively) incorporated herein by reference.

Example 6: Cas9 Orthologs

[0174] Applicants analyzed Cas9 orthologs to identify the relevant PAM sequences and the corresponding chimeric guide RNA. Having an expanded set of PAMs provides broader targeting across the genome and also significantly increases the number of unique target sites and provides potential for identifying novel Cas9s with increased levels of specificity in the genome.

[0175] The specificity of Cas9 orthologs can be evaluated by testing the ability of each Cas9 to tolerate mismatches between the guide RNA and its DNA target. For example, the specificity of SpCas9 has been characterized by testing the effect of mutations in the guide RNA on cleavage efficiency. Libraries of guide RNAs were made with single or multiple mismatches between the guide sequence and the target DNA. Based on these findings, target sites for SpCas9 can be selected based on the following guidelines:

[0176] To maximize SpCas9 specificity for editing a particular gene, one should choose a target site within the locus of interest such that potential ‘off-target’ genomic sequences abide by the following four constraints: First and foremost, they should not be followed by a PAM with either 5'-NGG or NAG sequences. Second, their global sequence similarity to the target sequence should be minimized. Third, a maximal number of mismatches should lie within the PAM-proximal region of the off-target site. Finally, a maximal number of mismatches should be consecutive or spaced less than four bases apart.

[0177] Similar methods can be used to evaluate the specificity of other Cas9 orthologs and to establish criteria for the selection of specific target sites within the genomes of target species. As mentioned previously phylogenetic analysis based on the protein sequence conservation of this set revealed five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids) (see FIGS. 19 and 20A-F). Further details on Cas orthologs can be found in U.S. application Ser. Nos. 61/836,101 and 61/835,936 (Attorney docket 44790.09.2022 and 4790.07.2022 and Broad Reference B1-2013/004E and B1-2013/004F respectively) incorporated herein by reference.

Example 7: Engineering of Plants (Micro-Algae) Using Cas9 to Target and Manipulate Plant Genes

[0178] Methods of Delivering Cas9

[0179] Method 1: Applicants deliver Cas9 and guide RNA using a vector that expresses Cas9 under the control of a constitutive promoter such as Hsp70A-Rbc S2 or Beta2-tubulin.

[0180] Method 2: Applicants deliver Cas9 and T7 polymerase using vectors that expresses Cas9 and T7 polymerase under the control of a constitutive promoter such as

Hsp70A-Rbc S2 or Beta2-tubulin. Guide RNA will be delivered using a vector containing T7 promoter driving the guide RNA.

[0181] Method 3: Applicants deliver Cas9 mRNA and in vitro transcribed guide RNA to algae cells. RNA can be in vitro transcribed. Cas9 mRNA will consist of the coding region for Cas9 as well as 3'UTR from Cop1 to ensure stabilization of the Cas9 mRNA.

[0182] For Homologous recombination, Applicants provide an additional homology directed repair template.

[0183] Sequence for a cassette driving the expression of Cas9 under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1.

(SEQ ID NO: 19)

TCTTTCTTGCCTATGACACTTCCAGCAAAAGGTAGGCGGGCTGCGAGA
CGGCTTCCCGCGCTGCGATGCAACACCGATGATGCTTCGACCCCCGAAG
CTCCTTCGGGGCTGCGATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTT
TTTTAAATAGCCAGGCCCCGATTGCAAGACATTATAGCGAGCTACCAA
AGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCCACTCGA
GCTTGTGATCGCACTCCGCTAAGGGGGCGCTCTTCTCTTCGTTTCAGT
CACAACCCGCAACATGTACCATACGATGTTCCAGATTACGCTTCGCCG
AAGAAAAAGCGCAAGGTGCAAGCGTCCGACAAGAAGTACAGCATCGGCCT
GGACATCGGCACCAACTCTGTGGGCGGGCGGTGATCACCAGCAGGTACA
AGGTGCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGGCACAGC
ATCAAGAAGAACCTGATCGGAGCCCTGTGTTTCGACAGCGCGAAACAGC
CGAGGCCACCCGGCTGAAGAGAACCAGCAGAAAGATACACCAGACGGA
AGAACCGGATCTGTATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAG
GTGGACGACAGCTTCTCCACAGACTGGAAGAGTCTTCTCGTGGGAAGA
GGATAAGAAGCAGCAGCGGCACCCCATCTTCGGCAACATCGTGGACGAGG
TGGCCTACCACGAGAAGTACCCACCATCTACCACTGAGAAAGAACTG
GTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGC
CCACATGATCAAGTTCCGGGGCCACTTCTGATCGAGGGCGACCTGAACC
CCGACAACAGCGACGTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTAC
AACCAGCTGTTTCGAGGAAAACCCCATCAACGCCAGCGCGTGGACGCCAA
GGCCATCTGTCTGCCAGACTGAGCAAGAGCAGACGGCTGGAAAATCTGA
TCGCCAGCTGCCCGCGAGAAGAAGATGGCTGTTCGGCAACCTGATT
GCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGC
CGAGGATGCCAAATGCAGCTGAGCAAGACACCTACGACGACGACCTGG
ACAACCTGCTGGCCAGATCGGCGACCACTACGCGACCTGTTTGTGGCC
GCCAAGAACCTGTCCGACGCCATCTGTGAGCGACATCCTGAGAGTGAA
CACCAGATCACCAAGGCCCCCTGAGCGCTCTATGATCAAGAGATACG
ACGAGCACCAAGGACCTGACCTGCTGAAAGCTCTCGTGGCGAGCAG
CTGCCTGAGAAGTACAAGAGATTTTCTTCGACCAGAGCAAGAAGCGCTA
CGCCGGCTACATTAGCGCGAGGCCAGCCAGGAAGAGTTCTACAAGTTCA

-continued

TCAAGCCCATCCTGGAAAAGATGGACGGCACCGAGGAACCTGCTCGTGAAG
CTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACCTTCGACAACGGCAG
CATCCCCCACCAGATCCACCTGGGAGAGCTGCACGCCATTCTGCGGCGGC
AGGAAGATTTTACCATTCTCTGAAGGACAACCGGGAAAAGATCGAGAAG
ATCTGACCTTCCGCTATCCCTACTACGTGGGCCCTCTGGCCAGGGGAAA
CAGCAGATTTCGCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCT
GGAACCTCGAGGAAGTGGTGGACAAGGGCGCTTCCGCCAGAGCTTCATC
GAGCGGATGACCAACTTCGATAAGAACCTGCCCAACGAGAAGGTGCTGCC
CAAGCACAGCCTGCTGTACGAGTACTTACCGTGTATAACGAGCTGACCA
AAGTGAAATACGTGACCGAGGGAATGAGAAGCCCGCTTCTGAGCGGC
GAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGT
GACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAAATCGAGTCTTCG
ACTCCGTGGAATCTCCGCGTGGAGATCGGTTCAACGCCTCCTGGGC
ACATACCAGATCTGCTGAAAATTATCAAGGACAAGGACTTCTTGACAA
TGAGGAAAACGAGGACATCTGGAAGATATCGTGTGACCTGACACTGT
TTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCCACCTG
TTCGACGACAAAGTGATGAAGCAGCTGAAGCGCGGAGATACACCGGCTG
GGGACGGCTGAGCCGGAAGCTGATCAACGCGATCCGGGACAAGCAGTCCG
GCAAGAGAATCCTGGATTCTCTGAAGTCCGACGGETTCCGCAACAGAAAC
TTCATGCAGCTGATCCACGACGACAGCCTGACCTTTAAGAGGACATCCA
GAAAGCCAGGTGTCCGGCCAGGGCGATAGCCTGCACGACACATTGCCA
ATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATCCTGCAGACAGTGAAG
GTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCGAGAACAT
CGTGTATCGAAATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGA
ACAGCCGCGAGAGAATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGC
AGCCAGATCCTGAAAGAACACCCCGTGGAAAAACCCAGCTGCAGAACGA
GAAGCTGTACCTGTACTACCTGGAGAATGGGCGGATATGTACGTGGACC
AGGAACCTGGACATCAACCGGCTGTCCGACTACGATGTGGACCATATCGTG
CCTCAGAGCTTCTGAAGGACGACTCCATCGACAACAAGGTGCTGACCAG
AAGCGACAAGAACCAGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTG
TGAAGAAGATGAAGAATACTGGCGCGAGCTGCTGAACGCCAAGCTGATT
ACCCAGAGAAAGTTCGACAATCTGACCAAGGCCGAGAGAGGCTGCCTGA
GCGAAGTGGATAAGGCCGCTTCATCAAGAGACAGCTGGTGGAAACCCGG
CAGATCACAAAGCAGTGGCAGATCCTGGACTCCCGGATGAACACTAA
GTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCTGA
AGTCCAAGCTGGTGTCCGATTTCGGAAGGATTTCCAGTTTACAAAGTG
CGCGAGATCAACAATAACACACGCCACGACGCTACCTGAACGCCGT
CGTGGGAACCGCCCTGATCAAAAAGTACCTAAGCTGGAAAGCGAGTTTCG
TGTAACGGCGACTACAAGGTGTACGACGTGCGGAAGATGATCGCCAAGAGC

-continued

GAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGCAACAT
 CATGAACTTTTTCAAGACCGAGATTACCTGGCCAACGGCGAGATCCGGA
 AGCGGCCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGAT
 AAGGGCCGGGATTTTGCCACCGTGCGGAAAGTGTGAGCATGCCCAAGT
 GAATATCGTGAAAAAGACCGAGGTGCAGACAGGCGGCTTCAGCAAAGAGT
 CTATCTGCCCAAGAGGAACAGCGATAAGCTGATCGCCAGAAAGAAGGAC
 TGGGACCCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCCTATTC
 TGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAACTGAAGA
 GTGTGAAAGAGCTGCTGGGGATCACCATCATGGAAGAAGCAGCTTCGAG
 AAGAATCCCATCGACTTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAA
 GGACCTGATCATCAAGCTGCCTAAGTACTCCCTGTTTCGAGCTGGAAAACG
 GCCGAAGAGAATGCAAGCCTCTGCCGCGCAACTGCAGAAGGGAACGAA
 CTGGCCCTGCCCTCCAATATGTGAACCTTCTGTACCTGGCCAGCCACTA
 TGAGAAGCTGAAGGGCTCCCCGAGGATAATGAGCAGAAACAGCTGTTTG
 TGGAACAGCACAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAG
 TTCTCCAAGAGAGTGATCTGGCCGACGCTAATCTGGACAAAGTGTCTGC
 CGCCTACAACAAGCACCGGGGATAAGCCCATCAGAGAGCAGGCCGAGAATA
 TCATCCACCTGTTTACCCTGACCAATCTGGGAGCCCCGCGCCTTCAAG
 TACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAGAGCT
 TGGTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACA
 CGGATCGACCTGTCTCAGCTGGGAGGCGACAGCCCCAAGAAGAAGAGAAA
 GGTGGAGGCCAGCTAAGGATCCGGCAAGACTGGCCCCGCTTGGCAACGCA
 ACAGTGAGCCCCCTCCCTAGTGTGTTTGGGGATGTGACTATGTATTCTGTG
 GTTGCCCAACGGGTCAACCCGAACAGATTGATACCCGCTTGGCATTTC
 TGTCAGAAATGTAACGTCAGTTGATGGTACT

[0184] Sequence for a cassette driving the expression of T7 polymerase under the control of beta-2 tubulin promoter, followed by the 3' UTR of Cop1:

(SEQ ID NO: 20)

TCTTTCTTGCCTATGACACTTCCAGCAAAAGGTAGGGCGGGTTCGAGA
 CGGCTTCCCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCGAAG
 CTCCTTCCGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGCGAGCGCTG
 TTAAATAGCCAGGCCCCGATTGCAAAGACATTATAGCGAGCTACCAAA
 GCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCCACTCGAG
 CTTGTGATCGCACTCCGCTAAGGGGGCGCTCTCTCTTCGTTTCAGTC
 ACAACCCGCAAAATgccttaagaagaaggaaggttaacacgattaaca
 tcgctaagaacgacttctctgacatcgaaactggctgctatcccgttcaac
 actoggtgaccattacggtgagcgttttagctcgcaacagttggccctt
 gagcatgagctcttacgagatgggtgaagcacgcttccgcaagatgtttga

-continued

gcgtcaacttaagctggtgaggttgcggataaacgctgccccaagccta
 catcactaccctactccctaagatgattgcacgcatcaacgactggtttg
 aggaagtgaagctaagcgcggaagcgcccagcagccaccagttcctgc
 aagaaatcaagccgaagccgtagcgtacatcaccattaagaccactctg
 gcttgccctaaccagtgctgacaatacaaccgttcaggctgtagcaagcgc
 aatcggtcgggcatctgaggacgaggtcgcttcggctcgatccgtgacc
 ttgaagctaagcacttcaagaaaaacgagaggaacaactcaacaagcgcg
 tagggcacgctctacaagaaagcatttatgcaagagtcgaggagacatgct
 ctctaagggtctactcggtggcgaggcgtggtcttcgtggcataaaggaa
 actctattcatgtaggagtacgctgcacgagatgctcattgagtcaccc
 ggaatggttagcttacaccgcaaaatgctggcgtagtaggtcaagactc
 tgagactatcgaaactcgacactgaatcgctgaggctatcgcaaccctg
 caggtgcgctggctggcatctctccgatgttccacettgctgagttcct
 cctaagccgtggactggcattactggtggtggctattgggctaaccgctg
 tcgtcctctggcgctggtgctactcacagtaagaaagcactgatgcgct
 acgaagacgtttacatgcctgaggtgtacaagcgattaacattgcgcaa
 aacaccgcatggaaaatcaacaagaaagtcctagcggctcgcaaccgta
 caccaagtgggaagcattgtccggtcgaggacatctttgcgattgagcgtg
 aagaactcccgatgaaaccggaagacatcgacatgaatcctgaggactca
 ccgctgggaaacgtgctgccgctgctgtgtaccgcaaggacaaggtcgc
 aagctcgcgctatcagccttgagttcatgcttgagcaagccaataagtt
 tgctaaccataaggccataggttcccttacacatggactggcgcggtcg
 tgtttacgctgtgtcaatgttcaaccgcgaaggttaacgatatgacaaa
 gactgcttacgctggcgaaaggttaaccaatcggttaagggaaggttactac
 tgggtgaaaaatccacggtgcaaacgtgtcggggtgctgacaaggttccgtt
 ccctgagcgcatcaagttcattgaggaaaaccacgagaacatcatggctt
 gcgctaagctctccactggagaacacttggtgggctgagcaagattctccg
 ttctgcttccctgcgttctgctttgagtacgctggggtacagcaccacgg
 cctgagctataactgctcccacgctggcggtttgacgggtcttgcctggt
 catccagcacttctccgcatgaccgagatgaggtaggtggctgcgcggt
 taacttgcttccctagtgaaccggttcaggacatctacgggattgttgtta
 agaaagtcaacgagattctacaagcagacgcaatcaatgggaccgataac
 gaagtgttaccgtgaccgatgagaacactgggtgaaatctctgagaaggt
 caagctgggactaaggcactggctgggtcaatggctggcttacgggtgta
 ctgcagtgtagtaagcgacagtcacgctggcttacgggtccaaag
 agttcggttccgctcaacaagtgctggaagataaccattcagccagctatt
 gattccggcaagggtctgatgttccactcagccgaatcaggctgctggata
 catggctaagctgatttgggaatctgtgagcgtgacggtggtagctgcgg
 ttgaagcaatgaactggcttaagctcgtgctgaagctgctggctgctgag

-continued

gtcaagataagaagactggagagattcttcgcaagcgttgcgctgtgca
 ttgggttaactcctgatggtttccctgtgtggcaggaatacaagaagccta
 ttcagacgcgcttgaaactgatgttcctcggtcagttccgcttacagcct
 accattaacaccaacaagatagcgagattgatgcacacaacaggagtc
 tggatcgcctcctaactttgtacacagccaagcggtagccaccttcgta
 agactgtagtgtgggcacacgagaagtaacggaatcgaatcttttgactg
 attcagcactccttcggtagcattccggctgacgctgcgaacctgttcaa
 agcagtgccgaaactatggttgacacatatgagtccttgatgtactgg
 ctgatactacgaccagttcgagaccagageacgagtcctcaattggacaaa
 atgcccagcacttcgggttaaaggtaacttgaaacctccgtgacatcttaga
 gtcggacttcgcttcgctgaaggATCCGGCAAGACTGGCCCCGCTTGGC
 AACGCAACAGTGAGCCCCCTCCTAGTGTGTTTGGGGATGTGACTATGTAT
 TCGTGTGTTGGCCAACGGGTCAACCCGAACAGATTGATACCCGCTTGGC
 ATTCCTGTGAGAAATGTAACGTGAGTTGATGGTACT

[0185] Sequence of guide RNA driven by the T7 promoter
 (T7 promoter, Ns represent targeting sequence):

(SEQ ID NO: 21)

gaaatTAATACGACTCACTATANNNNNNNNNNNNNNNNNNNNNNgttttaga
 gctaGAAAtagcaagttaaaataaggctagtcggttatcaacttgaaaaa
 gtggcaccgagtcggtgctttttt

[0186] Gene Delivery:

[0187] *Chlamydomonas reinhardtii* strain CC-124 and CC-125 from the *Chlamydomonas* Resource Center will be used for electroporation. Electroporation protocol follows standard recommended protocol from the GeneArt *Chlamydomonas* Engineering kit.

[0188] Also, Applicants generate a line of *Chlamydomonas reinhardtii* that expresses Cas9 constitutively. This can be done by using pChlamy1 (linearized using PvuI) and selecting for hygromycin resistant colonies. Sequence for pChlamy1 containing Cas9 is below. In this way to achieve gene knockout one simply needs to deliver RNA for the guideRNA. For homologous recombination Applicants deliver guideRNA as well as a linearized homologous recombination template.

pChlamy1-Cas9:

(SEQ ID NO: 22)

TGCGGTATTTACACCGCATCAGGTGGCACTTTTCGGGAAATGTGCGCG
 GAACCCCTATGTTTATTTTCTAANTACATTCAATATTTTATCCGCTCA
 TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAATGA
 AGTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTA
 CCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTT
 CATCCATAGTTGCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAG
 GGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGGAGACCCACGCTC
 ACCGGCTCCAGATTATCAGCAATAAACCGCCAGCCGGAAGGGCCGAGC
 GCAGAAGTGGTCTGCAAGTTATCCGCCTCCATCCAGTCTATTAATTGTT

-continued

GCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTT
 GTTGCCATTTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGG
 CTCATTAGCTCCGGTTCCTCAACGATCAAGCGGAGTTACATGATCCCCCA
 TGTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTGAGA
 AGTAAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAA
 TTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGGT
 ACTCAACCAAGTCATTCTGAGAATAGTTTTATGCGGCGACCGAGTTGCTC
 TTGCCCGGCGTCAATACGGGATAATACCGGCCACATAGCAGAACTTTAA
 AAGTCTCATCATTTGGAACGTTCTTCGGGGCGAAAACCTCAAGGATC
 TTACCGCTGTTGAGATCCAGTTCGATGTAAACCACTCGTGACCCCACTG
 ATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAG
 GAAGGCAAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAATGTTGA
 ATACTCATACTCTTCGTTTCAATATTTTTGAAGCATTATCAGGGTTA
 TTGTCTCATGACCAAAATCCCTTAACGTGAGTTTTCTGTTCCACTGAGCGT
 CAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTG
 CGCGTAATCTGCTGCTTGCAAAACAAAAAACCCGCTACCGAGCGTGGT
 TTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACGGCT
 TCAGCAGAGCGCAGATACCAATACTGTTCTTCTAGTGTAGCCGTAGTTA
 GGCCACCACTTCAAGAACTCTGTAGCACCGCTACATACCTCGCTCTGCT
 AATCCTGTTACCAGTGGCTGTTGCCAGTGGCGATAAGTCGTGTGTACCGG
 GTTGAGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGTGGGCTGAA
 CGGGGGGTTCTGTGCACACAGCCAGCTTGAGAGCGAACGACCTACCCGAA
 CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGG
 GAGAAAGGCGGACAGGTATCCGGTAAGCGGCGAGGTGGAACAGGAGAGC
 GCACGAGGGAGCTTCAGGGGGAACGCTGGTATCTTTATAGTCTGTCTC
 GGGTTTCGCCACGCTGACTTGAGCGTCGATTTTTTGTGATGCTCGTCAGG
 GGGGCGGAGCCTATGGAAAACGCCGCAACGCGGCCCTTTTACGGTTCC
 TGGCCTTTTGCTGGCCTTTTGCTCACATGTTGTTCTTCGCTATCCCTCG
 ATTTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCG
 CCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGTTCC
 CTGAGGCTTGACATGATTGGTGCGTATGTTTGTATGAAGCTACAGGACTG
 ATTTGGCGGGCTATGAGGGCGGGGAAGCTCTGGAAGGGCCGCGATGGGG
 CGCGCGCGCTCCAGAAGGCGCCATACGGCCGCTGGCGGCACCCATCCGG
 TATAAAGCCCGCGACCCCGAACGGTGACCTCCACTTTCAGCGCAAAACG
 AGCACTTATACATACGCGACTATTCTGCCGCTATTTACATTTAACCCTC
 AGCTTTAGCTTTTAAAGATCCCATCAAGCTTGCATGCCGGGCGCGCAGAA
 GGAGCGCAGCCAAACAGGATGATGTTTTTGTATGGGGTTTTTGTAGCACT
 TGCAACCCCTTATCCGAAGCCCCCTGGCCACAAAGGCTAGGCGCCAATG
 CAAGCAGTTCGATGCAGCCCTGGAGCGGTGCCCTCTGTATAAACCGC

-continued

CAGGGGGCCTATGTTCTTACTTTTTTACAAGAGAAGTCACTCAACATCT
 TAAACUGGCCAGGTGAGTCGACGAGCAAGCCCGCGGATCAGGCAGCGTG
 CTTGCAGATTTGACTTGCAACGCCCGCATTTGTGTCGACGAAGGCTTTTGG
 CTCCTCTGTGCGTGTCTCAAGCAGCATCTAACCTGCGTCGCGGTTTCCA
 TTTGCGAGAGATTCGAGGTACCATGTACCCATACGATGTTCCAGATTACG
 CTTGCGCCGAAGAAAAGCGCAAGGTCGAAGCGTCCGACAAGAAGTACAGC
 ATCGGCCTGGACATCGGCACCAACTCTGTGGCTGGGCCGTGATCACCGA
 CGAGTACAAGGTGCCAGCAAGAAATTCAGGTGCTGGGCAACACCGACC
 GGCACAGCATCAAGAAGAACCTGATTTGCGAGCCCTGCTTTGTTGACAG
 CGGGCAACAGCCGAGGCCACCCGCTGAAGAGAACCGCCAGAAGAAGAT
 ACACCAGACGGAAGAACCGGATCTGCTATCTGCAAGAGATCTTCAGCAAC
 GAGATGGCCAAAGTGGACGACAGCTTCTTCCACAGACTGGAAGAGTCTTC
 CCTGTGGAAGAGGATAAGAAGCACGAGCGGCACCCATCTTCGGAACA
 TCGTGGACGAGGTGGCTTACCACGAGAAGTACCCACCATCTACCACCTG
 AGAAAGAACTGGTGGACAGCACCGACAGGCCGACCTGCGGCTGATCTA
 TCTGGCCCTGGCCACATGATCAAGTTCGGGGCCACTTCTGATCGAGG
 GCGACCTGAACCCGACAAACAGCGACGTGGACAAGCTGTTATCCAGCTG
 GTGCAGACCTACAACAGCTGTTGAGGAAAACCCATCAACGCCAGCGG
 CGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGACGGC
 TGGAAAATCTGATCGCCAGCTGCCCGGCGAGAAGAAGATGGCCTGTTC
 GGCAACCTGATTGCCCTGAGCTGGGCTGACCCCACTTCAAGAGCAA
 CTTGACCTGGCCGAGGATGCCAACTGCAGCTGAGCAAGGACACCTACG
 ACGACGACCTGGACAACCTGCTGGCCAGATCGGCGACCAGTACGCCGAC
 CTGTTTCTGGCCGCCAAGAACCTGTCCGACGCATCTCTGCTGAGCGACAT
 CCTGAGAGTGAACACCGAGATCACCAGGCCCCCTGAGCGCCTCTATGA
 TCAAGAGATACGACGAGCACCACCAGGACCTGACCTGCTGAAAGCTCTC
 GTGCGGCAGCAGCTGCCGAGAAGTACAAAGAGATTTTTTCGACCAGAGC
 AAGAACGGCTACGCCGGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTT
 CTTACAAGTTTCATCAAGCCCATCCTTGAAAAGATGGACGGCACCGAGG
 AACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGGAAGCAGCGGACC
 TTCGACAACGGCAGCATCCCCACAGATCCACGGGAGAGCTTTCACGC
 CATTCTGCGGCGGCAGGAAGATTTTACCCATTCTGAAAGACAACCGGG
 AAAAGATCGAGAAGATCCTGACCTTCCGCATCCCTACTACGTGGGCCT
 CTGGCCAGGGGAAACAGCAGATTGCGCTGGATGACCAGAAAGAGCAGGA
 AACCATCACCCCTGGAACCTCGAGGAAGTGGTGACAAGGGCGCTTCCG
 CCGAGAGCTTGATCGAGCGGATGACCAACTTCGATAAGAACCTGCCAAC
 GAGAAGGTGCTTTCAAGCACAGCTGCTGTACGAGTACTTCACCGTGTA
 TAACGAGCTGACCAAAGTGAAATACGTGACCGAGGAATGAGAAAGCCCG
 CCTTCTGAGCGGCAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAG

-continued

ACCAACCGGAAAGTGACCGTGAAGCAGCTGAAAGAGGACTACTTCAAGAA
 AATCGAGTGCTTCGACTCCGTGGAAATCTCCGGCGTGGAAGATCGGTTCA
 ACGCCTCCCTGGGCACATACCAGATCTGCTGAAAATTATCAAGGACAAG
 GACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCTGTCT
 GACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAA
 CCTATGCCACCTTTTTCGACGACAAAGTGATGAAGCAGCTGAAGCGCGG
 GAGATACACCGGCTGGGGCAGGCTGAGCCGGAAGCTGATCAACGGCATCC
 GGGACAAGCAGTCCGGCAAGACAATCTGGATTTCTGAAGTCCGACGGC
 TTCGCCAACAGAACTTGATGGAGCTGATCCACGACGACAGCCTGACCTT
 TAAAGAGGACATCCAGAAAGCCAGGTGTCCGGCCAGGGCGATAGCCTGC
 ACGAGCACATTGCCAATCTGGCCGGCAGCCCCGCCATTAAGAAGGGCATC
 CTGCAGACAGTGAAGGTGGTGGACGAGCTCGTTTGAAGTGATGGGCCGG
 CACAAGCCCGAGAACATTTCTGATCGAAATGGCCAGAGAGAACCAGACC
 ACCCAGAAGGGACAGAAGAACAGCCGCGAGAGAATGAAGCGGATCGAAGA
 GGGCATCAAAGAGCTGGGCAGCCAGATCTGAAAGAACACCCCGTGAAA
 ACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAAATGGG
 CGGGATATGTACGTGGACCAGGAACCTGGAGATCAACCGGCTGTCCGACTA
 CGATGTGGACCATATCTGTCCTCAGAGCTTTCTGAAGGACGACTCCATCG
 ACAACAAGGTGCTGACCAGAAGCGACAAGAACCGGGGCAAGAGCGACAAC
 GTGCCCTCCGAAGAGGTCTGGAAGAAGATGAAGAAGACTGGCGGCAGCTG
 CTGAACGCCAAGCTGATTTTACCCAGAGAAGTTGCACAATCTGACCAAG
 GCCGAGAGAGGCGGCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAG
 ACAGCTGGTGGAAACCCGGCAGATCACAAGCAGTGGCACAGATCCTGG
 ACTCCCGGATGAACACTAAGTACGACGAGAATGACAAGCTGATCCGGGAA
 GTGAAAGTGATCACCTGAAGTCCAAGCTGGTGTCCGATTTCCGGAAGGA
 TTTCCAGTTTTTACAAAGTGCGCGAGATCAACAATACCACCACGCCACG
 ACGCCTACCTGAACGCCGTCTGTTGGGAACCGCCCTGATCAAAAAGTACCTT
 AAGCTGGAAGCGAGTTCTGTGACGGCGACTACAAGGTGTACGACGTGCG
 GAAGATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGT
 ACTTCTTCTACAGCAACATCATGAACTTTTTCAAGACCGAGATTACCTTG
 GCCAACCGCGAGATCCGGAAGCGGCTCTGATCGAGACAAACGGCGAAAC
 CGGGGAGATCGTGTGGGATAAGGGCCGGGATTTTGCACCGTGCAGAAAG
 TGCTGAGCATGCCCCAAGTGAATATCTGTAAGAACCGAGGTGACAGACA
 GGCGGCTTCAGCAAGAGTCTATCTGCCCCAAGAGGAACAGCGATAAGCT
 GATCGCCAGAAAGAAGGACTGGGACCGAAGAAGTACGGCGGCTTCGACAG
 CCCCACCGTGGCCTATTCTGTGCTGGTGGTGGCCAAAGTGGAAGGGCA
 AGTCCAAGAAACTGAAGAGTGTGAAGAGCTGCTGGGGATCACCATCATG
 GAAAGAAGCAGCTTCGAGAAGAATCCCATCGACTTTCTGGAAGCCAAGGG
 CTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCTTAAGTACTCCC

-continued

TGTTTCGAGCTGGAAAACGGCCGGAAGAGATGCTGGCCTCTGCCGGCGAA
 CTGCAGAAGGGAAACGAACTGGTTTTGTTTTCCAAATATGTGAATTCCT
 GTACCTGGCCAGCCACTATGAGAAGCTGAAGGGTCCCCGAGGATAATG
 AGCAGAAACAGCTGTTTGTGGAACAGCACAAGCACTACCTGGACGAGATC
 ATCGAGCAGATCAGCGAGTCTCCAAGAGAGTGATCCTGGCCGACGCTAA
 TCTGGACAAAGTGCTGTCCGCTACAACAAGCACCGGATAAGCCCATCA
 GAGAGCAGGCCGAGAATATCATCCACCTGTTTACCTGACCAATCTGGGA
 GCCCCTGCCGCTTCAAGTACTTTGACACCACCATCGACCGAAGAGGTA
 CACCAGCACCAAGAGGTGCTGGAGCCACCTGATCCACCAGAGCATCA
 CCGGCTGTACGAGACAGGATCGACCTGTCTCAGCTGGGAGGCGACAGC
 CCCAAGAAGAAGAGAAAGGTGGAGGCCAGCTAACATATGATTGGAATGTC
 TTTCCGCGTATGACACTTCCAGCAAAAGGTAGGCGGGCTGCGAGACGGC
 TTCCCGGCGCTGATGCAACACCGATGATGCTTCGACCCCCGAAGCTCC
 TTCGGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTA
 AATAGCCAGGCCCCGATTGCAAGACATTATAGCGAGCTACCAAGCCA
 TATTCAAACACTAGATCACTACCACTTCTACACAGGCCACTCGAGGTG
 TGATCGCACTCCGCTAAGGGGGCGCTCTTCTCTTCTGTTTCAGTCACAA
 CCCCAGAACATGACACAAGAATCCCTGTTACTTCTCGACCGTATTGATTC
 GGATGATTCTACGCGAGCGTGGGAACGACAGGAATTCTGGGAGGTGA
 GTCGACGAGCAAGCCCGCGGATCAGGCAGCGTGCTTGAGATTGACTT
 GCAACGCCCGCATTGTGTGACGAAGGCTTTTGCTCTCTGTCGCTGTC
 TCAAGCAGCATCTAACCTGCGTCCGCTTCCATTGACGCGCTGGCC
 CGCCGAGCCCTGGAGGAGCTCGGGTGGCGGTGCCGCCGTGGGCGGTTT
 TGCCCGCGAGAGCACCAACCCGTAAGTGTGCGGAGCCCGCCCGGTG
 ATCAAGCTGTTTCGGCGAGCACTGTTGCGGTCCGGAGACCTCGCGTTTCG
 GAGTTTCGGAGCGTACGCGTCTGGCGGACGCCCGGTGCCGTGCC
 CGCTCTCGCGCGCGGAGCTGCGGCCCGCACCGGAGCTGGCCGTG
 GCCCTACCTGGTGATGAGCCGATGACCGGACCACTGGCGGTCCGCGA
 TGGACGGCAGACCGACCGGAACGCGCTGCTCGCCCTGGCCCGGAACCTC
 GGCCGGGTGCTCGGCCGCTGCACAGGGTGCCGCTGACCGGAACACCGT
 GCTCACCCCCATTCCGAGGTCTTCCCGAACTGCTGCGGGAACCGCGC
 CGCGACCGCTCGAGGACACCGCGGTGGGCTACCTCTCGCCCCGGCTG
 CTGGACCGCTGGAGGACTGGCTGCCGACGTGGACACGCTGCTGGCCG
 CCGCAACCCCGGTTCTGTCACGGGACCTGCACGGGACCAACATCTTCG
 TGGACCTGGCCGCGACCGAGGTACCGGATTTCTGTCGACTTCACCGACG
 TTTCTATCGGGGAGACTCCGCTACAGCCTTTGGTGCAACTGCATCTCAA
 CGCCTTCCGGGCGCACCAGGATCTGGCCGCTGCTCGACGGGGCGC
 AGTGGAAGCGGACCGAGGACTTCGCCCCGAACTGCTCGCCTTCACTTC
 CTGCACGACTTCGAGGTGTTGAGGAGACCCCGGGATCTCTCCGGCTTC

-continued

ACCGATCCGGAGGAAGTGGCGAGTTCCTCTGGGGGCCCGGACACCGC
 CCCCAGCGCTGATAAGGATCCGGCAAGACTTTGGCCCCGCTTTGGCAAC
 GCAACAGTGAGCCCCCTAGTGTGTTTGGGGATGTGACTATGTATTCTG
 GTGTGGCCAAACGGGTCAACCCGAACACATTGATACCCGCGTTTGGCATT
 TCCTGTGAGAATGTTTAAAGTCATTTTGATGGTACT.

[0189] For all modified *Chlamydomonas reinhardtii* cells, Applicants use PCR, SURVEYOR nuclease assay, and DNA sequencing to verify successful modification.

[0190] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

REFERENCES

- [0191] 1. Umov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* 11, 636-646 (2010).
- [0192] 2. Bogdanove, A. J. & Voytas, D. F. TAL effectors: customizable proteins for DNA targeting. *Science* 333, 1843-1846 (2011).
- [0193] 3. Stoddard, B. L. Homing endonuclease structure and function. *Q. Rev. Biophys.* 38, 49-95 (2005).
- [0194] 4. Bae, T. & Schneewind, O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* 55, 58-63 (2006).
- [0195] 5. Sung, C. K., Li, H., Claverys, J. P. & Morrison, D. A. An rpsL cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*. *Appl. Environ. Microbiol.* 67, 5190-5196 (2001).
- [0196] 6. Sharan, S. K., Thomason, L. C., Kuznetsov, S. G. & Court, D. L. Recombineering: a homologous recombination-based method of genetic engineering. *Nat. Protoc.* 4, 206-223 (2009).
- [0197] 7. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816-821 (2012).
- [0198] 8. Deveau, H., Garneau, J. E. & Moineau, S. CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol.* 64, 475-493 (2010).
- [0199] 9. Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327, 167-170 (2010).
- [0200] 10. Terns, M. P. & Terns, R. M. CRISPR-based adaptive immune systems. *Curr. Opin. Microbiol.* 14, 321-327 (2011).
- [0201] 11. van der Oost, J., Jore, M. M., Westra, E. R., Lundgren, M. & Brouns, S. J. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends. Biochem. Sci.* 34, 401-407 (2009).

- [0202] 12. Brouns, S. J. et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321, 960-964 (2008).
- [0203] 13. Carte, J., Wang, R., Li, H., Terns, R. M. & Terns, M. P. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev.* 22, 3489-3496 (2008).
- [0204] 14. Deltcheva, E. et al. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471, 602-607 (2011).
- [0205] 15. Hatoum-Aslan, A., Maniv, I. & Marraffini, L. A. Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. *Proc. Natl. Acad. Sci. U.S.A.* 108, 21218-21222 (2011).
- [0206] 16. Haurwitz, R. E., Jinek, M., Wiedenheft, B., Zhou, K. & Doudna, J. A. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. *Science* 329, 1355-1358 (2010).
- [0207] 17. Deveau, H. et al. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. *J. Bacteriol.* 190, 1390-1400 (2008).
- [0208] 18. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* (2012).
- [0209] 19. Makarova, K. S., Aravind, L., Wolf, Y. I. & Koonin, E. V. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. *Biol. Direct.* 6, 38 (2011).
- [0210] 20. Barrangou, R. RNA-mediated programmable DNA cleavage. *Nat. Biotechnol.* 30, 836-838 (2012).
- [0211] 21. Brouns, S. J. Molecular biology. A Swiss army knife of immunity. *Science* 337, 808-809 (2012).
- [0212] 22. Carroll, D. A CRISPR Approach to Gene Targeting. *Mol. Ther.* 20, 1658-1660 (2012).
- [0213] 23. Bikard, D., Hatoum-Aslan, A., Mucida, D. & Marraffini, L. A. CRISPR interference can prevent natural transformation and virulence acquisition during in vivo bacterial infection. *Cell Host Microbe* 12, 177-186 (2012).
- [0214] 24. Sapranas, R. et al. The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Res.* (2011).
- [0215] 25. Semenova, E. et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc. Natl. Acad. Sci. U.S.A.* (2011).
- [0216] 26. Wiedenheft, B. et al. RNA-guided complex from a bacterial immune system enhances target recognition through seed sequence interactions. *Proc. Natl. Acad. Sci. U.S.A.* (2011).
- [0217] 27. Zahner, D. & Hakenbeck, R. The *Streptococcus pneumoniae* beta-galactosidase is a surface protein. *J. Bacteriol.* 182, 5919-5921 (2000).
- [0218] 28. Marraffini, L. A., Dedent, A. C. & Schneewind, O. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol. Mol. Biol. Rev.* 70, 192-221 (2006).
- [0219] 29. Motamedi, M. R., Szigety, S. K. & Rosenberg, S. M. Double-strand-break repair recombination in *Escherichia coli*: physical evidence for a DNA replication mechanism in vivo. *Genes Dev.* 13, 2889-2903 (1999).
- [0220] 30. Hosaka, T. et al. The novel mutation K87E in ribosomal protein S12 enhances protein synthesis activity during the late growth phase in *Escherichia coli*. *Mol. Genet. Genomics* 271, 317-324 (2004).
- [0221] 31. Costantino, N. & Court, D. L. Enhanced levels of lambda Red-mediated recombinants in mismatch repair mutants. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15748-15753 (2003).
- [0222] 32. Edgar, R. & Qimron, U. The *Escherichia coli* CRISPR system protects from lambda lysogenization, lysogens, and prophage induction. *J. Bacteriol.* 192, 6291-6294 (2010).
- [0223] 33. Marraffini, L. A. & Sontheimer, E. J. Self versus non-self discrimination during CRISPR RNA-directed immunity. *Nature* 463, 568-571 (2010).
- [0224] 34. Fischer, S. et al. An archaeal immune system can detect multiple Protospacer Adjacent Motifs (PAMs) to target invader DNA. *J. Biol. Chem.* 287, 33351-33363 (2012).
- [0225] 35. Gudbergdottir, S. et al. Dynamic properties of the *Sulfolobus* CRISPR/Cas and CRISPR/Cmr systems when challenged with vector-borne viral and plasmid genes and protospacers. *Mol. Microbiol.* 79, 35-49 (2011).
- [0226] 36. Wang, H. H. et al. Genome-scale promoter engineering by coselection MAGE. *Nat Methods* 9, 591-593 (2012).
- [0227] 37. Cong, L. et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* In press (2013).
- [0228] 38. Mali, P. et al. RNA-Guided Human Genome Engineering via Cas9. *Science* In press (2013).
- [0229] 39. Hoskins, J. et al. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J. Bacteriol.* 183, 5709-5717 (2001).
- [0230] 40. Havarstein, L. S., Coomaraswamy, G. & Morrison, D. A. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. U.S.A.* 92, 11140-11144 (1995).
- [0231] 41. Horinouchi, S. & Weisblum, B. Nucleotide sequence and functional map of pC 194, a plasmid that specifies inducible chloramphenicol resistance. *J. Bacteriol.* 150, 815-825 (1982).
- [0232] 42. Horton, R. M. In Vitro Recombination and Mutagenesis of DNA: SOEing Together Tailor-Made Genes. *Methods Mol. Biol.* 15, 251-261 (1993).
- [0233] 43. Podbielski, A., Spellerberg, B., Woischnik, M., Pohl, B. & Luttmann, R. Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* 177, 137-147 (1996).
- [0234] 44. Husmann, L. K., Scott, J. R., Lindahl, G. & Stenberg, L. Expression of the Arp protein, a member of the M protein family, is not sufficient to inhibit phagocytosis of *Streptococcus pyogenes*. *Infection and immunity* 63, 345-348 (1995).
- [0235] 45. Gibson, D. G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* 6, 343-345 (2009).
- [0236] 46. Garneau J. E. et al. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468, 67-71 (4 Nov. 2010).
- [0237] 47. Barrangou R. et al. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. 2007 Mar. 23; 315(5819): 1709-12.

- [0238] 48. Ishino Y. et al. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J Bacteriol. 1987 December; 169(12):5429-33.
- [0239] 49. Mojica F. J. M et al. Biological significance of a family of regularly spaced repeats in the genomes of

Archaea, Bacteria and mitochondria. Molecular Microbiology (2000) 36(1), 244-246.

- [0240] 50. Jansen R. et al. Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology (2002) 43(6), 1565-1575.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 181

<210> SEQ ID NO 1

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Unknown

<220> FEATURE:

<223> OTHER INFORMATION: Description of Unknown: CRISPR1 Cas9 target site sequence

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (1)..(20)

<223> OTHER INFORMATION: a, c, t or g

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (21)..(22)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 1

nnnnnnnnnn nnnnnnnnnn nnagaaw

27

<210> SEQ ID NO 2

<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Unknown

<220> FEATURE:

<223> OTHER INFORMATION: Description of Unknown: CRISPR1 Cas9 target site sequence

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (1)..(12)

<223> OTHER INFORMATION: a, c, t or g

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (13)..(14)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 2

nnnnnnnnnn nnnnagaaw

19

<210> SEQ ID NO 3

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Unknown

<220> FEATURE:

<223> OTHER INFORMATION: Description of Unknown: CRISPR1 Cas9 target site sequence

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (1)..(20)

<223> OTHER INFORMATION: a, c, t or g

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (21)..(22)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 3

nnnnnnnnnn nnnnnnnnnn nnagaaw

27

<210> SEQ ID NO 4

<211> LENGTH: 18

-continued

<212> TYPE: DNA
<213> ORGANISM: Unknown
<220> FEATURE:
<223> OTHER INFORMATION: Description of Unknown: CRISPR1 Cas9 target
site sequence
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(11)
<223> OTHER INFORMATION: a, c, t or g
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (12)..(13)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 4

nnnnnnnnnn nnnagaaw 18

<210> SEQ ID NO 5
<211> LENGTH: 137
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 5

nnnnnnnnnn nnnnnnnnnn gttttgtac tctcaagatt tagaaataaa tcttgcagaa 60
gctacaaaga taaggcttca tgccgaaatc aacaccctgt cattttatgg caggggtgtt 120
tcgttattta atttttt 137

<210> SEQ ID NO 6
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 6

nnnnnnnnnn nnnnnnnnnn gttttgtac tctcagaaat gcagaagcta caaagataag 60
gcttcatgcc gaaatcaaca cctgtcatt ttatggcagg gtgttttcgt tatttaattt 120
ttt 123

<210> SEQ ID NO 7
<211> LENGTH: 110
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 7

nnnnnnnnnn nnnnnnnnnn gttttgtac tctcagaaat gcagaagcta caaagataag 60

-continued

gcttcacgcc gaaatcaaca cctgtcatt ttatggcagg gtgttttttt 110

<210> SEQ ID NO 8
<211> LENGTH: 102
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 8

nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc 60

cgttatcaac ttgaaaaagt ggcaccgagt cggcgctttt tt 102

<210> SEQ ID NO 9
<211> LENGTH: 88
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 9

nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc 60

cgttatcaac ttgaaaaagt gttttttt 88

<210> SEQ ID NO 10
<211> LENGTH: 76
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(20)
<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 10

nnnnnnnnnn nnnnnnnnnn gttttagagc tagaaatagc aagttaaaat aaggctagtc 60

cgttatcatt tttttt 76

<210> SEQ ID NO 11
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 11

gttttagagc ta 12

<210> SEQ ID NO 12
<211> LENGTH: 31

-continued

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 12

tagcaagtta aaataaggct agtccgtttt t 31

<210> SEQ ID NO 13
<211> LENGTH: 12
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 13

guuuuagagc ua 12

<210> SEQ ID NO 14
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

ggacatcgat gtcacctcca atgactaggg tgg 33

<210> SEQ ID NO 15
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

cattggaggt gacatcgatg tcctcccat tgg 33

<210> SEQ ID NO 16
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

ggaagggcct ggtccgagc agaagaagaa ggg 33

<210> SEQ ID NO 17
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

ggtggcgaga ggggccgaga ttgggtgttc agg 33

<210> SEQ ID NO 18
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

atgcaggagg gtggcgagag gggccgagat tgg 33

<210> SEQ ID NO 19
<211> LENGTH: 4677

-continued

```

<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
        polynucleotide

<400> SEQUENCE: 19

tcttttcttgc gctatgacac ttccagcaaa aggtaggggcg ggctgcgaga cggcttcccg      60
gcgctgcatg caacaccgat gatgcttcga cccccgaag ctcttcggg gctgcatggg      120
cgctccgatg ccgctccagg gcgagcgctg tttaaatagc caggcccccg attgcaaaga      180
cattatagcg agctaccaaa gccatattca aacacctaga tctactaccac ttctacacag      240
gccactcgag cttgtgatcg cactccgcta agggggcgcc tcttctctt cgtttcagtc      300
acaaccgcga aacatgtacc catacgatgt tccagattac gcttcgccga agaaaaagcg      360
caaggtcgaa gcgtccgaca agaagtacag catcggcctg gacatcggca ccaactctgt      420
gggctggggc gtgatcaccg acgagtacaa ggtgcccagc aagaaattca aggtgctggg      480
caacaccgac cggcacagca tcaagaagaa cctgatcgga gccctgctgt tcgacagcgg      540
cgaaacagcc gaggcccccc ggctgaagag aaccgccaga agaagatata ccagacggaa      600
gaaccggatc tgctatctgc aagagatctt cagcaacgag atggccaagg tggacgacag      660
cttcttccac agactggaag agtccttctt ggtggaagag gataagaagc acgagcggca      720
ccccatcttc ggcaacatcg tggacgaggt ggcctaccac gagaagtacc ccaccatcta      780
ccacctgaga aagaaactgg tggacagcac cgacaaggcc gacctgcggc tgatctatct      840
ggccctggcc cacatgatca agttccgggg ccacttctctg atcgagggcg acctgaacct      900
cgacaacagc gacgtggaca agctgttcat ccagctggtg cagacctaca accagctggt      960
cgaggaaaaa cccatcaacg ccagcggcgt ggacgccaa ggcctcctgt ctgccagact     1020
gagcaagagc agacggctgg aaaatctgat cgcccagctg cccggcgaga agaagaatgg     1080
cctgttcggc aacctgattg ccttgagcct gggcctgacc cccaacttca agagcaactt     1140
cgacctggcc gaggatgcca aactgcagct gagcaaggac acctacgacg acgacctgga     1200
caacctgctg gccagatcg gcgaccagta cgccgacctg tttctggccg ccaagaacct     1260
gtccgacgcc atcctgctga gcgacatcct gagagtgaac accgagatca ccaaggcccc     1320
cctgagcgcc tctatgatca agagatacga cgagcaccac caggacctga cctgctgaa     1380
agctctctg cggcagcagc tgcctgagaa gtacaaagag attttctctg accagagcaa     1440
gaacggctac gccggctaca ttgacggcgg agccagccag gaagagttct acaagttcat     1500
caagcccatc ctgaaaaaga tggacggcac cgaggaactg ctctgaagc tgaacagaga     1560
ggacctgctg cggaagcagc ggaccttcga caacggcagc atccccacc agatccacct     1620
gggagagctg cacgccattc tgcggcggca ggaagathtt taccatttc tgaaggacaa     1680
ccgggaaaaa atcgagaaga tctgacctt ccgcatcccc tactacgtgg gccctctggc     1740
caggggaaac agcagattcg cctggatgac cagaaagagc gaggaaacca tcacctctg     1800
gaacttcgag gaagtgtggt acaagggcgc ttccgccagc agcttcatcg agcggatgac     1860
caacttcgat aagaacctgc ccaacgagaa ggtgctgccc aagcacagcc tgctgtacga     1920
gtacttcacc gtgtataacg agctgaccaa agtgaaatac gtgaccgagg gaatgagaaa     1980
gcccgccttc ctgagcggcg agcagaaaaa ggccatcgtg gacctgctgt tcaagaccaa     2040

```

-continued

| | | | | | | |
|-------------|------------|-------------|-------------|-------------|-------------|------|
| ccggaaagtg | accgtgaagc | agctgaaaga | ggactacttc | aagaaaatcg | agtgcctcga | 2100 |
| ctccgtggaa | atctccggcg | tggaagatcg | gttcaacgcc | tccttgggca | cataccacga | 2160 |
| tctgctgaaa | attatcaagg | acaaggactt | cctggacaat | gaggaaaacg | aggacattct | 2220 |
| ggaagataac | gtgctgaccc | tgacactgtt | tgaggacaga | gagatgatcg | aggaacggct | 2280 |
| gaaaacctat | gcccacctgt | tcgacgacaa | agtgatgaag | cagctgaagc | ggcggagata | 2340 |
| caccggctgg | ggcaggctga | gccggaagct | gatcaacggc | atccgggaca | agcagtcggg | 2400 |
| caagacaatc | ctggatttcc | tgaagtcga | cggtctcgcc | aacagaaact | tcatgcagct | 2460 |
| gatccacgac | gacagcctga | cctttaaaga | ggacatccag | aaagcccagg | tgtccggcca | 2520 |
| gggcgatagc | ctgcacgagc | acattgccaa | tctggccggc | agccccgcca | ttaagaaggg | 2580 |
| catcctgcag | acagtgaagg | tggtagacga | gctcgtgaaa | gtgatgggccc | ggcacaagcc | 2640 |
| cgagaacatc | gtgatcgaaa | tggccagaga | gaaccagacc | accagaagg | gacagaagaa | 2700 |
| cagccgcgag | agaatgaagc | ggatcgaaga | gggcatcaaa | gagctgggca | gccagatcct | 2760 |
| gaaagaacac | cccgtggaaa | acaccagct | gcagaacgag | aagctgtacc | tgtactacct | 2820 |
| gcagaatggg | cgggatatgt | acgtggacca | ggaactggac | atcaaccggc | tgtccgacta | 2880 |
| cgatgtggac | catatcgtgc | ctcagagctt | tctgaaggac | gactccatcg | acaacaagggt | 2940 |
| gctgaccaga | agcgacaaga | accggggcaa | gagcgacaac | gtgccctccg | aagaggtcgt | 3000 |
| gaagaagatg | aagaactact | ggcggcagct | gctgaacgcc | aagctgatta | cccagagaaa | 3060 |
| gttcgacaat | ctgaccaagg | ccgagagagg | cggcctgagc | gaactggata | aggccggctt | 3120 |
| catcaagaga | cagctggtgg | aaaccgggca | gatcacaaaag | cacgtggcac | agatcctgga | 3180 |
| ctcccggatg | aactactaag | acgacgagaa | tgacaagctg | atccgggaag | tgaaagtgat | 3240 |
| cacctgaag | tcgaagctgg | tgtccgattt | ccggaaggat | ttccagtttt | acaaagtgcg | 3300 |
| cgagatcaac | aactaccacc | acgcccacga | cgcctacctg | aacgccgtcg | tggaaccgc | 3360 |
| cctgatcaaa | aagtacccta | agctggaaaag | cgagttcgtg | tacggcgact | acaaggtgta | 3420 |
| cgagctgcgg | aagatgatcg | ccaagagcga | gcaggaaatc | ggcaaggcta | ccgccaagta | 3480 |
| cttctttctac | agcaacatca | tgaacttttt | caagaccgag | attaccctgg | ccaacggcga | 3540 |
| gatccggaag | cggcctctga | tcgagacaaa | cggcgaaaacc | ggggagatcg | tgtgggataa | 3600 |
| gggcggggat | tttccaccg | tgcggaaaag | gctgagcatg | ccccaaagta | atatcgtgaa | 3660 |
| aaagaccgag | gtgcagacag | gcggcttcag | caaagagtct | atcctgcccc | agaggaacag | 3720 |
| cgataagctg | atgccagaa | agaaggactg | ggaccctaag | aagtacggcg | gcttcgacag | 3780 |
| ccccaccgtg | gcctattctg | tgtggtggt | ggccaaagtg | gaaaagggca | agtccaagaa | 3840 |
| actgaagagt | gtgaaagagc | tgtctgggat | caccatcatg | gaaagaagca | gcttcgagaa | 3900 |
| gaatcccatc | gactttcttg | aagccaaggg | ctacaaagaa | gtgaaaaagg | acctgatcat | 3960 |
| caagtgcct | aagtactccc | tgttcgagct | ggaaaacggc | cggaagagaa | tgtctgcctc | 4020 |
| tgcggcgcaa | ctgcagaagg | gaaacgaact | ggcctgccc | tccaaatatg | tgaacttcct | 4080 |
| gtacctggcc | agccactatg | agaagtgaa | gggtccccc | gaggataatg | agcagaaaca | 4140 |
| gctgtttgtg | gaacagcaca | agcactacct | ggacgagatc | atcgagcaga | tcagcgagtt | 4200 |
| ctccaagaga | gtgatcctgg | ccgacgctaa | tctggacaaa | gtgctgtccg | cctacaacaa | 4260 |
| gcaccgggat | aagcccatca | gagagcaggc | cgagaatatc | atccacctgt | ttacctgac | 4320 |

-continued

| | |
|--|------|
| caatctggga gcccctgccg ccttcaagta ctttgacacc accatcgacc ggaagaggta | 4380 |
| caccagcacc aaagagggtgc tggacgccac cctgatccac cagagcatca ccggcctgta | 4440 |
| cgagacacgg atcgacctgt ctcagctggg aggcgacagc cccaagaaga agagaaagggt | 4500 |
| ggaggccagc taaggatccg gcaagactgg ccccgccttg caacgcaaca gtgagcccct | 4560 |
| ccctagtgtg tttggggatg tgactatgta ttcgtgtgtt ggccaacggg tcaaccggaa | 4620 |
| cagattgata cccgccttgg catttcctgt cagaatgtaa cgtcagttga tgggtact | 4677 |

<210> SEQ ID NO 20

<211> LENGTH: 3150

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 20

| | |
|--|------|
| tctttcttgc gctatgacac ttccagcaaa aggtagggcg ggctgcgaga cggcttcccg | 60 |
| gcgctgcatg caacaccgat gatgcttga cccccgaag ctcttcggg gctgcatggg | 120 |
| cgctccgatg ccgctccagg gcgagcgctg tttaaatagc caggccccg attgcaaaga | 180 |
| cattatagcg agctacaaaa gccatattca aacacctaga tctactaccac ttctacacag | 240 |
| gccactcgag cttgtgatcg cactccgta agggggcgcc tcttctctt cgtttcagtc | 300 |
| acaaccgcga aacatgccta agaagaagag gaaggttaac acgattaaca tcgctaagaa | 360 |
| cgacttctct gacatcgaa cggctgctat cccgttcaac actctggctg accattacgg | 420 |
| tgagcgttta gctcgcaac agttggccct tgagcatgag tcttacgaga tgggtgaagc | 480 |
| acgcttccgc aagatgtttg agcgtcaact taaagctggg gaggttcggg ataacgctgc | 540 |
| cgccaagcct ctcactacta cctactccc taagatgatt gcacgcacca acgactgggt | 600 |
| tgaggaaagt aaagctaagc gcggcaagcg cccgacagcc ttccagttcc tgcaagaaat | 660 |
| caagccggaa gccgtagcgt acatcaccat taagaccact ctggcttgcc taaccagtgc | 720 |
| tgacaataca accgttcagg ctgtagcaag cgcaatcggt cgggccattg aggacgaggc | 780 |
| tcgcttcggt cgtatccgtg accttgaagc taagcacttc aagaaaaacg ttgaggaaca | 840 |
| actcaacaag cgcgtagggc acgtctacaa gaaagcattt atgcaagttg tcgaggtgta | 900 |
| catgctctct aagggtctac tcgggtggcg ggcgtggtct tcgtggcata aggaagactc | 960 |
| tattcatgta ggagtacgct gcacgcagat gctcattgag tcaaccggaa tggttagctt | 1020 |
| acaccgcaa aatgctggcg tagtaggtca agactctgag actatcgaa tcgcacctga | 1080 |
| atacgtgag gctatcgcaa cccgtgcagg tgcgtggct ggcatctctc cgatgttcca | 1140 |
| accttgcgta gttctccta agccgtggac tggcattact ggtgggtggc attgggctaa | 1200 |
| cggctgctct cctctggcgc tgggtgcgtac tcacagtaag aaagcactga tgcgctacga | 1260 |
| agacgtttac atgcctgagg tgtacaaagc gattaacatt gcgcaaaaca ccgcattgaa | 1320 |
| aatcaacaag aaagtcttag cggtcgcaa cgtaatcacc aagtgaagc attgtccggt | 1380 |
| cgaggacatc cctgcgattg agcgtgaaga actcccgatg aaaccggaag acatcgacat | 1440 |
| gaatcctgag gctctcaccg cgtggaacg tgcgtccgct gctgtgtacc gcaaggacaa | 1500 |
| ggctcgcaag tctcgccgta tcagccttga gttcatgctt gagcaagcca ataagtttgc | 1560 |
| taaccataag gccatctggt tcccttacia catggactgg cgcggtcgtg ttacgctgt | 1620 |

-continued

```

gtcaatgttc aacccgcaag gtaacgatat gaccaaagga ctgcttacgc tggcgaaagg 1680
taaaccaatc ggtaaggaag gttactactg gctgaaaatc cacggtgcaa actgtgcggg 1740
tgtcgacaag gttccgttcc ctgagcgcat caagttcatt gaggaaaacc acgagaacat 1800
catggcttgc gctaagtctc cactggagaa cacttgggtg gctgagcaag attctccgtt 1860
ctgcttcctt gcgttctgct ttgagtacgc tggggtacag caccacggcc tgagctataa 1920
ctgctccctt ccgctggcgt ttgacgggtc ttgctctggc atccagcact tctcccgcat 1980
gctccgagat gaggtagggt gtcgcgcggt taacttgctt cctagtgaac ccgttcagga 2040
catctacggg attgttgcta agaaagtcaa cgagattcta caagcagacg caatcaatgg 2100
gaccgataac gaagtagtta ccgtgaccga tgagaacact ggtgaaatct ctgagaaagt 2160
caagctgggc actaaggcac tggctgggtc atggctgggt tacggtgtta ctgcagtggt 2220
gactaagcgt tcagtcatga cgctggctta cgggtccaaa gaggctcggt tccgtcaaca 2280
agtgtcggaa gataaccattc agccagctat tgattccggc aagggtctga tgttcactca 2340
gccgaatcag gctgctggat acatggctaa gctgatttgg gaatctgtga gcgtgacggg 2400
ggtagctcgc gttgaagcaa tgaactggct taagtctgct gctaagctgc tggctgctga 2460
gggtcaaatg aagaagactg gagagattct tcgcaagcgt tgcgctgtgc attgggtaac 2520
tcctgatggg ttcctgtgtg ggcagggaata caagaagcct attcagacgc gcttgaacct 2580
gatgttcctc ggtcagttcc gcttacagcc taccattaac accaacaag atagcgagat 2640
tgatgcacac aaacaggagt ctgggtatgc tcctaacttt gtacacagcc aagacggtag 2700
ccaccttcgt aagactgtag tgtgggcaca cgagaagtac ggaatcgaat cttttgcact 2760
gattcacgac tccttcggta cgattccggc tgacgctcgc aacctgttca aagcagtcgc 2820
cgaaactatg gttgacacat atgagtcttg tgatgtactg gctgatttct acgaccagtt 2880
cgctgaccag ttgcacgagt ctcaattgga caaaatgcc gacttccgg ctaaaggtaa 2940
cttgaaacct cgtgacatct tagagtcgga cttcgcgttc gcgtaaggat ccggcaagac 3000
tggccccgct tggcaacgca acagtgcgc cctccctagt gtgtttggg atgtgactat 3060
gtattcgtgt gttggccaac gggtaaccc gaacagattg ataccgcct tggcatttcc 3120
tgtcagaatg taacgtcagt tgatggtagt 3150

```

<210> SEQ ID NO 21

<211> LENGTH: 125

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (23)..(42)

<223> OTHER INFORMATION: a, c, t or g

<400> SEQUENCE: 21

```

gaaattaata cgactcacta tannnnnnnn nnnnnnnnnn nngttttaga gctagaaata 60
gcaagttaaa ataaggctag tccgttatca acttgaaaaa gtggcaccga gtcgggtgctt 120
ttttt 125

```

<210> SEQ ID NO 22

-continued

```

<211> LENGTH: 8452
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      polynucleotide

<400> SEQUENCE: 22

tgccgtatatt cacaccgcac caggtggcac ttttcgggga aatgtgcgcg gaacccttat    60
ttgtttatatt ttctaaatac attcaaatat gtatccgctc atgagattat caaaaaggat    120
cttcacctag atccttttaa attaaaaatg aagtttttaa tcaatctaaa gtatatatga    180
gtaaaacttg tctgacagtt accaatgctt aatcagtgag gcacctatct cagcgatctg    240
tctatttcgt tcaccatag ttgcctgact ccccgctcgt tagataacta cgatacggga    300
gggcttacca tctggcccca gtgctgcaat gataccgcga gaccacgct caccggctcc    360
agatttatca gcaataaacc agccagcccg aagggccgag cgcagaagtg gtcctgcaac    420
tttatccgcc tccatccagt ctattaattg ttgccgggaa gctagagtaa gtagttcgcc    480
agttaatagt ttgcgcaacg ttgttgccat tgctacagcg atcgtggtgt caccgctcgc    540
gtttggtatg gcttcattca gctccggttc ccaacgatca aggcgagtta catgatcccc    600
catgttgtgc aaaaaagcgg ttagctcctt cggtcctccg atcgttgtca gaagtaagtt    660
ggccgcagtg ttatcactca tggttatggc agcactgcat aattctctta ctgtcatgcc    720
atccgtaaga tgctttttctg tgactggtga gtactcaacc aagtcattct gagaatagtg    780
tatgcggcga ccgagttgct ctggcccgcc gtcaatacgg gataataccg cgccacatag    840
cagaacttta aaagtgtcga tcattggaaa acgtttcttcg gggcgaaaaa tctcaaggat    900
cttaccgctg ttgagatcca gttcgatgta acccactcgt gcacccaact gatcttcagc    960
atcttttact ttcaccagcg tttctgggtg agcaaaaaa ggaaggcaaa atgccgcaaa   1020
aaaggaata agggcgacac ggaatgttg aatactcata ctcttccttt ttcaatatta   1080
ttgaagcatt tatcagggtt attgtctcat gaccaaatac ccttaacgtg agttttcgtt   1140
ccactgagcg tcagaccccg tagaaaagat caaaggatct tcttgagatc ctttttttct   1200
gcgcgtaatc tgctgcttgc aaacaaaaaa accaccgcta ccagcgggtg tttgtttgcc   1260
ggatcaagag ctaccaactc tttttccgaa ggtaactggc ttcagcagag cgcagatacc   1320
aaatactggt cttctagtgt agccgtagt ttggccaccac ttcaagaact ctgtagcacc   1380
gcctacatac ctgctcttgc taatctgtt accagtggct gttgccagtg gcgataagtc   1440
gtgtcttacc ggggttgact caagacgata gttaccggat aaggcgcagc ggtcgggctg   1500
aacggggggg tcgtgcacac agcccagctt ggagcgaacg acctacaccg aactgagata   1560
cctacagcgt gagctatgag aaagcgccac gcttcccgaa gggagaaaag cggacaggta   1620
tccgtaagc ggcagggctg gaacaggaga gcgcacgagg gagcttccag ggggaaacgc   1680
ctggtatctt tatagtctg tcgggtttcg ccacctctga cttgagcgtc gatttttgtg   1740
atgctcgtca gggggggcga gcctatggaa aaacgccagc aacgcggcct ttttacgggt   1800
cctggccttt tgctggcctt ttgctcacat gttctttcct gcgttatccc ctgattctgt   1860
ggataaccgt attaccgctt ttgagtgagc tgataccgct cgccgcagcc gaacgaccga   1920
gcgcagcgag tcagtgagcg aggaagcggc cgtgaggct tgacatgatt ggtgcgtatg   1980
tttgtatgaa gctacaggac tgatttggcg ggctatgagg gcgggggaag ctctggaagg   2040

```

-continued

| | | | | | | |
|------------|-------------|-------------|-------------|------------|-------------|------|
| gccgcgatgg | ggcgcgcggc | gtccagaagg | cgccatacgg | cccgtggcg | gcacccatcc | 2100 |
| ggtataaaag | cccgcgaccc | cgaacgggtga | cctccacttt | cagcgacaaa | cgagcactta | 2160 |
| tacatacgcg | actattctgc | cgctatacat | aaccactcag | ctagcttaag | atcccatcaa | 2220 |
| gcttgcatgc | cgggcgcgcc | agaaggagcg | cagccaaaacc | aggatgatgt | ttgatggggt | 2280 |
| atttgagcac | ttgcaaccct | tatccggaag | ccccctggcc | cacaaaggct | aggcgccaat | 2340 |
| gcaagcagtt | cgcatgcagc | ccttgagcg | gtgcctcct | gataaacgg | ccagggggcc | 2400 |
| tatgttcttt | acttttttac | aagagaagtc | actcaacatc | ttaaaatggc | caggtgagtc | 2460 |
| gacgagcaag | cccggcggat | caggcagcgt | gcttgagat | ttgacttgca | acgcccgcac | 2520 |
| tgtgtcgacg | aaggcttttg | gtcctctgt | cgctgtctca | agcagcatct | aaccctgcgt | 2580 |
| cgccttttcc | atttgagga | gattcgaggt | accatgtacc | catacgatgt | tccagattac | 2640 |
| gcttcgccga | agaaaaagcg | caaggctgaa | gcgtccgaca | agaagtacag | catcggcctg | 2700 |
| gacatcggca | ccaactctgt | gggctgggcc | gtgatcaccg | acgagtacaa | ggtgcccagc | 2760 |
| aagaaattca | agggtgctgg | caacaccgac | cggcacagca | tcaagaagaa | cctgatcgga | 2820 |
| gccctgctgt | tcgacagcgg | cgaaacagcc | gaggccaccc | ggctgaagag | aaccgccaga | 2880 |
| agaagataca | ccagacggaa | gaaccggatc | tgctatctgc | aagagatctt | cagcaacgag | 2940 |
| atggccaagg | tggacgacag | cttcttccac | agactggaag | agtcttctct | ggtggaagag | 3000 |
| gataagaagc | acgagcggca | ccccatcttc | ggcaacatcg | tggacgaggt | ggcctaccac | 3060 |
| gagaagtacc | ccaccatcta | ccacctgaga | aagaaaactgg | tggacagcac | cgacaaggcc | 3120 |
| gacctgcggc | tgatctatct | ggccctggcc | cacatgatca | agttccgggg | ccacttctctg | 3180 |
| atcgagggcg | acctgaaccc | cgacaacagc | gacgtggaca | agctgttcat | ccagctggtg | 3240 |
| cagacctaca | accagctgtt | cgaggaaaac | cccatcaacg | ccagcggcgt | ggacgccaa | 3300 |
| gccatcctgt | ctgccagact | gagcaagagc | agacggctgg | aaaatctgat | cgcccagctg | 3360 |
| cccggcgaga | agaagaatgg | cctgttcggc | aacctgattg | ccctgagcct | gggctgacc | 3420 |
| cccaacttca | agagcaactt | cgacctggcc | gaggatgcca | aactgcagct | gagcaaggac | 3480 |
| acctacgacg | acgacctgga | caacctgctg | gcccagatcg | gcgaccagta | cgccgacctg | 3540 |
| tttctggcgg | ccaagaacct | gtccgacgcc | atcctgctga | gcgacatcct | gagagtgaac | 3600 |
| accgagatca | ccaaggcccc | cctgagcggc | tctatgatca | agagatacga | cgagcaccac | 3660 |
| caggacctga | ccctgctgaa | agctctcgtg | cggcagcagc | tgctgagaa | gtacaaagag | 3720 |
| attttctctg | accagagcaa | gaacggctac | gccggctaca | ttgacggcgg | agccagccag | 3780 |
| gaagagttct | acaagttcat | caagcccatc | ctggaaaaga | tggacggcac | cgaggaaactg | 3840 |
| ctcgtgaagc | tgaacagaga | ggacctgctg | cggaagcagc | ggaccttcga | caacggcagc | 3900 |
| atccccaccc | agatccacct | gggagagctg | cacgccattc | tgccggcgga | ggaagatttt | 3960 |
| taccatttcc | tgaaggacaa | ccgggaaaag | atcgagaaga | tcctgacctt | ccgcattccc | 4020 |
| tactacgtgg | gccctctggc | caggggaaac | agcagattcg | cctggatgac | cagaaagagc | 4080 |
| gaggaaacca | tcacccctctg | gaacttcgag | gaagtgggtg | acaagggcgc | ttccgccacg | 4140 |
| agcttcatcg | agcggatgac | caacttcgat | aagaacctgc | ccaacgagaa | ggtgctgccc | 4200 |
| aagcacagcc | tgtgttacga | gtacttcacc | gtgtataacg | agctgaccaa | agtgaataac | 4260 |
| gtgaccgagg | gaatgagaaa | gccgccttc | ctgagcggcg | agcagaaaaa | ggccatcgtg | 4320 |

-continued

| | |
|--|------|
| gacctgctgt tcaagaccaa ccggaaagt accgtgaagc agctgaaaga ggactacttc | 4380 |
| aagaaaatcg agtgcttcga ctccgtggaa atctccggcg tggaagatcg gttcaacgcc | 4440 |
| tccctgggca cataccacga tctgctgaaa attatcaagg acaaggactt cctggacaat | 4500 |
| gaggaaaacg aggacattct ggaagatata gtgctgaccc tgacactgtt tgaggacaga | 4560 |
| gagatgatcg aggaacggct gaaaacctat gccacactgt tcgacgacaa agtgatgaag | 4620 |
| cagctgaagc ggcggagata caccggctgg ggcaggctga gccggaagct gatcaacggc | 4680 |
| atccgggaca agcagtcggg caagacaata ctggatttcc tgaagtccga cggttcgcc | 4740 |
| aacagaaact tcattgcagct gatccacgac gacagcctga cctttaaga ggacatccag | 4800 |
| aaagcccagg tgtccggcca gggcgatagc ctgcacgagc acattgccaa tctggccggc | 4860 |
| agccccgcca ttaagaaggg catcctgcag acagtgaagg tggaggagca gctcgtgaaa | 4920 |
| gtgatgggcc ggcacaagcc cgagaacata gtgatcgaaa tggccagaga gaaccagacc | 4980 |
| accagaagg gacagaagaa cagcccgagc agaatgaagc ggatcgaa gaagcatcaaa | 5040 |
| gagctgggca gccagatcct gaaagaacac cccgtggaaa acaccagct gcagaacgag | 5100 |
| aagctgtacc tgtactacct gcagaatggg cgggatatgt acgtggacca ggaactggac | 5160 |
| atcaaccggc tgtccgacta cgatgtggac catatcgtgc ctcagagctt tctgaaggac | 5220 |
| gactccatcg acaacaaggt gctgaccaga agcgacaaga accggggcaa gagcgacaac | 5280 |
| gtgccctccg aagaggtcgt gaagaagatg aagaactact ggcggcagct gctgaacgcc | 5340 |
| aagctgatta cccagagaaa gttcgacaat ctgaccaagg ccgagagagg cggcctgagc | 5400 |
| gaactggata aggccggctt catcaagaga cagctggtgg aaaccggca gatcacaaag | 5460 |
| cacgtggcac agatcctgga ctcccggatg aacactaagt acgacgagaa tgacaagctg | 5520 |
| atccgggaag tgaaagtgat caccctgaag tccaagctgg tgtccgattt ccggaaggat | 5580 |
| ttccagtttt acaaaagtgc cgagatcaac aactaccacc acgcccacga cgctacctg | 5640 |
| aacgccgtcg tgggaaccgc cctgatcaaa aagtacccta agctggaaag cgagtctctg | 5700 |
| tacggcgact acaaggtgta cgacgtgcgg aagatgatcg ccaagagcga gcaggaaatc | 5760 |
| ggcaaggcta ccgccaagta cttcttctac agcaacatca tgaacttttt caagaccgag | 5820 |
| attaccctgg ccaacggcga gatccggaag cggcctctga tcgagacaaa cggcgaaacc | 5880 |
| ggggagatcg tgtgggataa gggccgggat tttgccaccg tgcggaaagt gctgagcatg | 5940 |
| ccccaaagtga atatcgtgaa aaagaccgag gtgcagacag gggccttcag caaagagtct | 6000 |
| atcctgcca agaggaacag cgataagctg atcgccagaa agaaggactg ggaccctaag | 6060 |
| aagtacggcg gcttcgacag cccacactg gcctattctg tgctgggtgg ggccaaagtg | 6120 |
| gaaaagggca agtccaagaa actgaagagt gtgaaagagc tgctggggat caccatcatg | 6180 |
| gaaagaagca gcttcgagaa gaatcccatc gactttcttg aagccaaggg ctacaaagaa | 6240 |
| gtgaaaaagg acctgatcat caagctgcct aagtactccc tgctcgagct ggaaaacggc | 6300 |
| cggaagagaa tgctggcctc tgccggcgaa ctgcagaagg gaaacgaact ggccctgccc | 6360 |
| tccaaatatg tgaacttctt gtacctggcc agccactatg agaagctgaa gggctcccc | 6420 |
| gaggataatg agcagaaaca gctgtttgtg gaacagcaca agcactacct ggacgagatc | 6480 |
| atcgagcaga tcagcgagtt ctccaagaga gtgatcctgg ccgacgctaa tctggacaaa | 6540 |
| gtgctgtccg cctacaacaa gcaccgggat aagcccatca gagagcaggc cgagaatatc | 6600 |

-continued

```

atccacctgt ttacctgac caatctggga gcccctgccc ccttcaagta ctttgacacc 6660
accatcgacc ggaagaggta caccagcacc aaagagggtg tggacgccac cctgatccac 6720
cagagcatca ccggcctgta cgagacacgg atcgacctgt ctgagctggg aggcgacagc 6780
cccaagaaga agagaaagggt ggaggccagc taacatatga ttgcaatgtc tttcttgccg 6840
tatgacactt ccagcaaaag gtaggcgagg ctgcgagacg gcttcccggc gctgcatgca 6900
acaccgatga tgcttcgacc ccccgaagct ccttcggggc tgcattggcg ctccgatgcc 6960
gtccaggggc gagcgctgtt taaatagcca ggccccgat tgcaagaca ttatagcgag 7020
ctaccaaagc catattcaaa cacctagatc actaccactt ctacacaggc cactcgagct 7080
tgtgatcgca ctccgctaag gggggccctc ttcctcttcg tttcagtcac aaccgcgaaa 7140
catgacacaa gaatccctgt tactttctga ccgtattgat tcggatgatt cctacgcgag 7200
cctgcggaac gaccaggaat tctgggaggt gagtcgacga gcaagcccgg cggatcaggc 7260
agcgtgcttg cagatttgac ttgcaacgcc cgcatttgtt cgacgaaggc ttttggtccc 7320
tctgtcgtg tctcaagcag catctaacc tgcgtcgccg tttccatttg cagccgctgg 7380
cccgcgagc cctggaggag ctccggctgc cggcgccgcc ggtgctgcgg gtgcccggcg 7440
agagcaccaa ccccgactg gtccggcagc ccggcccggg gatcaagctg ttcggcgagc 7500
actgtgctg tccggagagc ctccgctcgg agtcggaggc gtacgcggtc ctggcgagc 7560
ccccggtgcc ggtgcccgc ctccctcgcc cggcgagctc gcggcccggc accggagcct 7620
ggccgtggcc ctacctggtg atgagccgga tgaccggcac cacctggcgg tccgcgatgg 7680
acggcacgac cgaccggaac gcgctgctc ccttgcccgc cgaactcggc cgggtgctcg 7740
gccggctgca cagggtgccc ctgaccggga acaccgtgct cccccccat tccgaggtct 7800
tcccgaact gctcgggaa cgcgcgcgg cgaccgtcga ggaccaccgc ggggtgggct 7860
acctctcgcc ccggtgctg gaccgcctgg aggactggct gccggacgtg gacacgtgc 7920
tggccggccg cgaaccccgg ttcgtccacg gcgacctgca cgggaccaac atcttcgtgg 7980
acctggccgc gaccgaggtc accgggatcg tcgacttcac cgacgtctat gcgggagact 8040
cccgtacag cctggtgcaa ctgcatctca acgccttcg gggcgaccgc gagatcctgg 8100
ccgcgctgct cgacggggcg cagtggaagc ggaccgagga cttcgcccgc gaactgctcg 8160
ccttcacctt cctgcacgac ttcgaggtgt tcgaggagac ccgctggat ctctccggct 8220
tcaccgatcc ggaggaaact gcgcagttcc tctgggggccc gccggacacc gccccggcg 8280
cctgataagg atccggcaag actggccccg cttggcaacg caacagtgag cccctcccta 8340
gtgtgtttgg ggatgtgact atgtattcgt gtgttgcca acgggtcaac ccgaacagat 8400
tgataccgc cttggcattt cctgtcagaa tgtaacgtca gttgatggta ct 8452

```

<210> SEQ ID NO 23

<211> LENGTH: 102

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

<400> SEQUENCE: 23

```

gttttagagc tatgctgttt tgaatgggcc caaacggaa gggcctgagt ccgagcagaa 60

```

-continued

gaagaagttt tagagctatg ctgttttgaa tggccccaaa ac 102

<210> SEQ ID NO 24
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

cggaggacaa agtacaaacg gcagaagctg gaggaggaag ggctgagtc cgagcagaag 60

aagaagggt cccatcacat caaccggtgg cgcattgcc 100

<210> SEQ ID NO 25
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

agctggagga ggaagggcct gagtccgagc agaagaagaa gggtcccac 50

<210> SEQ ID NO 26
<211> LENGTH: 30
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 26

gaguccgagc agaagaagaa guuuuagagc 30

<210> SEQ ID NO 27
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

agctggagga ggaagggcct gagtccgagc agaagagaag gggtcccat 49

<210> SEQ ID NO 28
<211> LENGTH: 53
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

ctggaggagg aagggcctga gtccgagcag aagaagaagg gctcccatca cat 53

<210> SEQ ID NO 29
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

ctggaggagg aagggcctga gtccgagcag aagagaagg gctcccatcac at 52

<210> SEQ ID NO 30
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

ctggaggagg aagggcctga gtccgagcag aagaagaag gggtcccatc acat 54

-continued

<210> SEQ ID NO 31
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 31

ctggaggagg aagggcctga gtccgagcag aagaagggt cccatcacat 50

<210> SEQ ID NO 32
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 32

ctggaggagg aagggcctga gcccgagcag aagggtccc atcacat 47

<210> SEQ ID NO 33
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 33

ctggaggagg aagggcctga gtccgagcag aagaagaagg gctcccat 48

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 34

gaguccgagc agaagaagau 20

<210> SEQ ID NO 35
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 35

gaguccgagc agaagaagua 20

<210> SEQ ID NO 36
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 36

gaguccgagc agaagaacaa 20

<210> SEQ ID NO 37
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 37

gaguccgagc agaagaugaa 20

<210> SEQ ID NO 38
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 38

gaguccgagc agaaguagaa

20

<210> SEQ ID NO 39

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 39

gaguccgagc agaugaagaa

20

<210> SEQ ID NO 40

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 40

gaguccgagc acaagaagaa

20

<210> SEQ ID NO 41

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 41

gaguccgagg agaagaagaa

20

<210> SEQ ID NO 42

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 42

gaguccgugc agaagaagaa

20

<210> SEQ ID NO 43

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 43

gagucggagc agaagaagaa

20

<210> SEQ ID NO 44

<211> LENGTH: 20

<212> TYPE: RNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

gagaccgagc agaagaagaa

20

<210> SEQ ID NO 45

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 45

-continued

aatgacaagc ttgctagcgg tggg 24

<210> SEQ ID NO 46
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 46

aaaacggaag ggcctgagtc cgagcagaag aagaagttt 39

<210> SEQ ID NO 47
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 47

aaacaggggc cgagattggg tggtcagggc agaggtttt 39

<210> SEQ ID NO 48
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 48

aaaacggaag ggcctgagtc cgagcagaag aagaagtt 38

<210> SEQ ID NO 49
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 49

aacggaggga ggggcacaga tgagaaactc agggtttttag 40

<210> SEQ ID NO 50
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 50

agcccttttt cttctgctcg gactcaggcc cttctctc 38

<210> SEQ ID NO 51
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 51

cagggaggga ggggcacaga tgagaaactc aggaggcccc 40

<210> SEQ ID NO 52

-continued

<211> LENGTH: 80
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 52

ggcaatgcgc caccggttga tgtgatggga gcccttctag gaggecccca gageagccac 60

tggggcctca acactcaggc 80

<210> SEQ ID NO 53
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 53

catcgatgtc ctccccattg gcctgcttcg tgg 33

<210> SEQ ID NO 54
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 54

ttcgtggcaa tgcgccaccg gttgatgtga tgg 33

<210> SEQ ID NO 55
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 55

tcgtggcaat gcgccaccg ttgatgtgat ggg 33

<210> SEQ ID NO 56
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 56

tccagcttct gccgtttgta ctttgtctc cgg 33

<210> SEQ ID NO 57
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 57

ggagggaggg gcacagatga gaaactcagg agg 33

<210> SEQ ID NO 58
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 58

aggggccgag attgggtggt cagggcagag agg 33

<210> SEQ ID NO 59
<211> LENGTH: 33

-continued

<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 59

caagcactga gtgccattag ctaaatgcat agg 33

<210> SEQ ID NO 60
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 60

aatgcatagg gtaccacca caggtgccag ggg 33

<210> SEQ ID NO 61
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 61

acacacatgg gaaagcctct gggccaggaa agg 33

<210> SEQ ID NO 62
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 62

ggaggaggta gtatacagaa acacagagaa gtagaat 37

<210> SEQ ID NO 63
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 63

agaatgtaga ggagtcacag aaactcagca ctagaaa 37

<210> SEQ ID NO 64
<211> LENGTH: 98
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 64

ggacgaaaca ccggaacctat tcaaaacagc atagcaagtt aaaataaggc tagtccgtta 60

tcaacttgaa aaagtggcac cgagtcggtg cttttttt 98

<210> SEQ ID NO 65
<211> LENGTH: 186
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
polynucleotide

<400> SEQUENCE: 65

ggacgaaaca ccggtagtagt taagtattgt tttatggctg ataaatttct ttgaatttct 60

ccttgattat ttgttataaa agttataaaa taatcttggtt ggaaccattc aaaacagcat 120

-continued

agcaagttaa aataaggcta gtcggttatc aacttgaaaa agtggcaccg agtcggtgct 180

tttttt 186

<210> SEQ ID NO 66

<211> LENGTH: 95

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 66

gggttttaga gctatgctgt tttgaatggt cccaaaacgg gtcttcgaga agacgtttta 60

gagctatgct gttttgaatg gtcccaaac ttttt 95

<210> SEQ ID NO 67

<211> LENGTH: 36

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (5)..(34)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 67

aaacnnnnnn nnnnnnnnnn nnnnnnnnnn nnnngt 36

<210> SEQ ID NO 68

<211> LENGTH: 36

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (7)..(36)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 68

taaaacnnnn nnnnnnnnnn nnnnnnnnnn nnnnnn 36

<210> SEQ ID NO 69

<211> LENGTH: 84

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 69

gtggaaagga cgaaacaccg ggtcttcgag aagacctgtt ttagagctag aaatagcaag 60

ttaaaataag gctagtcogt tttt 84

<210> SEQ ID NO 70

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

-continued

```

    oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (6)..(24)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 70
caccgnnnnn nnnnnnnnnn nnnn                                     24

<210> SEQ ID NO 71
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
    oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (5)..(23)
<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 71
aaacnnnnnn nnnnnnnnnn nnnn                                     24

<210> SEQ ID NO 72
<211> LENGTH: 46
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
    oligonucleotide
<220> FEATURE:
<221> NAME/KEY: modified_base
<222> LOCATION: (1)..(19)
<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 72
nnnnnnnnnn nnnnnnnnng uuauuguacu cucaagauuu auuuuuu         46

<210> SEQ ID NO 73
<211> LENGTH: 91
<212> TYPE: RNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
    oligonucleotide

<400> SEQUENCE: 73
guuacuuaaa ucuugcagaa gcuacaaaga uaaggcuuca ugccgaaauc aacaccugu   60
cauuuuuagg caggguuuu ucguauuuu a                                     91

<210> SEQ ID NO 74
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 74
ttttctagtg ctgagtttct gtgactcttc tacattctac ttctctgtgt ttctgtatac   60
tacctcctcc                                                         70

<210> SEQ ID NO 75
<211> LENGTH: 122
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

```

-continued

<400> SEQUENCE: 75

ggaggaaggg cctgagtccg agcagaagaa gaaggggtcc catcacatca accggtggcg 60
cattgccacg aagcaggcca atggggagga catcgatgtc acctccaatg actaggggtg 120
gc 122

<210> SEQ ID NO 76

<211> LENGTH: 48

<212> TYPE: RNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (3)..(32)

<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 76

acnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnguuiuaga gcuaugcu 48

<210> SEQ ID NO 77

<211> LENGTH: 67

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<223> OTHER INFORMATION: Description of Combined DNA/RNA Molecule:
Synthetic oligonucleotide

<400> SEQUENCE: 77

agcauagcaa guuaaaauaa ggctaguccg uuaucacuu gaaaaagugg caccgagucg 60
gugcuuu 67

<210> SEQ ID NO 78

<211> LENGTH: 62

<212> TYPE: RNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (1)..(20)

<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 78

nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaaagc aaguuaaaau aaggcuaguc 60
cg 62

<210> SEQ ID NO 79

<211> LENGTH: 73

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 79

tgaatggtcc caaaacggaa gggcctgagt ccgagcagaa gaagaagttt tagagctatg 60

-continued

ctgttttgaa tgg 73

<210> SEQ ID NO 80
<211> LENGTH: 69
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 80

ctggtcttcc acctctctgc cctgaacacc caatctcggc ccctctcgcc acctctctgc 60

atttctgtt 69

<210> SEQ ID NO 81
<211> LENGTH: 138
<212> TYPE: DNA
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 81

acccaagcac tgagtcccat tagctaaatg catagggtac caccacaggg tgccaggggc 60

ctttcccaaa gttcccagcc ccttctccaa cctttctctgg ccagagggt ttcccatgtg 120

tgtggctgga ccctttga 138

<210> SEQ ID NO 82
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
primer

<400> SEQUENCE: 82

aaaaccaccc ttctctctgg c 21

<210> SEQ ID NO 83
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
primer

<400> SEQUENCE: 83

ggagattgga gacacggaga g 21

<210> SEQ ID NO 84
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
primer

<400> SEQUENCE: 84

ctggaaagcc aatgcctgac 20

<210> SEQ ID NO 85
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
primer

-continued

<400> SEQUENCE: 85

ggcagcaaac tccttgctct

20

<210> SEQ ID NO 86

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 86

gtgctttgca gaggcctacc

20

<210> SEQ ID NO 87

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 87

cctggagcgc atgcagtagt

20

<210> SEQ ID NO 88

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 88

accttctgtg tttccaccat tc

22

<210> SEQ ID NO 89

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 89

ttggggagtg cacagacttc

20

<210> SEQ ID NO 90

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic primer

<400> SEQUENCE: 90

ggctccctgg gttcaaagta

20

<210> SEQ ID NO 91

<211> LENGTH: 21

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

-continued

primer

<400> SEQUENCE: 91

agaggggtct ggatgtcgta a 21

<210> SEQ ID NO 92

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic probe

<400> SEQUENCE: 92

tagctctaaa acttcttctt ctgctcggac 30

<210> SEQ ID NO 93

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic probe

<400> SEQUENCE: 93

ctagccttat ttaacttgc tatgctgttt 30

<210> SEQ ID NO 94

<211> LENGTH: 99

<212> TYPE: RNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (1)..(20)

<223> OTHER INFORMATION: a, c, u, g, unknown or other

<400> SEQUENCE: 94

nnnnnnnnnn nnnnnnnnnn guuuuagagc uagaaauagc aaguuaaaau aaggcuaguc 60

cguuaucaac uugaaaaagu ggcaccgagu cggugcuuu 99

<210> SEQ ID NO 95

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 95

tagcgggtaa gc 12

<210> SEQ ID NO 96

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 96

tcggtcacat gt 12

<210> SEQ ID NO 97

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 97

actccccgta gg 12

<210> SEQ ID NO 98

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 98

actgcgtggt aa 12

<210> SEQ ID NO 99

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 99

acgtcgctg at 12

<210> SEQ ID NO 100

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 100

taggtcgacc ag 12

<210> SEQ ID NO 101

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 101

ggcgtaatg at 12

<210> SEQ ID NO 102

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 102

tgctgcatgt ta 12

<210> SEQ ID NO 103

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 103

atggaaacgc at 12

<210> SEQ ID NO 104

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 104

gccgaattcc tc 12

-continued

<210> SEQ ID NO 105
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 105

gcatggtacg ga 12

<210> SEQ ID NO 106
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 106

cggtactctt ac 12

<210> SEQ ID NO 107
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 107

gcctgtgccg ta 12

<210> SEQ ID NO 108
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 108

tacggtaaagt cg 12

<210> SEQ ID NO 109
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 109

cacgaaatta cc 12

<210> SEQ ID NO 110
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 110

aaccaagata cg 12

<210> SEQ ID NO 111
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 111

gagtcgatac gc 12

<210> SEQ ID NO 112
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 112

-continued

gtctcagcat cg 12

<210> SEQ ID NO 113
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 113

tcgtcgggtg ca 12

<210> SEQ ID NO 114
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 114

actccgtagt ga 12

<210> SEQ ID NO 115
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 115

caggacgtcc gt 12

<210> SEQ ID NO 116
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 116

tcgtatccct ac 12

<210> SEQ ID NO 117
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 117

tttcaaggcc gg 12

<210> SEQ ID NO 118
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 118

cgccggtgga at 12

<210> SEQ ID NO 119
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 119

gaaccgtcc ta 12

<210> SEQ ID NO 120
<211> LENGTH: 12

-continued

<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 120

gattcatcag cg 12

<210> SEQ ID NO 121
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 121

acaccgtctc tc 12

<210> SEQ ID NO 122
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 122

atcgtgccct aa 12

<210> SEQ ID NO 123
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 123

gcgtcaatgt tc 12

<210> SEQ ID NO 124
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 124

ctccgtatct cg 12

<210> SEQ ID NO 125
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 125

ccgattcctt cg 12

<210> SEQ ID NO 126
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 126

tgcgcctcca gt 12

<210> SEQ ID NO 127
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 127

taacgtcgga gc 12

-continued

<210> SEQ ID NO 128
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 128

aaggtcgccc at 12

<210> SEQ ID NO 129
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 129

ctcggggact at 12

<210> SEQ ID NO 130
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 130

ttcgagcgat tt 12

<210> SEQ ID NO 131
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 131

tgagtcgtcg ag 12

<210> SEQ ID NO 132
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 132

tttacgcaga gg 12

<210> SEQ ID NO 133
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 133

aggaagtatc gc 12

<210> SEQ ID NO 134
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 134

actcgatacc at 12

<210> SEQ ID NO 135
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 135

cgctacatag ca 12

<210> SEQ ID NO 136

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 136

ttcataaccg gc 12

<210> SEQ ID NO 137

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 137

ccaaacgggt aa 12

<210> SEQ ID NO 138

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 138

cgattccttc gt 12

<210> SEQ ID NO 139

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 139

cgtcatgaat aa 12

<210> SEQ ID NO 140

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 140

agtggcgatg ac 12

<210> SEQ ID NO 141

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 141

cccctacggc ac 12

<210> SEQ ID NO 142

<211> LENGTH: 12

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 142

gccaaaccgc ac 12

-continued

<210> SEQ ID NO 143
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 143

tgggacacg gt 12

<210> SEQ ID NO 144
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 144

ttgactgcgg cg 12

<210> SEQ ID NO 145
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 145

actatgcgta gg 12

<210> SEQ ID NO 146
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 146

tcacccaaag cg 12

<210> SEQ ID NO 147
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 147

gcaggacgtc cg 12

<210> SEQ ID NO 148
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 148

acaccgaaaa cg 12

<210> SEQ ID NO 149
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 149

cgggtgtattg ag 12

<210> SEQ ID NO 150
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 150

-continued

cacgaggtat gc 12

<210> SEQ ID NO 151
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 151

taaagcgacc cg 12

<210> SEQ ID NO 152
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 152

cttagtcggc ca 12

<210> SEQ ID NO 153
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 153

cgaaaacgtg gc 12

<210> SEQ ID NO 154
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 154

egtgccctga ac 12

<210> SEQ ID NO 155
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 155

tttaccatcg aa 12

<210> SEQ ID NO 156
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 156

cgtagccatg tt 12

<210> SEQ ID NO 157
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 157

cccaaacggt ta 12

<210> SEQ ID NO 158
<211> LENGTH: 12

-continued

<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 158

gcgttatcag aa 12

<210> SEQ ID NO 159
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 159

tcgatggtaa ac 12

<210> SEQ ID NO 160
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 160

cgactttttg ca 12

<210> SEQ ID NO 161
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 161

tcgacgactc ac 12

<210> SEQ ID NO 162
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 162

acgcgtcaga ta 12

<210> SEQ ID NO 163
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 163

cgtacggcac ag 12

<210> SEQ ID NO 164
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 164

ctatgccgtg ca 12

<210> SEQ ID NO 165
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 165

cgcgtcagat at 12

-continued

<210> SEQ ID NO 166
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 166

aagatcggta gc 12

<210> SEQ ID NO 167
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 167

cttcgcaagg ag 12

<210> SEQ ID NO 168
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 168

gtcgtggact ac 12

<210> SEQ ID NO 169
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 169

ggtcgtcatc aa 12

<210> SEQ ID NO 170
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 170

gttaacagcg tg 12

<210> SEQ ID NO 171
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 171

tagctaaccg tt 12

<210> SEQ ID NO 172
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 172

agtaaaggcg ct 12

<210> SEQ ID NO 173
<211> LENGTH: 12
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

-continued

<400> SEQUENCE: 173

ggtaatttcg tg 12

<210> SEQ ID NO 174

<211> LENGTH: 15

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 174

cagaagaaga agggc 15

<210> SEQ ID NO 175

<211> LENGTH: 51

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 175

ccaatgggga ggacatcgat gtcacctcca atgactaggg tgggtgggcaa c 51

<210> SEQ ID NO 176

<211> LENGTH: 15

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 176

ctctggccac tccct 15

<210> SEQ ID NO 177

<211> LENGTH: 52

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 177

acatcgatgt cacctccaat gacaagcttg ctacgggtgg gcaaccacaa ac 52

<210> SEQ ID NO 178

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (6)..(25)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 178

caccgnnnnn nnnnnnnnnn nnnnn 25

<210> SEQ ID NO 179

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<220> FEATURE:

<221> NAME/KEY: modified_base

<222> LOCATION: (5)..(24)

<223> OTHER INFORMATION: a, c, t, g, unknown or other

<400> SEQUENCE: 179

-continued

aaacnnnnnnnn nnnnnnnnnn nnnnc

25

<210> SEQ ID NO 180

<211> LENGTH: 54

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 180

aacaccgggt cttcgagaag acctgtttta gagctagaaa tagcaagtta aaat

54

<210> SEQ ID NO 181

<211> LENGTH: 54

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 181

caaaacgggt cttcgagaag acgttttaga gctatgctgt tttgaatggt ccca

54

1-50. (canceled)**51.** A method of selecting one or more prokaryotic cell(s), comprising:

- (a) introducing to a population of prokaryotic cells an engineered, programmable, non-naturally occurring CRISPR-Cas system comprising a Cas protein and one or more guide RNAs that target genomic loci of DNA molecules encoding one or more gene products in prokaryotic cell(s) of the population, wherein the one or more guide RNAs comprise a guide sequence, a tracr sequence, and a tracr mate sequence, and wherein the Cas protein and the one or more guide RNAs do not naturally occur together;

- (b) allowing the Cas protein to cleave the genomic loci of the DNA molecules encoding the one or more gene products,

wherein cleavage of the genomic loci induces cell death, thereby allowing one or more prokaryotic cell(s) having one or more mutations in the genomic loci to be selected.

52. The method of claim **51**, wherein the Cas protein is a type II CRISPR system Cas protein.

53. The method of claim **52**, wherein the Cas protein is Cas9 enzyme.

54. The method of claim **53**, wherein the Cas9 enzyme is a *Streptococcus pyogenes* Cas9 enzyme.

55. The method of claim **51**, wherein the prokaryotic cells are *S. pneumoniae*.

56. The method of claim **51**, wherein the one or more guide RNAs target an antibiotic resistance gene.

* * * * *