(12) STANDARD PATENT (11) Application No. **AU 2020221274 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Crispr/Cas fusion proteins and systems

(51) International Patent Classification(s)
C12N 9/22 (2006.01) ***C12N 15/10*** (2006.01)

(21) Application No: **2020221274** (22) Date of Filing: **2020.02.13**

(87) WIPO No: **WO20/168102**

(30) Priority Data

(31) Number	(32) Date	(33) Country
62/806,708	**2019.02.15**	**US**

(43) Publication Date: **2020.08.20**
(44) Accepted Journal Date: **2024.02.08**

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(56) Related Art
WO 2018/027078 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 August 2020 (20.08.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/168102 A1

(51) International Patent Classification:
C12N 9/22 (2006.01) *C12N 15/10* (2006.01)

(21) International Application Number:
PCT/US2020/018145

(22) International Filing Date:
13 February 2020 (13.02.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/806,708 15 February 2019 (15.02.2019) US

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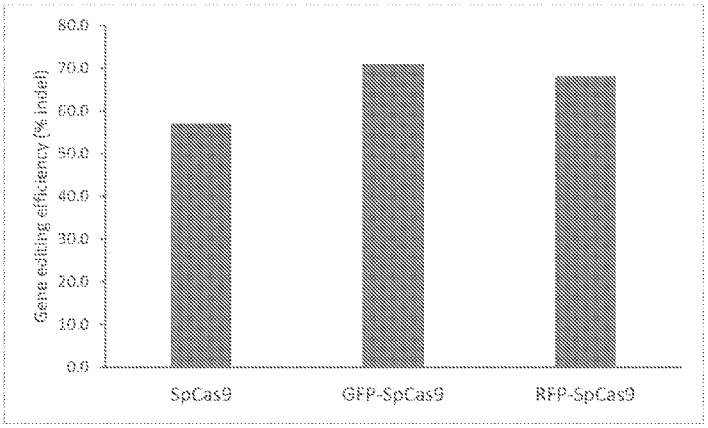
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: CRISPR/CAS FUSION PROTEINS AND SYSTEMS

FIG. 1



(57) Abstract: Engineered Cas9 systems are disclosed herein.

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CRISPR/CAS FUSION PROTEINS AND SYSTEMS

RELATED APPLICATIONS

[0001] The present application claims the benefit of priority of U.S. Provisional Application No. 62/806,708, filed February 15, 2019, the entirety of which is incorporated herein by reference.

FIELD

[0002] The present disclosure relates to engineered Cas9 systems, nucleic acids encoding said systems, and methods of using said systems for genome modification.

BACKGROUND

[0003] Many different types of peptide linkers have been tested to fuse GFP to Cas9, but typically result in lower activity of the underlying Cas9.

SUMMARY OF THE DISCLOSURE

[0004] Among the various aspects of the present disclosure include engineered Cas9 systems.

[0005] Other aspects and features of the disclosure are detailed bellow.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] **FIG. 1** shows that the Cas9 fusion proteins disclosed herein each retain the editing activity parallel to the level of SpCas9 protein.

[0007] **FIG. 2A** and **FIG. 2B** show that the editing efficiencies of the Cas9 fusion proteins disclosed herein were several-fold higher than that of the commercial proteins in all targets.

SEQUENCE LISTING

[0008] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 13, 2020, is named P19-027_WO-PCT_SL.txt and is 87,735 bytes in size.

DETAILED DESCRIPTION

[0009] Fusion of accessory proteins to CRISPR proteins creates a wide range of opportunities to localize various protein functionalities to defined locations within cells. Among other things, peptide linkers which enable fusion of heterologous proteins to CRISPR proteins in ways that preserve CRISPR functionality are disclosed.

(I) Engineered Cas9 Systems

[0010] One aspect of the present disclosure provides engineered Cas9 proteins and systems. For example, Cas9-marker fusion proteins are disclosed. In some aspects, systems include engineered Cas9 proteins and engineered guide RNAs, wherein each engineered guide RNA is designed to complex with a specific engineered Cas9 protein. These engineered Cas9 systems do not occur naturally.

(a) Engineered Cas9 Proteins

[0011] Cas9 protein is the single effector protein in Type II CRISPR systems, which are present in various bacteria. The engineered Cas9 protein disclosed herein can be from *Acaryochloris sp., Acetohalobium sp., Acidaminococcus sp., Acidithiobacillus sp., Acidothermus* sp., *Akkermansia sp., Alicyclobacillus sp., Allochromatium sp., Ammonifex sp., Anabaena sp., Arthrospira sp., Bacillus sp., Bifidobacterium sp., Burkholderiales sp., Caldicelulosiruptor sp., Campylobacter sp., Candidatus sp., Clostridium sp., Corynebacterium sp., Crocosphaera sp., Cyanothece sp., Exiguobacterium sp., Fibrobacter sp., Finegoldia sp., Francisella sp., Ktedonobacter sp., Lachnospiraceae sp., Lactobacillus sp., Listeria sp., Lyngbya sp., Marinobacter sp., Methanohalobium sp., Microscilla sp., Microcoleus sp., Microcystis sp., Mycoplasma sp., Natranaerobius sp., Neisseria sp., Nitratifractor sp., Nitrosococcus sp., Nocardiopsis sp., Nodularia sp., Nostoc sp., Oenococcus sp., Oscillatoria sp., Parasutterella sp., Pasteurella sp., Parvibaculum sp., Pelotomaculum sp., Petrotoga sp., Polaromonas sp., Prevotella sp., Pseudoalteromonas sp., Ralstonia sp., Rhodospirillum sp., Staphylococcus sp., Streptococcus sp., Streptomyces sp., Streptosporangium sp., Synechococcus sp., Thermosipho sp., Treponema sp., Verrucomicrobia* sp., and *Wolinella sp.*

[0012] Exemplary species that the Cas9 protein or other components may be from or derived from include *Acaryochloris* spp. (*e.g., Acaryochloris marina*), *Acetohalobium* spp. (*e.g.*, *Acetohalobium arabaticum*), *Acidaminococcus* spp., *Acidithiobacillus* spp. (*e.g.*, *Acidithiobacillus caldus, Acidithiobacillus ferrooxidans*), *Acidothermus* spp., *Akkermansia* spp., *Alicyclobacillus* spp. (*e.g.*, *Alicyclobacillus acidocaldarius*), *Allochromatium* spp. (*e.g.*, *Allochromatium vinosum*), *Ammonifex* spp. (*e.g.*, *Ammonifex degensii*), *Anabaena* spp. (*e.g.*, *Anabaena variabilis*), *Arthrospira* spp. (*e.g.*, *Arthrospira maxima, Arthrospira platensis*), *Bacillus* spp. (*e.g.*, *Bacillus pseudomycoides, Bacillus selenitireducens*), *Bifidobacterium* spp., *Burkholderiales* spp. (*e.g.*, *Burkholderiales bacterium*), *Caldicelulosiruptor spp.* (*e.g.*, *Caldicelulosiruptor becscii*), *Campylobacter* spp. (*e.g.*, *Campylobacter jejuni, Campylobacter lari*), *Candidatus* spp., (*e.g.*, *Candidatus Desulforudis*), *Clostridium* spp. (*e.g.*, *Clostridium botulinum, Clostridium difficile*), *Corynebacterium* spp. (*e.g.*, *Corynebacterium diphtheria*), *Crocosphaera* spp. (*e.g.*, *Crocosphaera watsonii*), *Cyanothece* spp., *Deltaproteobacterium* spp., *Exiguobacterium* spp. (*e.g.*, *Exiguobacterium sibiricum*), (*Fibrobacter* spp. (*e.g.*, *Fibrobacter succinogene*), *Finegoldia* spp. (*e.g.*, *Finegoldia magna*), *Francisella* spp. (*e.g.*, *Francisella novicida*), *Gammaproteobacterium, Ktedonobacter* spp. (*e.g.*, *Ktedonobacter racemifer*), *Lachnospiraceae* spp., *Lactobacillus* spp. (*e.g.*, *Lactobacillus buchneri, Lactobacillus delbrueckii, Lactobacillus gasseri, Lactobacillus salivarius*), *Listeria* spp. (e.g., *Listeria innocua*), *Leptotrichia* spp., *Lyngbya* spp., *Marinobacter* spp., *Methanohalobium* spp. (*e.g.*, *Methanohalobium evestigatum*), *Microcoleus* spp. (*e.g.*, *Microcoleus chthonoplastes*), *Microscilla* spp. (*e.g.*, *Microscilla marina*), *Microcystis* spp. (*e.g.*, *Microcystis aeruginosa*), *Mycoplasma* spp., *Natranaerobius* spp. (*e.g.*, *Natranaerobius thermophilus*), *Neisseria* spp. (*e.g.*, *Neisseria cinerea, Neisseria meningitidis*), *Nitratifractor* spp., *Nitrosococcus* spp. (*e.g.*, *Nitrosococcus halophilus, Nitrosococcus watsoni*), *Nocardiopsis* spp. (*e.g.*, *Nocardiopsis dassonvillei*), *Nodularia* spp. (*e.g.*, *Nodularia spumigena*), *Nostoc* spp., *Oenococcus* spp., *Oscillatoria* spp., *Parasutterella* spp., *Parvibaculum* spp. (*e.g.*, *Parvibaculum lavamentivorans*), *Pasteurella* spp. (*e.g.*, *Pasteurella multocida*), *Pelotomaculum* spp., (*e.g.*, *Pelotomaculum thermopropionicum*), *Petrotoga* spp. (*e.g.*, *Petrotoga mobilis*),

Planctomyces spp., Polaromonas spp. (*e.g.*, *Polaromonas naphthalenivorans*), *Prevotella* spp., *Pseudoalteromonas* spp. (*e.g.*, *Pseudoalteromonas haloplanktis*), *Ralstonia* spp., *Ruminococcus* spp., *Rhodospirillum* spp. (*e.g.*, *Rhodospirillum rubrum*), *Staphylococcus* spp. (*e.g.*, *Staphylococcus aureus*), *Streptococcus* spp. (*e.g.*, *Streptococcus pasteurianus, Streptococcus pyogenes, Streptococcus thermophilus*), *Sutterella* spp. (*e.g.*, *Sutterella wadsworthensis*), *Streptomyces* spp. (*e.g.*, *Streptomyces pristinaespiralis, Streptomyces viridochromogenes, Streptomyces viridochromogenes*), *Streptosporangium* spp. (*e.g.*,*Streptosporangium roseum, Streptosporangium roseum*), *Synechococcus* spp., *Thermosipho* spp. (*e.g.*, *Thermosipho africanus*), *Treponema* spp. (*e.g.*, *Treponema denticola*), and *Verrucomicrobia* spp., *Wolinella* spp. (*e.g.*, *Wolinella succinogenes*), and/or species delineated in bioinformatic surveys of genomic databases such as those disclosed in Makarova, Kira S., et al. "An updated evolutionary classification of CRISPR–Cas systems." Nature Reviews Microbiology 13.11 (2015): 722 and Koonin, Eugene V., Kira S. Makarova, and Feng Zhang. "Diversity, classification and evolution of CRISPR-Cas systems." Current opinion in microbiology 37 (2017): 67-78, each of which is hereby incorporated by reference herein in their entirety.

[0013] In some embodiments, the engineered Cas9 protein may be from *Streptococcus pyogenes*. In some embodiments, the engineered Cas9 protein may be from *Streptococcus thermophilus*. In some embodiments, the engineered Cas9 protein may be from *Neisseria meningitidis*. In some embodiments, the engineered Cas9 protein may be from *Staphylococcus aureus*. In some embodiments, the engineered Cas9 protein may be from *Campylobacter jejuni*.

[0014] Wild-type Cas9 proteins comprise two nuclease domains, *i.e.*, RuvC and HNH domains, each of which cleaves one strand of a double-stranded sequence. Cas9 proteins also comprise REC domains that interact with the guide RNA (*e.g.*, REC1, REC2) or the RNA/DNA heteroduplex (*e.g.*, REC3), and a domain that interacts with the protospacer-adjacent motif (PAM) (*i.e.*, PAM-interacting domain).

[0015] The Cas9 protein can be engineered to comprise one or more modifications (*i.e.*, a substitution of at least one amino acid, a deletion of at least one amino acid, an insertion of at least one amino acid) such that the Cas9 protein has altered activity, specificity, and/or stability.

[0016] For example, Cas9 protein can be engineered by one or more mutations and/or deletions to inactivate one or both of the nuclease domains. Inactivation of one nuclease domain generates a Cas9 protein that cleaves one strand of a double-stranded sequence (*i.e.*, a Cas9 nickase). The RuvC domain can be inactivated by mutations such as D10A, D8A, E762A, and/or D986A, and the HNH domain can be inactivated by mutations such as H840A, H559A, N854A, N856A, and/or N863A (with reference to the numbering system of *Streptococcus pyogenes* Cas9, SpyCas9). Inactivation of both nuclease domains generates a Cas9 protein having no cleavage activity (*i.e.*, a catalytically inactive or dead Cas9).

[0017] The Cas9 protein can also be engineered by one or more amino acid substitutions, deletions, and/or insertions to have improved targeting specificity, improved fidelity, altered PAM specificity, decreased off-target effects, and/or increased stability. Non-limiting examples of one or more mutations that improve targeting specificity, improve fidelity, and/or decrease off-target effects include N497A, R661A, Q695A, K810A, K848A, K855A, Q926A, K1003A, R1060A, and/or D1135E (with reference to the numbering system of SpyCas9).

[0018] In alternative embodiments, the Cas protein may be from a Type I CRISPR/Cas system. In some embodiments, the Cas protein may be a component of the Cascade complex of a Type-I CRISPR/Cas system. For example, the Cas protein may be a Cas3 protein. In some embodiments, the Cas protein may be from a Type III CRISPR/Cas system. In some embodiments, the Cas protein may be from a Type IV CRISPR/Cas system. In some embodiments, the Cas protein may be from a Type V CRISPR/Cas system. In some embodiments, the Cas protein may be from a Type VI CRISPR/Cas system. In some embodiments, the Cas protein may have an RNA cleavage activity. In various embodiments, the Cas protein may be classified as Cas9, Cas12a (a.k.a. Cpf1), Cas12b, Cas12c, Cas12d, Cas12e (a.k.a. CasX), Cas13a, or Cas13b.

(i) Heterologous domains

[0019] The Cas9 protein can be engineered to comprise at least one heterologous domain, *i.e.*, Cas9 is fused to one or more heterologous domains. In situations in which two or more heterologous domains are fused with Cas9, the two or more heterologous domains can be the same or they can be different. The one or more heterologous domains can be fused to the N terminal end, the C terminal end, an internal location, or combination thereof. The fusion can be direct via a chemical bond, or the linkage can be indirect via one or more linkers.

[0020] In certain preferred embodiments, the engineered Cas9 proteins described herein include one or more nuclear localization signals (NLS). Non-limiting examples of nuclear localization signals include PKKKRKV (SEQ ID NO:1), PKKKRRV (SEQ ID NO:2), KRPAATKKAGQAKKKK (SEQ ID NO:3), YGRKKRRQRRR (SEQ ID NO:4), RKKRRQRRR (SEQ ID NO:5), PAAKRVKLD (SEQ ID NO:6), RQRRNELKRSP (SEQ ID NO:7), VSRKRPRP (SEQ ID NO:8), PPKKARED (SEQ ID NO:9), PQPKKKPL (SEQ ID NO:10), SALIKKKKKMAP (SEQ ID NO:11), PKQKKRK (SEQ ID NO:12), RKLKKKIKKL (SEQ ID NO:13), REKKKFLKRR (SEQ ID NO:14), KRKGDEVDGVDEVAKKKSKK (SEQ ID NO:15), RKCLQAGMNLEARKTKK (SEQ ID NO:16), NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY (SEQ ID NO:17), and RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV (SEQ ID NO:18).

[0021] In one particular embodiment, the nuclear localization signal is selected from PKKKRKV (SEQ ID NO:1) and PAAKRVKLD (SEQ ID NO:6). In another particular embodiment, the engineered Cas9 protein includes both of PKKKRKV (SEQ ID NO:1) and PAAKRVKLD (SEQ ID NO:6). In another particular embodiment, the engineered Cas9 protein includes at least two of PKKKRKV (SEQ ID NO:1) and at least one of PAAKRVKLD (SEQ ID NO:6). In another particular embodiment, the engineered Cas9 protein includes two of PKKKRKV (SEQ ID NO:1) and one of PAAKRVKLD (SEQ ID NO:6).

[0022] In these and other preferred embodiments, the engineered Cas9 proteins include one or more marker domains. Marker

domains include fluorescent proteins and purification or epitope tags. Suitable fluorescent proteins include, without limit, green fluorescent proteins (*e.g.*, GFP, eGFP, GFP-2, tagGFP, turboGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, ZsGreen1), yellow fluorescent proteins (*e.g.*, YFP, EYFP, Citrine, Venus, YPet, PhiYFP, ZsYellow1), blue fluorescent proteins (*e.g.*, BFP, EBFP, EBFP2, Azurite, mKalama1, GFPuv, Sapphire, T-sapphire), cyan fluorescent proteins (*e.g.,* ECFP, Cerulean, CyPet, AmCyan1, Midoriishi-Cyan), red fluorescent proteins (*e.g.*, mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRed1, AsRed2, eqFP611, mRasberry, mStrawberry, Jred), orange fluorescent proteins (*e.g.*, mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato), or combinations thereof. The marker domain can comprise tandem repeats of one or more fluorescent proteins (*e.g.*, Suntag).

[0023] In one embodiment, the marker protein is selected from the following:

Marker Protein Sequence
MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTL KFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMP EGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNI LGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHY QQNTPIGDGPVLLPDNHYLSTQSKLSKDPNEKRDHMVLLEFVTAA GITLGMDELYK (SEQ ID NO:19) MVSKGEAVIKEFMRFKVHMEGSMNGHEFEIEGEGEGRPYEGTQT AKLKVTKGGPLPFSWDILSPQFMYGSRAFTKHPADIPDYYKQSFPE GFKWERVMNFEDGGAVTVTQDTSLEDGTLIYKVKLRGTNFPPDGP VMQKKTMGWEASTERLYPEDGVLKGDIKMALRLKDGGRYLADFK TTYKAKKPVQMPGAYNVDRKLDITSHNEDYTVVEQYERSEGRHST GGMDELYK (SEQ ID NO:20)

[0024] Non-limiting examples of suitable purification or epitope tags include 6xHis (SEQ ID NO:22), FLAG® (e.g., SEQ ID NO:21), HA, GST, Myc, SAM, and the like. Non-limiting examples of heterologous fusions which facilitate detection or enrichment of CRISPR complexes include streptavidin (Kipriyanov et al., Human Antibodies, 1995, 6(3):93-101.), avidin (Airenne et al., Biomolecular Engineering, 1999, 16(1-4):87-92), monomeric forms of avidin (Laitinen et al., Journal of Biological Chemistry, 2003, 278(6):4010-

4014), peptide tags which facilitate biotinylation during recombinant production (Cull et al., Methods in Enzymology, 2000, 326:430-440).

[0025] In addition to a nuclear localization signal(s) and a marker protein(s), in various embodiments the engineered Cas9 protein may also include one or more heterologous domains such as a cell-penetrating domain, a marker domain, a chromatin disrupting domain, an epigenetic modification domain (*e.g.*, a cytidine deaminase domain, a histone acetyltransferase domain, and the like), a transcriptional regulation domain, an RNA aptamer binding domain, or a non-Cas9 nuclease domain.

[0026] In some embodiments, the one or more heterologous domains can be a cell-penetrating domain. Examples of suitable cell-penetrating domains include, without limit, GRKKRRQRRRPPQPKKKRKV (SEQ ID NO:23), PLSSIFSRIGDPPKKKRKV (SEQ ID NO:24), GALFLGWLGAAGSTMGAPKKKRKV (SEQ ID NO:25), GALFLGFLGAAGSTMGAWSQPKKKRKV (SEQ ID NO:26), KETWWETWWTEWSQPKKKRKV (SEQ ID NO:27), YARAAARQARA (SEQ ID NO:28), THRLPRRRRRR (SEQ ID NO:29), GGRRARRRRRR (SEQ ID NO:30), RRQRRTSKLMKR (SEQ ID NO:31), GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO:32), KALAWEAKLAKALAKALAKHLAKALAKALKCEA (SEQ ID NO:33), and RQIKIWFQNRRMKWKK (SEQ ID NO:34).

[0027] In still other embodiments, the one or more heterologous domain can be a chromatin modulating motif (CMM). Non-limiting examples of CMMs include nucleosome interacting peptides derived from high mobility group (HMG) proteins (*e.g.*, HMGB1, HMGB2, HMGB3, HMGN1, HMGN2, HMGN3a, HMGN3b, HMGN4, and HMGN5 proteins), the central globular domain of histone H1 variants (*e.g.*, histone H1.0, H1.1, H1.2, H1.3, H1.4, H1.5, H1.6, H1.7, H1.8, H1.9, and H.1.10), or DNA binding domains of chromatin remodeling complexes (*e.g.*, SWI/SNF (SWItch/Sucrose Non-Fermentable), ISWI (Imitation SWItch), CHD (Chromodomain-Helicase-DNA binding), Mi-2/NuRD (Nucleosome Remodeling and Deacetylase), INO80, SWR1, and RSC complexes. In other embodiments, CMMs also can be derived from topoisomerases, helicases, or viral proteins. The source of the CMM can and will vary. CMMs can be from humans, animals (*i.e.*,

vertebrates and invertebrates), plants, algae, or yeast. Non-limiting examples of specific CMMs are listed in the table below. Persons of skill in the art can readily identify homologs in other species and/or the relevant fusion motif therein.

Protein	**Accession No.**	**Fusion Motif**
Human HMGN1	P05114	Full length
Human HMGN2	P05204	Full length
Human HMGN3a	Q15651	Full length
Human HMGN3b	Q15651-2	Full length
Human HMGN4	O00479	Full length
Human HMGN5	P82970	Nucleosome binding motif
Human HMGB1	P09429	Box A
Human histone H1.0	P07305	Globular motif
Human histone H1.2	P16403	Globular motif
Human CHD1	O14646	DNA binding motif
Yeast CHD1	P32657	DNA binding motif
Yeast ISWI	P38144	DNA binding motif
Human TOP1	P11387	DNA binding motif
Human herpesvirus 8 LANA	J9QSF0	Nucleosome binding motif
Human CMV IE1	P13202	Chromatin tethering motif
M. leprae DNA helicase	P40832	HhH binding motif

[0028] In yet other embodiments, the one or more heterologous domains can be an epigenetic modification domain. Non-limiting examples of suitable epigenetic modification domains include those with DNA deamination (*e.g.*, cytidine deaminase, adenosine deaminase, guanine deaminase), DNA methyltransferase activity (*e.g.*, cytosine methyltransferase), DNA demethylase activity, DNA amination, DNA oxidation activity, DNA helicase activity, histone acetyltransferase (HAT) activity (*e.g.*, HAT domain derived from E1A binding protein p300), histone deacetylase activity, histone

methyltransferase activity, histone demethylase activity, histone kinase activity, histone phosphatase activity, histone ubiquitin ligase activity, histone deubiquitinating activity, histone adenylation activity, histone deadenylation activity, histone SUMOylating activity, histone deSUMOylating activity, histone ribosylation activity, histone deribosylation activity, histone myristoylation activity, histone demyristoylation activity, histone citrullination activity, histone alkylation activity, histone dealkylation activity, or histone oxidation activity. In specific embodiments, the epigenetic modification domain can comprise cytidine deaminase activity, adenosine deaminase activity, histone acetyltransferase activity, or DNA methyltransferase activity.

[0029] In other embodiments, the one or more heterologous domains can be a transcriptional regulation domain (*i.e.*, a transcriptional activation domain or transcriptional repressor domain). Suitable transcriptional activation domains include, without limit, herpes simplex virus VP16 domain, VP64 (*i.e.*, four tandem copies of VP16), VP160 (*i.e.*, ten tandem copies of VP16), NFκB p65 activation domain (p65) , Epstein-Barr virus R transactivator (Rta) domain, VPR (*i.e.*, VP64+p65+Rta), p300-dependent transcriptional activation domains, p53 activation domains 1 and 2, heat-shock factor 1 (HSF1) activation domains, Smad4 activation domains (SAD), cAMP response element binding protein (CREB) activation domains, E2A activation domains, nuclear factor of activated T-cells (NFAT) activation domains, or combinations thereof. Non-limiting examples of suitable transcriptional repressor domains include Kruppel-associated box (KRAB) repressor domains, Mxi repressor domains, inducible cAMP early repressor (ICER) domains, YY1 glycine rich repressor domains, Sp1-like repressors, E(spl) repressors, IκB repressors, Sin3 repressors, methyl-CpG binding protein 2 (MeCP2) repressors, or combinations thereof. Transcriptional activation or transcriptional repressor domains can be genetically fused to the Cas9 protein or bound via noncovalent protein-protein, protein-RNA, or protein-DNA interactions.

[0030] In further embodiments, the one or more heterologous domains can be an RNA aptamer binding domain (Konermann *et al.*, Nature, 2015, 517(7536):583-588; Zalatan *et al.*, Cell, 2015, 160(1-2):339-50). Examples of suitable RNA aptamer protein domains include MS2 coat protein

(MCP), PP7 bacteriophage coat protein (PCP), Mu bacteriophage Com protein, lambda bacteriophage N22 protein, stem-loop binding protein (SLBP), Fragile X mental retardation syndrome-related protein 1 (FXR1), proteins derived from bacteriophage such as AP205, BZ13, f1, f2, fd, fr, ID2, JP34/GA, JP501, JP34, JP500, KU1, M11, M12, MX1, NL95, PP7, ϕCb5, ϕCb8r, ϕCb12r, ϕCb23r, Qβ, R17, SP-β, TW18, TW19, and VK, fragments thereof, or derivatives thereof.

[0031] In yet other embodiments, the one or more heterologous domains can be a non-Cas9 nuclease domain. Suitable nuclease domains can be obtained from any endonuclease or exonuclease. Non-limiting examples of endonucleases from which a nuclease domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. In some embodiments, the nuclease domain can be derived from a type II-S restriction endonuclease. Type II-S endonucleases cleave DNA at sites that are typically several base pairs away from the recognition/binding site and, as such, have separable binding and cleavage domains. These enzymes generally are monomers that transiently associate to form dimers to cleave each strand of DNA at staggered locations. Non-limiting examples of suitable type II-S endonucleases include BfiI, BpmI, BsaI, BsgI, BsmBI, BsmI, BspMI, FokI, MboII, and SapI. In some embodiments, the nuclease domain can be a FokI nuclease domain or a derivative thereof. The type II-S nuclease domain can be modified to facilitate dimerization of two different nuclease domains. For example, the cleavage domain of FokI can be modified by mutating certain amino acid residues. By way of non-limiting example, amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of FokI nuclease domains are targets for modification. In specific embodiments, the FokI nuclease domain can comprise a first FokI half- domain comprising Q486E, I499L, and/or N496D mutations, and a second FokI half-domain comprising E490K, I538K, and/or H537R mutations.

[0032] The one or more heterologous domains can be linked directly to the Cas9 protein via one or more chemical bonds (*e.g.*, covalent bonds), or the one or more heterologous domains can be linked indirectly to the Cas9 protein via one or more linkers.

[0033] A linker is a chemical group that connects one or more other chemical groups via at least one covalent bond. Suitable linkers include amino acids, peptides, nucleotides, nucleic acids, organic linker molecules (*e.g.*, maleimide derivatives, N-ethoxybenzylimidazole, biphenyl-3,4′,5-tricarboxylic acid, p-aminobenzyloxycarbonyl, and the like), disulfide linkers, and polymer linkers (*e.g.*, PEG). The linker can include one or more spacing groups including, but not limited to alkylene, alkenylene, alkynylene, alkyl, alkenyl, alkynyl, alkoxy, aryl, heteroaryl, aralkyl, aralkenyl, aralkynyl and the like. The linker can be neutral, or carry a positive or negative charge. Additionally, the linker can be cleavable such that the linker's covalent bond that connects the linker to another chemical group can be broken or cleaved under certain conditions, including pH, temperature, salt concentration, light, a catalyst, or an enzyme. In some embodiments, the linker can be a peptide linker. The peptide linker can be a flexible amino acid linker (*e.g.*, comprising small, non-polar or polar amino acids).

[0034] In one particular embodiment, the linker is selected from the following:

Linker Protein Sequence
AEAAAKEAAAKEAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA (SEQ ID NO:35)
SGGSSGGSSGSETPGTSESATPESSGGSSGGS (SEQ ID NO:36)

[0035] Other non-limiting examples of flexible linkers include LEGGGS (SEQ ID NO:37), TGSG (SEQ ID NO:38), GGSGGGSG (SEQ ID NO:39), $(GGGGS)_{1-4}$ (SEQ ID NO:40), and $(Gly)_{6-8}$ (SEQ ID NO:41). Alternatively, the peptide linker can be a rigid amino acid linker. Such linkers include $(EAAAK)_{1-4}$ (SEQ ID NO:42), $A(EAAAK)_{2-5}A$ (SEQ ID NO:43), PAPAP (SEQ ID NO:44), and $(AP)_{6-8}$ (SEQ ID NO:45). Additional examples of suitable linkers are well known in the art and programs to design linkers are readily available (Crasto *et al.*, Protein Eng., 2000, 13(5):309-312).

[0036] In some embodiments, the engineered Cas9 proteins can be produced recombinantly in cell-free systems, bacterial cells, or eukaryotic cells and purified using standard purification means. In other embodiments, the engineered Cas9 proteins are produced *in vivo* in eukaryotic cells of

interest from nucleic acids encoding the engineered Cas9 proteins (see section (II) below).

[0037] In embodiments in which the engineered Cas9 protein comprises nuclease or nickase activity, the engineered Cas9 protein can further comprise at least cell-penetrating domain, as well as at least one chromatin disrupting domain. In embodiments in which the engineered Cas9 protein is linked to an epigenetic modification domain, the engineered Cas9 protein can further comprise at least one cell-penetrating domain, as well as at least one chromatin disrupting domain. Furthermore, in embodiments in which the engineered Cas9 protein is linked to a transcriptional regulation domain, the engineered Cas9 protein can further comprise at least one cell-penetrating domain, as well as at least one chromatin disrupting domain and/or at least one RNA aptamer binding domain.

[0038] The various fusion protein components can be combined, from N-terminus to C-terminus, in any order. For example, wherein A represents the marker protein, B represents a nuclear localization signal, and C represents the Cas9 protein, the fusion protein can be arranged, from N-terminus to C-terminus, in the following manner: A-B-C; A-C-B; B-A-C; B-C-A; C-A-B; or C-B-A, wherein a linker ("-L-") may be disposed between any two items (e.g., A-L-B-C; A-B-L-C; A-L-B-L-C; and so on).

(b) Engineered guide RNAs

[0039] The engineered guide RNAs is designed to complex with a specific engineered Cas9 protein. A guide RNA comprises (i) a CRISPR RNA (crRNA) that contains a guide sequence at the 5' end that hybridizes with a target sequence and (ii) a transacting crRNA (tracrRNA) sequence that recruits the Cas9 protein. The crRNA guide sequence of each guide RNA is different (*i.e.*, is sequence specific). The tracrRNA sequence is generally the same in guide RNAs designed to complex with a Cas9 protein from a particular bacterial species.

[0040] The crRNA guide sequence is designed to hybridize with a target sequence (*i.e.*, protospacer) in a double-stranded sequence. In general, the complementarity between the crRNA and the target sequence is at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%. In specific embodiments, the complementarity is complete (*i.e.*, 100%). In

various embodiments, the length of the crRNA guide sequence can range from about 15 nucleotides to about 25 nucleotides. For example, the crRNA guide sequence can be about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In specific embodiments, the crRNA is about 19, 20, or 21 nucleotides in length. In one embodiment, the crRNA guide sequence has a length of 20 nucleotides.

[0041] The guide RNA comprises repeat sequence that forms at least one stem loop structure, which interacts with the Cas9 protein, and 3' sequence that remains single-stranded. The length of each loop and stem can vary. For example, the loop can range from about 3 to about 10 nucleotides in length, and the stem can range from about 6 to about 20 base pairs in length. The stem can comprise one or more bulges of 1 to about 10 nucleotides. The length of the single-stranded 3' region can vary. The tracrRNA sequence in the engineered guide RNA generally is based upon the coding sequence of wild type tracrRNA in the bacterial species of interest. The wild-type sequence can be modified to facilitate secondary structure formation, increased secondary structure stability, facilitate expression in eukaryotic cells, and so forth. For example, one or more nucleotide changes can be introduced into the guide RNA coding sequence (see Example 3, below). The tracrRNA sequence can range in length from about 50 nucleotides to about 300 nucleotides. In various embodiments, the tracrRNA can range in length from about 50 to about 90 nucleotides, from about 90 to about 110 nucleotides, from about 110 to about 130 nucleotides, from about 130 to about 150 nucleotides, from about 150 to about 170 nucleotides, from about 170 to about 200 nucleotides, from about 200 to about 250 nucleotides, or from about 250 to about 300 nucleotides.

[0042] In general, the engineered guide RNA is a single molecule (*i.e.*, a single guide RNA or sgRNA), wherein the crRNA sequence is linked to the tracrRNA sequence. In some embodiments, however, the engineered guide RNA can be two separate molecules. A first molecule comprising the crRNA that contains 3' sequence (comprising from about 6 to about 20 nucleotides) that is capable of base pairing with the 5' end of a second molecule, wherein the second molecule comprises the tracrRNA that

contains 5' sequence (comprising from about 6 to about 20 nucleotides) that is capable of base pairing with the 3' end of the first molecule.

[0043] In some embodiments, the tracrRNA sequence of the engineered guide RNA can be modified to comprise one or more aptamer sequences (Konermann *et al.*, Nature, 2015, 517(7536):583-588; Zalatan *et al.*, Cell, 2015, 160(1-2):339-50). Suitable aptamer sequences include those that bind adaptor proteins chosen from MCP, PCP, Com, SLBP, FXR1, AP205, BZ13, f1, f2, fd, fr, ID2, JP34/GA, JP501, JP34, JP500, KU1, M11, M12, MX1, NL95, PP7, ϕCb5, ϕCb8r, ϕCb12r, ϕCb23r, Qβ, R17, SP-β, TW18, TW19, VK, fragments thereof, or derivatives thereof. Those of skill in the art appreciate that the length of the aptamer sequence can vary.

[0044] In other embodiments, the guide RNA can further comprise at least one detectable label. The detectable label can be a fluorophore (*e.g.*, FAM, TMR, Cy3, Cy5, Texas Red, Oregon Green, Alexa Fluors, Halo tags, or suitable fluorescent dye), a detection tag (*e.g.*, biotin, digoxigenin, and the like), quantum dots, or gold particles.

[0045] The guide RNA can comprise standard ribonucleotides and/or modified ribonucleotides. In some embodiment, the guide RNA can comprise standard or modified deoxyribonucleotides. In embodiments in which the guide RNA is enzymatically synthesized (*i.e.*, *in vivo* or *in vitro*), the guide RNA generally comprises standard ribonucleotides. In embodiments in which the guide RNA is chemically synthesized, the guide RNA can comprise standard or modified ribonucleotides and/or deoxyribonucleotides. Modified ribonucleotides and/or deoxyribonucleotides include base modifications (*e.g.*, pseudouridine, 2-thiouridine, N6-methyladenosine, and the like) and/or sugar modifications (*e.g.*, 2'-O-methy, 2'-fluoro, 2'-amino, locked nucleic acid (LNA), and so forth). The backbone of the guide RNA can also be modified to comprise phosphorothioate linkages, boranophosphate linkages, or peptide nucleic acids.

(c) PAM Sequence

[0046] In some embodiments, the target sequence may be adjacent to a protospacer adjacent motif (PAM), a short sequence recognized by a CRISPR/Cas9 complex. In some embodiments, the PAM may be adjacent to or within 1, 2, 3, or 4, nucleotides of the 3' end of the target

sequence. The length and the sequence of the PAM may depend on the Cas9 protein used. For example, the PAM may be selected from a consensus or a particular PAM sequence for a specific Cas9 protein or Cas9 ortholog, including those disclosed in FIG. 1 of Ran et al., Nature, 520: 186-191 (2015), which is incorporated herein by reference. In some embodiments, the PAM may comprise 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. Non-limiting exemplary PAM sequences include NGG, NGGNG, NG, NAAAAN, NNAAAAW, NNNNACA, GNNNCNNA, and NNNNGATT (wherein N is defined as any nucleotide, and W is defined as either A or T). In some embodiments, the PAM sequence may be NGG. In some embodiments, the PAM sequence may be NGGNG. In some embodiments, the PAM sequence may be NNAAAAW.

[0047] It will be understood that different CRISPR proteins recognize different PAM sequences. For example, PAM sequences for Cas9 proteins include 5'-NGG, 5'-NGGNG, 5'-NNAGAAW, 5'-NNNNGATT, 5-NNNNRYAC, 5'-NNNNCAAA, 5'-NGAAA, 5'-NNAAT, 5'-NNNRTA, 5'-NNGG, 5'-NNNRTA, 5'-MMACCA, 5'-NNNNGRY, 5'-NRGNK, 5'-GGGRG, 5'-NNAMMMC, and 5'-NNG, and PAM sequences for Cas12a proteins include 5'-TTN and 5'-TTTV, wherein N is defined as any nucleotide, R is defined as either G or A, W is defined as either A or T, Y is defined an either C or T, and V is defined as A, C, or G. In general, Cas9 PAMs are located 3' of the target sequence, and Cas12a PAMs are located 5' of the target sequence. Various PAM sequences and the CRISPR proteins that recognize them are known in the art, *e.g.*, U.S. Patent Application Publication 2019/0249200; Leenay, Ryan T., et al. "Identifying and visualizing functional PAM diversity across CRISPR-Cas systems." Molecular cell 62.1 (2016): 137-147; and Kleinstiver, Benjamin P., et al. "Engineered CRISPR-Cas9 nucleases with altered PAM specificities." Nature 523.7561 (2015): 481, each of which are incorporated by reference herein in their entirety.

[0048] Additionally or alternatively, the PAM for each of the engineered Cas9 systems disclosed herein is presented below.

PAM Sequences	
Engineered Cas9system	PAM (5'-3')*

Bacillus smithii Cas9 (BsmCas9)	NNNNCAAA
Lactobacillus rhamnosus Cas9 (LrhCas9)	NGAAA
Parasutterella excrementihominis Cas9 (PexCas9)	NGG
Mycoplasma canis Cas9 (McaCas9)	NNGG
Mycoplasma gallisepticum Cas9 (MgaCas9)	NNAAT
Akkermansia glycaniphila Cas9 (AglCas9)	NNNRTA
Akkermansia muciniphila Cas9 (AmuCas9)	MMACCA
Oenococcus kitaharae Cas9 (OkiCas9)	NNG
Bifidobacterium bombi Cas9 (BboCas9)	NNNNGRY
Acidothermus cellulolyticus Cas9 (AceCas9)	NGG
Alicyclobacillus hesperidum Cas9 (AheCas9)	NGG
Wolinella succinogenes Cas9 (WsuCas9)	NGG
Nitratifractor salsuginis Cas9 (NsaCas9)	NRGNK
Ralstonia syzygii Cas9 (RsyCas9)	GGGRG
Corynebacterium diphtheria Cas9 (CdiCas9)	NNAMMMC

* K is G or T; M is A or C; R is A or G; Y is C or T; and N is A, C, G, or T.

See, e.g., U.S. Patent Application Publication No. 2019/0249200 (hereby incorporated by reference herein in its entirety.

(II) Nucleic Acids

[0049] A further aspect of the present disclosure provides nucleic acids encoding the engineered Cas9 systems described above in section (I). The systems can be encoded by single nucleic acids or multiple nucleic acids. The nucleic acids can be DNA or RNA, linear or circular, single-stranded or double-stranded. The RNA or DNA can be codon optimized for efficient translation into protein in the eukaryotic cell of interest. Codon optimization programs are available as freeware or from commercial sources.

[0050] In some embodiments, nucleic acid encodes a protein having at least about 75%, at least about 80%, at least about 85%, at least

about 90%, at least about 95%, or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO:48, 49, or 50. In certain embodiments, the nucleic acid encoding the engineered Cas9 protein can have at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to the DNA sequence of SEQ ID NO:48, 49, or 50. In certain embodiments, the DNA encoding the engineered Cas9 protein has the DNA sequence of SEQ ID NO:48, 49, or 50. In additional embodiments, the nucleic acid encodes a protein having at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to the amino acid sequence of SEQ ID NO:48, 49, or 50.

[0051] In some embodiments, the nucleic acid encoding the engineered Cas9 protein can be RNA. The RNA can be enzymatically synthesized *in vitro*. For this, DNA encoding the engineered Cas9 protein can be operably linked to a promoter sequence that is recognized by a phage RNA polymerase for *in vitro* RNA synthesis. For example, the promoter sequence can be a T7, T3, or SP6 promoter sequence or a variation of a T7, T3, or SP6 promoter sequence. The DNA encoding the engineered protein can be part of a vector, as detailed below. In such embodiments, the *in vitro*-transcribed RNA can be purified, capped, and/or polyadenylated. In other embodiments, the RNA encoding the engineered Cas9 protein can be part of a self-replicating RNA (Yoshioka *et al.*, Cell Stem Cell, 2013, 13:246-254). The self-replicating RNA can be derived from a noninfectious, self-replicating Venezuelan equine encephalitis (VEE) virus RNA replicon, which is a positive-sense, single-stranded RNA that is capable of self-replicating for a limited number of cell divisions, and which can be modified to code proteins of interest (Yoshioka *et al.*, Cell Stem Cell, 2013, 13:246-254).

[0052] In other embodiments, the nucleic acid encoding the engineered Cas9 protein can be DNA. The DNA coding sequence can be operably linked to at least one promoter control sequence for expression in the cell of interest. In certain embodiments, the DNA coding sequence can be operably linked to a promoter sequence for expression of the engineered Cas9 protein in bacterial (*e.g.*, *E. coli*) cells or eukaryotic (*e.g.*, yeast, insect, or mammalian) cells. Suitable bacterial promoters include, without limit, T7

promoters, *lac* operon promoters, *trp* promoters, *tac* promoters (which are hybrids of *trp* and *lac* promoters), variations of any of the foregoing, and combinations of any of the foregoing. Non-limiting examples of suitable eukaryotic promoters include constitutive, regulated, or cell- or tissue-specific promoters. Suitable eukaryotic constitutive promoter control sequences include, but are not limited to, cytomegalovirus immediate early promoter (CMV), simian virus (SV40) promoter, adenovirus major late promoter, Rous sarcoma virus (RSV) promoter, mouse mammary tumor virus (MMTV) promoter, phosphoglycerate kinase (PGK) promoter, elongation factor (ED1)-alpha promoter, ubiquitin promoters, actin promoters, tubulin promoters, immunoglobulin promoters, fragments thereof, or combinations of any of the foregoing. Examples of suitable eukaryotic regulated promoter control sequences include without limit those regulated by heat shock, metals, steroids, antibiotics, or alcohol. Non-limiting examples of tissue-specific promoters include B29 promoter, CD14 promoter, CD43 promoter, CD45 promoter, CD68 promoter, desmin promoter, elastase-1 promoter, endoglin promoter, fibronectin promoter, Flt-1 promoter, GFAP promoter, GPIIb promoter, ICAM-2 promoter, INF-β promoter, Mb promoter, Nphsl promoter, OG-2 promoter, SP-B promoter, SYN1 promoter, and WASP promoter. The promoter sequence can be wild type or it can be modified for more efficient or efficacious expression. In some embodiments, the DNA coding sequence also can be linked to a polyadenylation signal (*e.g.*, SV40 polyA signal, bovine growth hormone (BGH) polyA signal, *etc.*) and/or at least one transcriptional termination sequence. In some situations, the engineered Cas9 protein can be purified from the bacterial or eukaryotic cells.

[0053] In still other embodiments, the engineered guide RNA can be encoded by DNA. In some instances, the DNA encoding the engineered guide RNA can be operably linked to a promoter sequence that is recognized by a phage RNA polymerase for *in vitro* RNA synthesis. For example, the promoter sequence can be a T7, T3, or SP6 promoter sequence or a variation of a T7, T3, or SP6 promoter sequence. In other instances, the DNA encoding the engineered guide RNA can be operably linked to a promoter sequence that is recognized by RNA polymerase III (Pol III) for expression in eukaryotic cells of interest. Examples of suitable Pol III

promoters include, but are not limited to, mammalian U6, U3, H1, and 7SL RNA promoters.

[0054] In various embodiments, the nucleic acid encoding the engineered Cas9 protein can be present in a vector. In some embodiments, the vector can further comprise nucleic acid encoding the engineered guide RNA. Suitable vectors include plasmid vectors, viral vectors, and self-replicating RNA (Yoshioka *et al.*, Cell Stem Cell, 2013, 13:246-254). In some embodiments, the nucleic acid encoding the complex or fusion protein can be present in a plasmid vector. Non-limiting examples of suitable plasmid vectors include pUC, pBR322, pET, pBluescript, and variants thereof. In other embodiments, the nucleic acid encoding the complex or fusion protein can be part of a viral vector (*e.g.*, lentiviral vectors, adeno-associated viral vectors, adenoviral vectors, and so forth). The plasmid or viral vector can comprise additional expression control sequences (*e.g.*, enhancer sequences, Kozak sequences, polyadenylation sequences, transcriptional termination sequences, *etc.*), selectable marker sequences (*e.g.*, antibiotic resistance genes), origins of replication, and the like. Additional information about vectors and use thereof can be found in "Current Protocols in Molecular Biology" Ausubel *et al.*, John Wiley & Sons, New York, 2003 or "Molecular Cloning: A Laboratory Manual" Sambrook & Russell, Cold Spring Harbor Press, Cold Spring Harbor, NY, 3rd edition, 2001.

(III) Eukaryotic Cells

[0055] Another aspect of the present disclosure comprises eukaryotic cells comprising at least one engineered Cas9 system as detailed above in section (I) and/or at least one nucleic acid encoding and engineered Cas9 protein and/or engineered guide RNA as detailed above in section (II).

[0056] The eukaryotic cell can be a human cell, a non-human mammalian cell, a non-mammalian vertebrate cell, an invertebrate cell, a plant cell, or a single cell eukaryotic organism. Examples of suitable eukaryotic cells are detailed below in section (IV)(c). The eukaryotic cell can be *in vitro*, *ex vivo*, or *in vivo*.

[0057] By way of example, in some embodiments, the eukaryotic cell, or a population of eukaryotic cells, is a T-cell, a $CD8^+$ T-cell, a $CD8^+$ naive T cell, a central memory T cell, an effector memory T-cell, a $CD4^+$ T-

cell, a stem cell memory T-cell, a helper T-cell, a regulatory T-cell, a cytotoxic T-cell, a natural killer T-cell, a hematopoietic stem cell, a long term hematopoietic stem cell, a short term hematopoietic stem cell, a multipotent progenitor cell, a lineage restricted progenitor cell, a lymphoid progenitor cell, a pancreatic progenitor cell, an endocrine progenitor cell, an exocrine progenitor cell, a myeloid progenitor cell, a common myeloid progenitor cell, an erythroid progenitor cell, a megakaryocyte erythroid progenitor cell, a monocytic precursor cell, an endocrine precursor cell, an exocrine cell, a fibroblast, a hepatoblast, a myoblast, a macrophage, an islet beta-cell, a cardiomyocyte, a blood cell, a ductal cell, an acinar cell, an alpha cell, a beta cell, a delta cell, a PP cell, a cholangiocyte, a retinal cell, a photoreceptor cell, a rod cell, a cone cell, a retinal pigmented epithelium cell, a trabecular meshwork cell, a cochlear hair cell, an outer hair cell, an inner hair cell, a pulmonary epithelial cell, a bronchial epithelial cell, an alveolar epithelial cell, a pulmonary epithelial progenitor cell, a striated muscle cell, a cardiac muscle cell, a muscle satellite cell, a myocyte, a neuron, a neuronal stem cell, a mesenchymal stem cell, an induced pluripotent stem (iPS) cell, an embryonic stem cell, a monocyte, a megakaryocyte, a neutrophil, an eosinophil, a basophil, a mast cell, a reticulocyte, a B cell, e.g. a progenitor B cell, a Pre B cell, a Pro B cell, a memory B cell, a plasma B cell, a gastrointestinal epithelial cell, a biliary epithelial cell, a pancreatic ductal epithelial cell, an intestinal stem cell, a hepatocyte, a liver stellate cell, a Kupffer cell, an osteoblast, an osteoclast, an adipocyte (e.g., a brown adipocyte, or a white adipocyte), a preadipocyte, a pancreatic precursor cell, a pancreatic islet cell, a pancreatic beta cell, a pancreatic alpha cell, a pancreatic delta cell, a pancreatic exocrine cell, a Schwann cell, or an oligodendrocyte, or a population of such cells.

(IV) Methods for Modifying Chromosomal Sequences

[0058] A further aspect of the present disclosure encompasses methods for modifying a chromosomal sequence in eukaryotic cells. In general, the methods comprise introducing into the eukaryotic cell of interest at least one engineered Cas9 system as detailed above in section (I) and/or at least one nucleic acid encoding said engineered Cas9 system as detailed above in section (II).

[0059] In embodiments in which the engineered Cas9 protein comprises nuclease or nickase activity, the chromosomal sequence modification can comprise a substitution of at least one nucleotide, a deletion of at least one nucleotide, an insertion of at least one nucleotide. In some iterations, the method comprises introducing into the eukaryotic cell one engineered Cas9 system comprising nuclease activity or two engineered Cas9 systems comprising nickase activity and no donor polynucleotide, such that the engineered Cas9 system or systems introduce a double-stranded break in the target site in the chromosomal sequence and repair of the double-stranded break by cellular DNA repair processes introduces at least one nucleotide change (*i.e.*, indel), thereby inactivating the chromosomal sequence (*i.e.*, gene knock-out). In other iterations, the method comprises introducing into the eukaryotic cell one engineered Cas9 system comprising nuclease activity or two engineered Cas9 systems comprising nickase activity, as well as the donor polynucleotide, such that the engineered Cas9 system or systems introduce a double-stranded break in the target site in the chromosomal sequence and repair of the double-stranded break by cellular DNA repair processes leads to insertion or exchange of sequence in the donor polynucleotide into the target site in the chromosomal sequence (i.e., gene correction or gene knock-in).

[0060] In embodiments, in which the engineered Cas9 protein comprises epigenetic modification activity or transcriptional regulation activity, the chromosomal sequence modification can comprise a conversion of at least one nucleotide in or near the target site, a modification of at least one nucleotide in or near the target site, a modification of at least one histone protein in or near the target site, and/or a change in transcription in or near the target site in the chromosomal sequence.

(a) Introduction into the Cell

[0061] As mentioned above, the method comprises introducing into the eukaryotic cell at least one engineered Cas9 system and/or nucleic acid encoding said system (and optional donor polynucleotide). The at least one system and/or nucleic acid/donor polynucleotide can be introduced into the cell of interest by a variety of means.

[0062] In some embodiments, the cell can be transfected with the appropriate molecules (*i.e.*, protein, DNA, and/or RNA). Suitable transfection methods include nucleofection (or electroporation), calcium phosphate-mediated transfection, cationic polymer transfection (*e.g.*, DEAE-dextran or polyethylenimine), viral transduction, virosome transfection, virion transfection, liposome transfection, cationic liposome transfection, immunoliposome transfection, nonliposomal lipid transfection, dendrimer transfection, heat shock transfection, magnetofection, lipofection, gene gun delivery, impalefection, sonoporation, optical transfection, and proprietary agent-enhanced uptake of nucleic acids. Transfection methods are well known in the art (*see*, *e.g.*, "Current Protocols in Molecular Biology" Ausubel *et al.*, John Wiley & Sons, New York, 2003 or "Molecular Cloning: A Laboratory Manual" Sambrook & Russell, Cold Spring Harbor Press, Cold Spring Harbor, NY, 3rd edition, 2001). In other embodiments, the molecules can be introduced into the cell by microinjection. For example, the molecules can be injected into the cytoplasm or nuclei of the cells of interest. The amount of each molecule introduced into the cell can vary, but those skilled in the art are familiar with means for determining the appropriate amount.

[0063] The various molecules can be introduced into the cell simultaneously or sequentially. For example, the engineered Cas9 system (or its encoding nucleic acid) and the donor polynucleotide can be introduced at the same time. Alternatively, one can be introduced first and then the other can be introduced later into the cell.

[0064] In general, the cell is maintained under conditions appropriate for cell growth and/or maintenance. Suitable cell culture conditions are well known in the art and are described, for example, in Santiago *et al.*, Proc. Natl. Acad. Sci. USA, 2008, 105:5809-5814; Moehle *et al.* Proc. Natl. Acad. Sci. USA, 2007, 104:3055-3060; Urnov *et al.*, Nature, 2005, 435:646-651; and Lombardo *et al.*, Nat. Biotechnol., 2007, 25:1298-1306. Those of skill in the art appreciate that methods for culturing cells are known in the art and can and will vary depending on the cell type. Routine optimization may be used, in all cases, to determine the best techniques for a particular cell type.

(b) Optional Donor Polynucleotide

[0065] In embodiments in which the engineered Cas9 protein comprises nuclease or nickase activity, the method can further comprise introducing at least one donor polynucleotide into the cell. The donor polynucleotide can be single-stranded or double-stranded, linear or circular, and/or RNA or DNA. In some embodiments, the donor polynucleotide can be a vector, *e.g.*, a plasmid vector.

[0066] The donor polynucleotide comprises at least one donor sequence. In some aspects, the donor sequence of the donor polynucleotide can be a modified version of an endogenous or native chromosomal sequence. For example, the donor sequence can be essentially identical to a portion of the chromosomal sequence at or near the sequence targeted by the engineered Cas9 system, but which comprises at least one nucleotide change. Thus, upon integration or exchange with the native sequence, the sequence at the targeted chromosomal location comprises at least one nucleotide change. For example, the change can be an insertion of one or more nucleotides, a deletion of one or more nucleotides, a substitution of one or more nucleotides, or combinations thereof. As a consequence of the "gene correction" integration of the modified sequence, the cell can produce a modified gene product from the targeted chromosomal sequence.

[0067] In other aspects, the donor sequence of the donor polynucleotide can be an exogenous sequence. As used herein, an "exogenous" sequence refers to a sequence that is not native to the cell, or a sequence whose native location is in a different location in the genome of the cell. For example, the exogenous sequence can comprise protein coding sequence, which can be operably linked to an exogenous promoter control sequence such that, upon integration into the genome, the cell is able to express the protein coded by the integrated sequence. Alternatively, the exogenous sequence can be integrated into the chromosomal sequence such that its expression is regulated by an endogenous promoter control sequence. In other iterations, the exogenous sequence can be a transcriptional control sequence, another expression control sequence, an RNA coding sequence, and so forth. As noted above, integration of an exogenous sequence into a chromosomal sequence is termed a "knock in."

[0068] As can be appreciated by those skilled in the art, the length of the donor sequence can and will vary. For example, the donor sequence can vary in length from several nucleotides to hundreds of nucleotides to hundreds of thousands of nucleotides.

[0069] Typically, the donor sequence in the donor polynucleotide is flanked by an upstream sequence and a downstream sequence, which have substantial sequence identity to sequences located upstream and downstream, respectively, of the sequence targeted by the engineered Cas9 system. Because of these sequence similarities, the upstream and downstream sequences of the donor polynucleotide permit homologous recombination between the donor polynucleotide and the targeted chromosomal sequence such that the donor sequence can be integrated into (or exchanged with) the chromosomal sequence.

[0070] The upstream sequence, as used herein, refers to a nucleic acid sequence that shares substantial sequence identity with a chromosomal sequence upstream of the sequence targeted by the engineered Cas9 system. Similarly, the downstream sequence refers to a nucleic acid sequence that shares substantial sequence identity with a chromosomal sequence downstream of the sequence targeted by the engineered Cas9 system. As used herein, the phrase “substantial sequence identity” refers to sequences having at least about 75% sequence identity. Thus, the upstream and downstream sequences in the donor polynucleotide can have about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity with sequence upstream or downstream to the target sequence. In an exemplary embodiment, the upstream and downstream sequences in the donor polynucleotide can have about 95% or 100% sequence identity with chromosomal sequences upstream or downstream to the sequence targeted by the engineered Cas9 system.

[0071] In some embodiments, the upstream sequence shares substantial sequence identity with a chromosomal sequence located immediately upstream of the sequence targeted by the engineered Cas9 system. In other embodiments, the upstream sequence shares substantial sequence identity with a chromosomal sequence that is located within about

one hundred (100) nucleotides upstream from the target sequence. Thus, for example, the upstream sequence can share substantial sequence identity with a chromosomal sequence that is located about 1 to about 20, about 21 to about 40, about 41 to about 60, about 61 to about 80, or about 81 to about 100 nucleotides upstream from the target sequence. In some embodiments, the downstream sequence shares substantial sequence identity with a chromosomal sequence located immediately downstream of the sequence targeted by the engineered Cas9 system. In other embodiments, the downstream sequence shares substantial sequence identity with a chromosomal sequence that is located within about one hundred (100) nucleotides downstream from the target sequence. Thus, for example, the downstream sequence can share substantial sequence identity with a chromosomal sequence that is located about 1 to about 20, about 21 to about 40, about 41 to about 60, about 61 to about 80, or about 81 to about 100 nucleotides downstream from the target sequence.

[0072] Each upstream or downstream sequence can range in length from about 20 nucleotides to about 5000 nucleotides. In some embodiments, upstream and downstream sequences can comprise about 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2800, 3000, 3200, 3400, 3600, 3800, 4000, 4200, 4400, 4600, 4800, or 5000 nucleotides. In specific embodiments, upstream and downstream sequences can range in length from about 50 to about 1500 nucleotides.

(c) Cell Types

[0073] A variety of eukaryotic cells are suitable for use in the methods disclosed herein. For example, the cell can be a human cell, a non-human mammalian cell, a non-mammalian vertebrate cell, an invertebrate cell, an insect cell, a plant cell, a yeast cell, or a single cell eukaryotic organism. In some embodiments, the cell can be a one cell embryo. For example, a non-human mammalian embryo including rat, hamster, rodent, rabbit, feline, canine, ovine, porcine, bovine, equine, and primate embryos. In still other embodiments, the cell can be a stem cell such as embryonic stem cells, ES-like stem cells, fetal stem cells, adult stem cells, and the like. In one embodiment, the stem cell is not a human embryonic stem cell. Furthermore,

the stem cells may include those made by the techniques disclosed in WO2003/046141, which is incorporated herein in its entirety, or Chung *et al.* (Cell Stem Cell, 2008, 2:113-117). The cell can be *in vitro* (*i.e.*, in culture), *ex vivo* (*i.e.*, within tissue isolated from an organism), or *in vivo* (*i.e.*, within an organism). In exemplary embodiments, the cell is a mammalian cell or mammalian cell line. In particular embodiments, the cell is a human cell or human cell line.

[0074] By way of example, in some embodiments, the eukaryotic cell, or a population of eukaryotic cells, is a T-cell, a $CD8^+$ T-cell, a $CD8^+$ naive T cell, a central memory T cell, an effector memory T-cell, a $CD4^+$ T-cell, a stem cell memory T-cell, a helper T-cell, a regulatory T-cell, a cytotoxic T-cell, a natural killer T-cell, a hematopoietic stem cell, a long term hematopoietic stem cell, a short term hematopoietic stem cell, a multipotent progenitor cell, a lineage restricted progenitor cell, a lymphoid progenitor cell, a pancreatic progenitor cell, an endocrine progenitor cell, an exocrine progenitor cell, a myeloid progenitor cell, a common myeloid progenitor cell, an erythroid progenitor cell, a megakaryocyte erythroid progenitor cell, a monocytic precursor cell, an endocrine precursor cell, an exocrine cell, a fibroblast, a hepatoblast, a myoblast, a macrophage, an islet beta-cell, a cardiomyocyte, a blood cell, a ductal cell, an acinar cell, an alpha cell, a beta cell, a delta cell, a PP cell, a cholangiocyte, a retinal cell, a photoreceptor cell, a rod cell, a cone cell, a retinal pigmented epithelium cell, a trabecular meshwork cell, a cochlear hair cell, an outer hair cell, an inner hair cell, a pulmonary epithelial cell, a bronchial epithelial cell, an alveolar epithelial cell, a pulmonary epithelial progenitor cell, a striated muscle cell, a cardiac muscle cell, a muscle satellite cell, a myocyte, a neuron, a neuronal stem cell, a mesenchymal stem cell, an induced pluripotent stem (iPS) cell, an embryonic stem cell, a monocyte, a megakaryocyte, a neutrophil, an eosinophil, a basophil, a mast cell, a reticulocyte, a B cell, e.g. a progenitor B cell, a Pre B cell, a Pro B cell, a memory B cell, a plasma B cell, a gastrointestinal epithelial cell, a biliary epithelial cell, a pancreatic ductal epithelial cell, an intestinal stem cell, a hepatocyte, a liver stellate cell, a Kupffer cell, an osteoblast, an osteoclast, an adipocyte (e.g., a brown adipocyte, or a white adipocyte), a preadipocyte, a pancreatic precursor cell, a pancreatic islet cell, a pancreatic

beta cell, a pancreatic alpha cell, a pancreatic delta cell, a pancreatic exocrine cell, a Schwann cell, or an oligodendrocyte, or a population of such cells.

[0075] Non-limiting examples of suitable mammalian cells or cell lines include human embryonic kidney cells (HEK293, HEK293T); human cervical carcinoma cells (HELA); human lung cells (W138); human liver cells (Hep G2); human U2-OS osteosarcoma cells, human A549 cells, human A-431 cells, and human K562 cells; Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells; mouse myeloma NS0 cells, mouse embryonic fibroblast 3T3 cells (NIH3T3), mouse B lymphoma A20 cells; mouse melanoma B16 cells; mouse myoblast C2C12 cells; mouse myeloma SP2/0 cells; mouse embryonic mesenchymal C3H-10T1/2 cells; mouse carcinoma CT26 cells, mouse prostate DuCuP cells; mouse breast EMT6 cells; mouse hepatoma Hepa1c1c7 cells; mouse myeloma J5582 cells; mouse epithelial MTD-1A cells; mouse myocardial MyEnd cells; mouse renal RenCa cells; mouse pancreatic RIN-5F cells; mouse melanoma X64 cells; mouse lymphoma YAC-1 cells; rat glioblastoma 9L cells; rat B lymphoma RBL cells; rat neuroblastoma B35 cells; rat hepatoma cells (HTC); buffalo rat liver BRL 3A cells; canine kidney cells (MDCK); canine mammary (CMT) cells; rat osteosarcoma D17 cells; rat monocyte/macrophage DH82 cells; monkey kidney SV-40 transformed fibroblast (COS7) cells; monkey kidney CVI-76 cells; African green monkey kidney (VERO-76) cells. An extensive list of mammalian cell lines may be found in the American Type Culture Collection catalog (ATCC, Manassas, VA).

(V) Applications

[0076] The compositions and methods disclosed herein can be used in a variety of therapeutic, diagnostic, industrial, and research applications. In some embodiments, the present disclosure can be used to modify any chromosomal sequence of interest in a cell, animal, or plant in order to model and/or study the function of genes, study genetic or epigenetic conditions of interest, or study biochemical pathways involved in various diseases or disorders. For example, transgenic organisms can be created that model diseases or disorders, wherein the expression of one or more nucleic acid sequences associated with a disease or disorder is altered. The disease model can be used to study the effects of mutations on the organism,

study the development and/or progression of the disease, study the effect of a pharmaceutically active compound on the disease, and/or assess the efficacy of a potential gene therapy strategy.

[0077] In other embodiments, the compositions and methods can be used to perform efficient and cost effective functional genomic screens, which can be used to study the function of genes involved in a particular biological process and how any alteration in gene expression can affect the biological process, or to perform saturating or deep scanning mutagenesis of genomic loci in conjunction with a cellular phenotype. Saturating or deep scanning mutagenesis can be used to determine critical minimal features and discrete vulnerabilities of functional elements required for gene expression, drug resistance, and reversal of disease, for example.

[0078] In further embodiments, the compositions and methods disclosed herein can be used for diagnostic tests to establish the presence of a disease or disorder and/or for use in determining treatment options. Examples of suitable diagnostic tests include detection of specific mutations in cancer cells (*e.g.*, specific mutation in EGFR, HER2, and the like), detection of specific mutations associated with particular diseases (*e.g.*, trinucleotide repeats, mutations in β-globin associated with sickle cell disease, specific SNPs, *etc.*), detection of hepatitis, detection of viruses (*e.g.*, Zika), and so forth.

[0079] In additional embodiments, the compositions and methods disclosed herein can be used to correct genetic mutations associated with a particular disease or disorder such as, *e.g.*, correct globin gene mutations associated with sickle cell disease or thalassemia, correct mutations in the adenosine deaminase gene associated with severe combined immune deficiency (SCID), reduce the expression of HTT, the disease-causing gene of Huntington's disease, or correct mutations in the rhodopsin gene for the treatment of retinitis pigmentosa. Such modifications may be made in cells *ex vivo*.

[0080] In still other embodiments, the compositions and methods disclosed herein can be used to generate crop plants with improved traits or increased resistance to environmental stresses. The present disclosure can also be used to generate farm animal with improved traits or production

animals. For example, pigs have many features that make them attractive as biomedical models, especially in regenerative medicine or xenotransplantation.

[0081] In still other embodiments, the compositions and methods disclosed herein can be used to determine chromosome identity and location within a living cell or chemically fixed cell (such as formalin fixation used in formalin-fixed paraffin embedded clinical samples). For example, a CRIPSR complex linked via a peptide sequence disclosed herein to a fluorescent protein maybe targeted in single or multiple copies to a genetic locus, and such complexes detected by microscopy to determine chromosomal locus copy number and/or location. Example genetic loci for tracking might include centromeric regions, telomeric regions, or other repetitive regions of the genome to which multiple copies of a single identical CRISPR complex may bind.

DEFINITIONS

[0082] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, Dictionary of Microbiology and Molecular Biology (2nd Ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0083] When introducing elements of the present disclosure or the preferred embodiments(s) thereof, the articles “a”, “an”, “the” and “said" are intended to mean that there are one or more of the elements. The terms “comprising”, “including” and “having” are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[0084] The term “about” when used in relation to a numerical value, x, for example means x ± 5%.

[0085] As used herein, the terms “complementary” or “complementarity” refer to the association of double-stranded nucleic acids by

base pairing through specific hydrogen bonds. The base paring may be standard Watson-Crick base pairing (*e.g.*, 5'-A G T C-3' pairs with the complementary sequence 3'-T C A G-5'). The base pairing also may be Hoogsteen or reversed Hoogsteen hydrogen bonding. Complementarity is typically measured with respect to a duplex region and thus, excludes overhangs, for example. Complementarity between two strands of the duplex region may be partial and expressed as a percentage (*e.g.*, 70%), if only some (*e.g.*, 70%) of the bases are complementary. The bases that are not complementary are "mismatched." Complementarity may also be complete (*i.e.*, 100%), if all the bases in the duplex region are complementary.

[0086] As used herein, the term "CRISPR/Cas system" or "Cas9 system" refers to a complex comprising a Cas9 protein (*i.e.*, nuclease, nickase, or catalytically dead protein) and a guide RNA.

[0087] The term "endogenous sequence," as used herein, refers to a chromosomal sequence that is native to the cell.

[0088] As used herein, the term "exogenous" refers to a sequence that is not native to the cell, or a chromosomal sequence whose native location in the genome of the cell is in a different chromosomal location.

[0089] A "gene," as used herein, refers to a DNA region (including exons and introns) encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites, and locus control regions.

[0090] The term "heterologous" refers to an entity that is not endogenous or native to the cell of interest. For example, a heterologous protein refers to a protein that is derived from or was originally derived from an exogenous source, such as an exogenously introduced nucleic acid sequence. In some instances, the heterologous protein is not normally produced by the cell of interest.

[0091] The term "nickase" refers to an enzyme that cleaves one strand of a double-stranded nucleic acid sequence (*i.e.*, nicks a double-stranded sequence). For example, a nuclease with double strand cleavage activity can be modified by mutation and/or deletion to function as a nickase and cleave only one strand of a double-stranded sequence.

[0092] The term "nuclease," as used herein, refers to an enzyme that cleaves both strands of a double-stranded nucleic acid sequence.

[0093] The terms "nucleic acid" and "polynucleotide" refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogs of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*, phosphorothioate backbones). In general, an analog of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analog of A will base-pair with T.

[0094] The term "nucleotide" refers to deoxyribonucleotides or ribonucleotides. The nucleotides may be standard nucleotides (*i.e.*, adenosine, guanosine, cytidine, thymidine, and uridine), nucleotide isomers, or nucleotide analogs. A nucleotide analog refers to a nucleotide having a modified purine or pyrimidine base or a modified ribose moiety. A nucleotide analog may be a naturally occurring nucleotide (*e.g.*, inosine, pseudouridine, etc.) or a non-naturally occurring nucleotide. Non-limiting examples of modifications on the sugar or base moieties of a nucleotide include the addition (or removal) of acetyl groups, amino groups, carboxyl groups, carboxymethyl groups, hydroxyl groups, methyl groups, phosphoryl groups, and thiol groups, as well as the substitution of the carbon and nitrogen atoms of the bases with other atoms (*e.g.*, 7-deaza purines). Nucleotide analogs also include dideoxy nucleotides, 2'-O-methyl nucleotides, locked nucleic acids (LNA), peptide nucleic acids (PNA), and morpholinos.

[0095] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues.

[0096] The terms "target sequence," "target chromosomal sequence," and "target site" are used interchangeably to refer to the specific

sequence in chromosomal DNA to which the engineered Cas9 system is targeted, and the site at which the engineered Cas9 system modifies the DNA or protein(s) associated with the DNA.

[0097] Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the “BestFit” utility application. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found on the GenBank website.

[0098] As various changes could be made in the above-described cells and methods without departing from the scope of the invention, it is intended that all matter contained in the above description and in the examples given below, shall be interpreted as illustrative and not in a limiting sense.

EXAMPLES

[0099] The following examples illustrate certain aspects of the disclosure.

Example 1: Human cell gene editing using GFP-SpCas9 and RFP-SpCas9 fusion proteins

[0100] Human K562 cells (0.35×10^6) were transfected with 60 pmol of SpCas9, GFP-SpCas9, or RFP-SpCas9 recombinant protein and 180 pmol of an in vitro transcribed single guide RNA (sgRNA) targeting the human EMX1 locus with the guide sequence 5'-GCUCCCAUCACAUCAACCGG-3'. Transfection was carried out using Nucleofection Solution V and an Amaxa instrument. Cells were maintained at 37°C and 5% CO_2 for three days before harvested for gene editing analysis. Genomic DNA was prepared using QuickExtract DNA extraction solution. Targeted EMX1 region was PCR amplified using primers consisting of target-specific sequences and next generation sequencing (NGS) adaptors. The forward primer is 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNAGTCTTCCCATCAGGCTCTCA-3' (SEQ ID NO:46) and the reverse primer is GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNAGAGTCCAGCTTGGGCC-3' (SEQ ID NO:47), where the target-specific sequences are underlined, and N represents A, T, G, or C. PCR amplicons were analyzed by NGS using the Illumina MiSeq to determine the editing efficiency of each Cas9 protein. The results displayed in **FIG. 1** show that the GFP-SpCas9 and RFP-SpCas9 fusing proteins each retain the editing activity parallel to the level by SpCas9 protein.

[0101] Table 1 presents the human codon optimized DNA and protein sequences of engineered Cas9/NLS proteins, wherein the NLS sequences are presented in bold text and the linker between the marker protein and Cas9 is presented in underlined text.

Table 1. Engineered Cas9 Systems

Amino acid sequence of GFP-SpCas9 fusion protein

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYL
STQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKVDAEAAAKEAAAK
EAAAKEAAAKALEAEAAAKEAAAKEAAAKEAAAKA**PAAKRVKLD**GGGGS
TGMDKKYSIGLDIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIG
ALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHR
LEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLR
LIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINAS
GVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNF
DLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRV
NTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGY
AGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIP
HQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFA
WMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSL
LYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQL
KEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILE
DIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLIN
GIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDS
LHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQ
KGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRD
MYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVP
SEEVVKKMKNYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQL
VETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFY
KVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAK
SEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDK
GRDFATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDW
DPKKYGGFDSPTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKN
PIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALP
SKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVI
LADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDR
KRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDEF**PKKKRKV**GGGGS**PK**
KKRKV (SEQ ID NO: 48)

Underlined: Linker between GFP and SpCas9
Bold: Nuclear localization signals

Amino acid sequence of RFP-SpCas9 fusion protein

MVSKGEAVIKEFMRFKVHMEGSMNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFSWDILSPQFMYGSRAFTKHPADIPDYYKQSFPEGFKWERVM
NFEDGGAVTVTQDTSLEDGTLIYKVKLRGTNFPPDGPVMQKKTMGWEAS
TERLYPEDGVLKGDIKMALRLKDGGRYLADFKTTYKAKKPVQMPGAYNV
DRKLDITSHNEDYTVVEQYERSEGRHSTGGMDELYKVDSGGSSGGSSG
SETPGTSESATPESSGGSSGGS**PAAKRVKLD**GGGGSTGMDKKYSIGLDI

GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEAT
RLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKK
HERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLS
KSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSK
DTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSAS
MIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQE
EFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAIL
RRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETIT
PWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNEL
TKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECF
DSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFED
REMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKT
ILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAG
SPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRE
RMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDI
NRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMK
NYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHV
AQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHH
AHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRKMIAKSEQEIGKATA
KYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKV
LSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDS
PTVAYSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYK
EVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLAS
HYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLS
AYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLD
ATLIHQSITGLYETRIDLSQLGGDEF**PKKKRKV**GGGGS**PKKKRKV** (SEQ
ID NO: 49)

Underlined: Linker between RFP and SpCas9
Bold: Nuclear localization signals

Amino acid sequence of GFP-eSpCas9 fusion protein

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIF
FKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYL
STQSKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYKVD<u>SGGSSGGSSG
SETPGTSESATPESSGGSSGGS</u>**PAAKRVKLD**GGGGSTGMDKKYSIGLDI
GTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEAT
RLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKK
HERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFR
GHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLS
KSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSK
DTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSAS
MIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQE
EFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAIL
RRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETIT

PWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNEL
TKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECF
DSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFED
REMIEERLKTYAHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKT
ILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAG
SPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQKGQKNSRE
RMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDI
NRLSDYDVDHIVPQSFLADDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMK
NYWRQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHV
AQILDSRMNTKYDENDKLIREVKVITLKSKLVSDFRKDFQFYKVREINNYHH
AHDAYLNAVVGTALIKKYPALESEFVYGDYKVYDVRKMIAKSEQEIGKATA
KYFFYSNIMNFFKTEITLANGEIRKAPLIETNGETGEIVWDKGRDFATVRKV
LSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDS
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EVKKDLIIKLPKYSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLAS
HYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISEFSKRVILADANLDKVLS
AYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLD
ATLIHQSITGLYETRIDLSQLGGDEF**PKKKRKV**GGGGS**PKKKRKV** (SEQ
ID NO:50)

Underlined: Linker between GFP and eSpCas9
Bold: Nuclear localization signals

[0102] Human codon optimized DNA sequences used to produce the three proteins are as follows:

Human codon optimized GFP-SpCas9 DNA sequence

ATGGTTAGCAAAGGTGAAGAACTGTTTACAGGTGTTGTTCCGATTCTG
GTTGAACTGGATGGTGATGTTAATGGCCACAAATTTTCAGTTAGCGGT
GAAGGCGAAGGTGATGCAACCTATGGTAAACTGACCCTGAAATTTATC
TGTACCACCGGCAAACTGCCGGTTCCGTGGCCGACACTGGTTACCAC
ACTGACCTATGGTGTTCAGTGTTTTAGCCGTTATCCGGATCACATGAAA
CAGCACGATTTTTTCAAAAGCGCAATGCCGGAAGGTTATGTTCAAGAA
CGTACCATCTTCTTCAAAGATGACGGCAACTATAAAACCCGTGCCGAA
GTTAAATTTGAAGGTGATACCCTGGTGAATCGCATTGAACTGAAAGGC
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TACGACCATCGACCGCAAGCGCTATACCAGCACCAAAGAGGTCCTGG
ACGCGACCCTGATCCACCAGAGCATTACCGGCTTATACGAAACCCGTA
TTGATTTGAGCCAACTGGGTGGCGATGAATTCCCGAAAAAAAAGCGCA
AAGTTGGTGGCGGTGGTAGCCCGAAAAAGAAACGTAAAGTG (SEQ ID
NO:62)

Human codon optimized RFP-SpCas9 DNA sequence

ATGGTTAGCAAAGGTGAAGCCGTGATTAAAGAATTTATGCGCTTTAAG
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Human codon optimized GFP-eSpCas9 DNA sequence

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GCGCAAAGTTGGTGGCGGTGGTAGCCCGAAAAAGAAACGTAAAGTG (SEQ ID NO:64)

Example 2: Editing efficiency comparison with commercial products

[0103] Two commercial GFP-SpCas9 fusion protein products, GenCrispr NLS-Cas9-EGFP Nuclease and ArciTect Cas9-eGFP Nuclease, were purchased from GenScript (Piscataway, NJ) and Stemcell Technologies (Vancouver, Canada), respectively. Human U2OS cells (0.2 x 10^6) and HEK293 cells (0.3 x 10^6) were transfected with 50 pmol of GenCrispr NLS-Cas9-EGFP Nuclease, or ArciTect Cas9-eGFP Nuclease, or the GFP-SpCas9 protein of the current invention, in combination with 150 pmol each of four chemically synthesized sgRNAs targeting the Human EMX1, HEKSite4, VEGFA3, HPRT loci. The guide sequences are: 5'-GAGUCCGAGCAGAAGAAGAA-3' (EMX1) (SEQ ID NO:51), 5'-GGCACUGCGGCUGGAGGUGG-3' (HEKSite4) (SEQ ID NO:52), 5'GGUGAGUGAGUGUGUGCGUG-3' (VEGFA3), and 5'-GGUCACUUUUAACACACCCA-3' (HPRT) (SEQ ID NO:53). Transfection was carried out using Nucleofection Solution V and an Amaxa instrument. Cells were maintained at 37°C and 5% CO_2 for three days before harvested for gene editing analysis. Genomic DNA was prepared using QuickExtract DNA extraction solution. Each targeted genomic region was PCR amplified using a pair of primers consisting of target-specific sequences and next generation sequencing (NGS) adaptors. The primers are listed in the following table:

NGS primer sequences	
Target	Primer sequence (5'-3')
EMX1	Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNCCCCAGTGGCTGCTCT (SEQ ID NO:54) Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNCCAGGCCTCCCCAAAGC (SEQ ID NO:55)
HEKSite4	Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNGGAACCCAGGTAGCCAGAGA (SEQ ID NO:56)

	Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNGG GGTGGGGTCAGACGT (SEQ ID NO:57)
VEGF A3	Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNGCC CATTCCCTCTTTAGCCA (SEQ ID NO:58) Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNGG AGCAGGAAAGTGAGGTTAC (SEQ ID NO:59)
HPRT	Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNAAT GGACACATGGGTAGTCAGG (SEQ ID NO:60) Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNGG CTTATATCCAACACTTCGTGGG (SEQ ID NO:61)

[0104] PCR amplicons were analyzed by NGS using the Illumina MiSeq to determine the editing efficiency of each Cas9 protein. The results in **FIG. 2A** and **FIG. 2B** show that the editing efficiencies by the GFP-SpCas9 protein of the current invention were several-fold higher than that of the commercial proteins in all targets.

CLAIMS

1. A fusion protein comprising at least one nuclear localization signal and a Cas9 protein linked to at least one marker protein, wherein the marker protein is linked to the Cas9 protein directly or indirectly via a linker having a sequence set forth in SEQ ID NO:35,

wherein the nuclear localization signal, the marker protein, the linker, and the Cas9 protein are arranged in the following order from N-terminus to C-terminus:

marker protein – linker – nuclear localization signal – Cas9 protein.

2. The fusion protein of claim 1, further comprising at least one heterologous domain and optionally wherein the at least one heterologous domain is a cell-penetrating domain, a chromatin modulating motif, an epigenetic modification domain, a transcriptional regulation domain, an RNA aptamer binding domain, or combination thereof.

3. The fusion protein of claim 1 or 2, wherein the fusion protein is a nuclease and cleaves both strands of a double-stranded sequence, is a nickase and cleaves one strand of a double-stranded sequence, or has no nuclease or nickase activity, and/or wherein the cas9 protein is from *Streptococcus pyogenes, Streptococcus thermophilus*, *Neisseria meningitidis*, *Staphylococcus aureus*, or *Campylobacter jejuni*.

4. The fusion protein of any one of claims 1 to 3, wherein the at least one marker protein has an amino acid sequence having at least 90% sequence identity with SEQ ID NO:19 or 20 or wherein the at least one marker protein has an amino acid sequence set forth in SEQ ID NO:19 or 20.

5. The fusion protein of any one of claims 1 to 4, wherein the fusion protein has an amino acid sequence having at least 90% sequence identity with SEQ ID NO:48, 49, or 50 or the fusion protein has an amino acid sequence as set forth in SEQ ID NO:48, 49, or 50.

6. The fusion protein of claim 5, wherein the fusion protein has an amino acid sequence having at least 95% sequence identity with SEQ ID NO:48, 49, or 50.

7. The fusion protein of claim 6, wherein the fusion protein has an amino acid sequence having at least 98% sequence identity with SEQ ID NO:48, 49, or 50.

8. The fusion protein of claim 7, wherein the fusion protein has an amino acid sequence having at least 99% sequence identity with SEQ ID NO:48, 49, or 50.

9. A system comprising the fusion protein of any one of claims 1 to 8 and an engineered guide RNA.

10. The system of claim 9, wherein (a) the engineered guide RNA is a single molecule and/or (b) the engineered guide RNA sequence is optimized to facilitate base-paring within the engineered guide RNA, minimize base-paring within the engineered guide RNA, increase stability of the engineered guide RNA, facilitate transcription of the engineered guide RNA in a eukaryotic cell, or a combination thereof.

11. A plurality of nucleic acids encoding:
(a) the fusion protein of any one of claims 1 to 8 or
(b) the system of claim 9 or 10, wherein the plurality of nucleic acids comprises at least one nucleic acid encoding the fusion protein, and at least one nucleic acid encoding the engineered guide RNA.

12. The plurality of nucleic acids of claim 11, wherein (a) the at least one nucleic acid encoding the fusion protein is RNA or DNA (b) the at least one nucleic acid encoding the fusion protein is codon optimized for expression in a eukaryotic cell and the eukaryotic cell is optionally a human cell, a non-human mammalian cell, a non-mammalian vertebrate cell, an invertebrate cell, a plant cell, or a single cell eukaryotic organism, (c) the at least one nucleic acid encoding the engineered guide RNA is DNA, (d) the at least one nucleic acid encoding the fusion protein is operably linked to a phage promoter sequence for *in vitro* RNA synthesis or protein expression in a bacterial cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a phage promoter sequence for *in vitro* RNA synthesis, or (e) the at least one nucleic acid encoding the fusion protein is

operably linked to a eukaryotic promoter sequence for expression in a eukaryotic cell, and the at least one nucleic acid encoding the engineered guide RNA is operably linked to a eukaryotic promoter sequence for expression in a eukaryotic cell.

13. At least one vector comprising the plurality of nucleic acids of claim 11 or 12, optionally wherein the at least one vector is a plasmid vector, a viral vector, or a self-replicating viral RNA replicon.

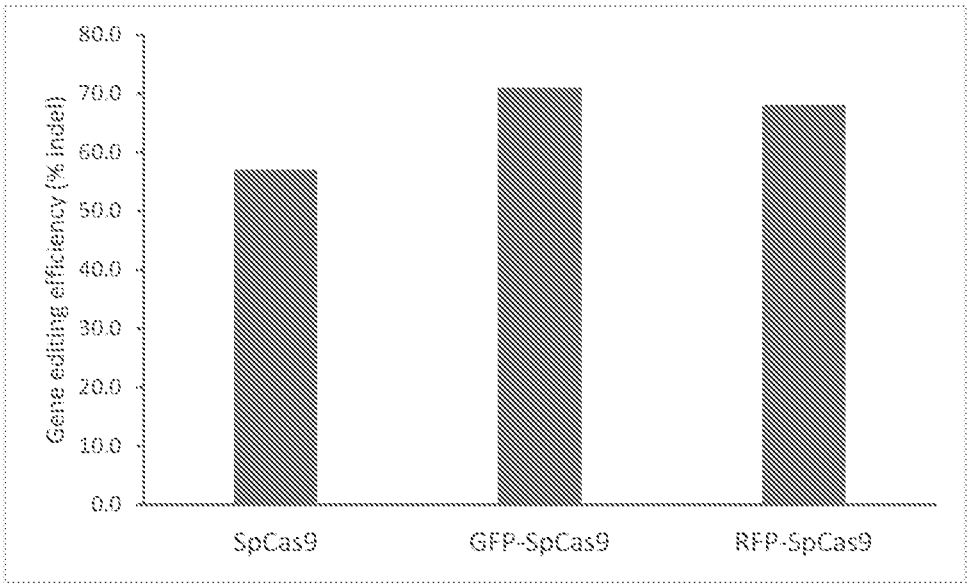
14. A eukaryotic cell comprising at least one system comprising a fusion protein as defined in any of claims 1 to 8, a system as defined in claim 9 or 10, a plurality of nucleic acids as defined in claim 11 or 12, or at least one vector as defined in claim 13.

15. The eukaryotic cell of claim 14, which is a human cell, a non-human mammalian cell, a plant cell, a non-mammalian vertebrate cell, an invertebrate cell, or a single cell eukaryotic organism and/or which is *in vivo*, *ex vivo*, or *in vitro*.

16. A method for determining chromosome identity and location within a living eukaryotic cell or chemically fixed eukaryotic cell, the method comprising introducing the fusion protein, system, plurality of nucleic acids, or vector of any one of claims 1 to 13 into the living or chemically fixed eukaryotic cell and detecting a signal from the marker protein.

17. The method of claim 16, wherein the eukaryotic cell is a human cell, a non-human mammalian cell, a plant cell, a non-mammalian vertebrate cell, an invertebrate cell, or a single cell eukaryotic organism and/or wherein the eukaryotic cell is *in vivo, ex vivo,* or *in vitro*.

FIG. 1


80.0
70.0
60.0
50.0
40.0
30.0
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10.0
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Gene editing efficiency (% indel)
SpCas9
GFP-SpCas9
RFP-SpCas9

FIG. 2A

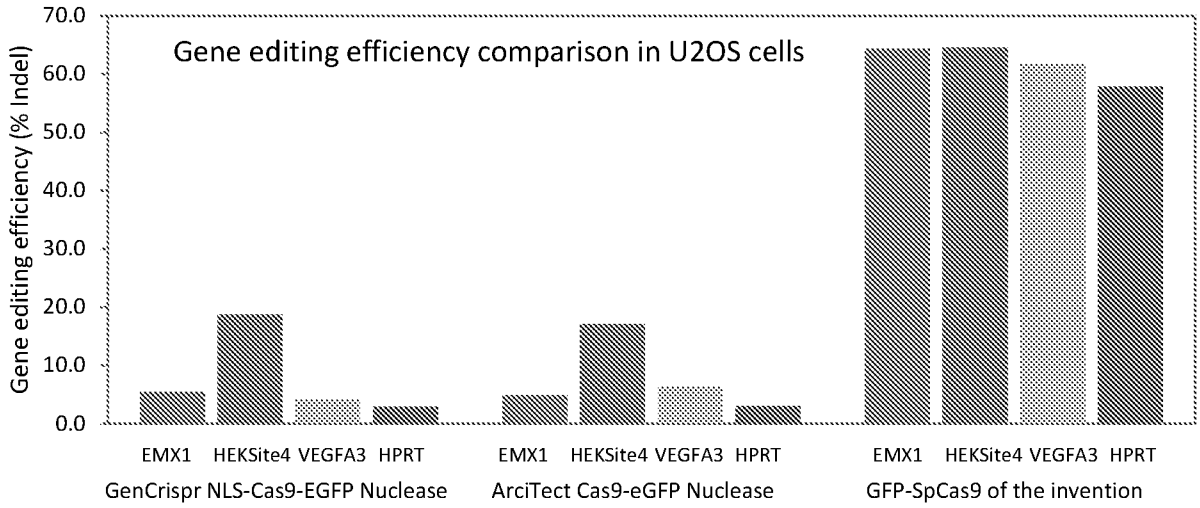
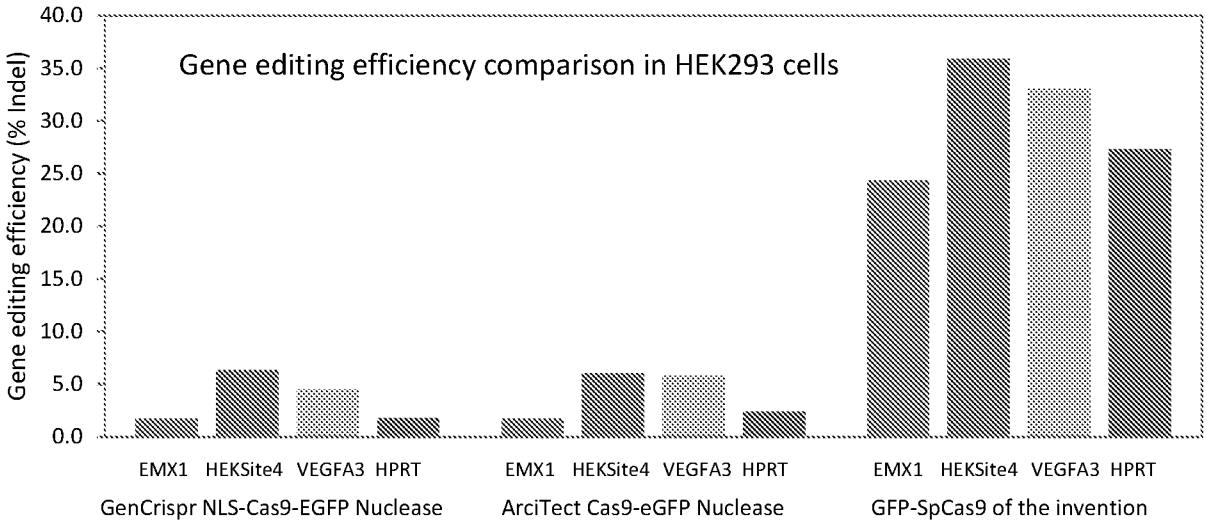

Gene editing efficiency comparison in U2OS cells
Gene editing efficiency (% Indel)
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
EMX1 HEKSite4 VEGFA3 HPRT
EMX1 HEKSite4 VEGFA3 HPRT
EMX1 HEKSite4 VEGFA3 HPRT
GenCrispr NLS-Cas9-EGFP Nuclease
ArciTect Cas9-eGFP Nuclease
GFP-SpCas9 of the invention

FIG. 2B


Gene editing efficiency comparison in HEK293 cells
Gene editing efficiency (% Indel)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
EMX1 HEKSite4 VEGFA3 HPRT
EMX1 HEKSite4 VEGFA3 HPRT
EMX1 HEKSite4 VEGFA3 HPRT
GenCrispr NLS-Cas9-EGFP Nuclease
ArciTect Cas9-eGFP Nuclease
GFP-SpCas9 of the invention

SEQUENCE LISTING

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<120> CRISPR/CAS FUSION PROTEINS AND SYSTEMS

<130> P19-027 WO-PCT

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Lys Ser Lys Lys
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Lys

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20 25 30

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1 5 10 15

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            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Lys Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
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20 25 30

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35 40 45

Val Thr Lys Gly Gly Pro Leu Pro Phe Ser Trp Asp Ile Leu Ser Pro
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Gln Phe Met Tyr Gly Ser Arg Ala Phe Thr Lys His Pro Ala Asp Ile
65 70 75 80

Pro Asp Tyr Tyr Lys Gln Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg
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Val Met Asn Phe Glu Asp Gly Gly Ala Val Thr Val Thr Gln Asp Thr
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Ser Leu Glu Asp Gly Thr Leu Ile Tyr Lys Val Lys Leu Arg Gly Thr
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Asn Phe Pro Pro Asp Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp
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Glu Ala Ser Thr Glu Arg Leu Tyr Pro Glu Asp Gly Val Leu Lys Gly
145 150 155 160

Asp Ile Lys Met Ala Leu Arg Leu Lys Asp Gly Gly Arg Tyr Leu Ala
165 170 175

Asp Phe Lys Thr Thr Tyr Lys Ala Lys Lys Pro Val Gln Met Pro Gly
180 185 190

Ala Tyr Asn Val Asp Arg Lys Leu Asp Ile Thr Ser His Asn Glu Asp
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Gly Gly Met Asp Glu Leu Tyr Lys
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1 5 10 15

Lys Arg Lys Val
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<210> 24
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1 5 10 15

Arg Lys Val

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Ala Trp Ser Gln Pro Lys Lys Lys Arg Lys Val
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Lys Lys Arg Lys Val
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Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
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Leu Ala Lys His Leu Ala Lys Ala Leu Ala Lys Ala Leu Lys Cys Glu
            20   25   30

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Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Ala
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Ser Glu Ser Ala Thr Pro Glu Ser Ser Gly Gly Ser Ser Gly Gly Ser
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1 5

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Ala Ala Ala Lys
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repeating units"

<400> 45
Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro Ala Pro
1 5 10 15

<210> 46
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (34)..(39)
<223> a, t, g or c

<400> 46
tcgtcggcag cgtcagatgt gtataagaga cagnnnnnna gtcttcccat caggctctca 60

<210> 47
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (35)..(40)
<223> a, t, g or c

<400> 47
gtctcgtggg ctcggagatg tgtataagag acagnnnnnn agagtccagc ttgggcc 57

<210> 48
<211> 1692
<212> PRT
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polypeptide"

<400> 48
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

```
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20                  25                  30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Lys Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220
```

```
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Val
225                 230                 235                 240

Asp Ala Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala
                245                 250                 255

Lys Glu Ala Ala Ala Lys Ala Leu Glu Ala Glu Ala Ala Ala Lys Glu
            260                 265                 270

Ala Ala Ala Lys Glu Ala Ala Ala Lys Glu Ala Ala Ala Lys Ala Pro
        275                 280                 285

Ala Ala Lys Arg Val Lys Leu Asp Gly Gly Gly Gly Ser Thr Gly Met
    290                 295                 300

Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser Val Gly
305                 310                 315                 320

Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe Lys
                325                 330                 335

Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile Gly
            340                 345                 350

Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu Lys
        355                 360                 365

Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys Tyr
    370                 375                 380

Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser Phe
385                 390                 395                 400

Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys His
                405                 410                 415

Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr His
            420                 425                 430

Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp Ser
        435                 440                 445
```

```
Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His Met
    450                 455                 460

Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro Asp
465                 470                 475                 480

Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr Asn
                485                 490                 495

Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp Ala Lys
            500                 505                 510

Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn Leu
        515                 520                 525

Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn Leu
    530                 535                 540

Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe Asp
545                 550                 555                 560

Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp Asp
                565                 570                 575

Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp Leu
            580                 585                 590

Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp Ile
        595                 600                 605

Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser Met
    610                 615                 620

Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu Lys Ala
625                 630                 635                 640

Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe Asp
                645                 650                 655
```

```
Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser Gln
            660                 665                 670

Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met Asp Gly
        675                 680                 685

Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu Arg Lys
    690                 695                 700

Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His Leu Gly
705                 710                 715                 720

Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe Leu
                725                 730                 735

Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile Pro
            740                 745                 750

Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp Met
        755                 760                 765

Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu Val
    770                 775                 780

Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr Asn
785                 790                 795                 800

Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His Ser Leu
                805                 810                 815

Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val Lys Tyr
            820                 825                 830

Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln Lys
        835                 840                 845

Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val Thr Val
    850                 855                 860

Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp Ser
865                 870                 875                 880
```

```
Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu Gly Thr
               885                 890                 895


Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp Asn
            900                 905                 910


Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu Thr Leu
        915                 920                 925


Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala His
    930                 935                 940


Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg Tyr Thr
945                 950                 955                 960


Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp Lys
               965                 970                 975


Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe Ala
            980                 985                 990


Asn Arg Asn Phe Met Gln Leu Ile  His Asp Asp Ser Leu  Thr Phe Lys
        995                 1000                1005


Glu Asp  Ile Gln Lys Ala Gln  Val Ser Gly Gln Gly  Asp Ser Leu
    1010                1015                1020


His Glu  His Ile Ala Asn Leu  Ala Gly Ser Pro Ala  Ile Lys Lys
    1025                1030                1035


Gly Ile  Leu Gln Thr Val Lys  Val Val Asp Glu Leu  Val Lys Val
    1040                1045                1050


Met Gly  Arg His Lys Pro Glu  Asn Ile Val Ile Glu  Met Ala Arg
    1055                1060                1065


Glu Asn  Gln Thr Thr Gln Lys  Gly Gln Lys Asn Ser  Arg Glu Arg
    1070                1075                1080
```

```
Met Lys  Arg Ile Glu Glu Gly  Ile Lys Glu Leu Gly  Ser Gln Ile
    1085                 1090                 1095

Leu Lys  Glu His Pro Val Glu  Asn Thr Gln Leu Gln  Asn Glu Lys
    1100                 1105                 1110

Leu Tyr  Leu Tyr Tyr Leu Gln  Asn Gly Arg Asp Met  Tyr Val Asp
    1115                 1120                 1125

Gln Glu  Leu Asp Ile Asn Arg  Leu Ser Asp Tyr Asp  Val Asp His
    1130                 1135                 1140

Ile Val  Pro Gln Ser Phe Leu  Lys Asp Asp Ser Ile  Asp Asn Lys
    1145                 1150                 1155

Val Leu  Thr Arg Ser Asp Lys  Asn Arg Gly Lys Ser  Asp Asn Val
    1160                 1165                 1170

Pro Ser  Glu Glu Val Val Lys  Lys Met Lys Asn Tyr  Trp Arg Gln
    1175                 1180                 1185

Leu Leu  Asn Ala Lys Leu Ile  Thr Gln Arg Lys Phe  Asp Asn Leu
    1190                 1195                 1200

Thr Lys  Ala Glu Arg Gly Gly  Leu Ser Glu Leu Asp  Lys Ala Gly
    1205                 1210                 1215

Phe Ile  Lys Arg Gln Leu Val  Glu Thr Arg Gln Ile  Thr Lys His
    1220                 1225                 1230

Val Ala  Gln Ile Leu Asp Ser  Arg Met Asn Thr Lys  Tyr Asp Glu
    1235                 1240                 1245

Asn Asp  Lys Leu Ile Arg Glu  Val Lys Val Ile Thr  Leu Lys Ser
    1250                 1255                 1260

Lys Leu  Val Ser Asp Phe Arg  Lys Asp Phe Gln Phe  Tyr Lys Val
    1265                 1270                 1275

Arg Glu  Ile Asn Asn Tyr His  His Ala His Asp Ala  Tyr Leu Asn
    1280                 1285                 1290
```

```
Ala Val  Val Gly Thr Ala Leu  Ile Lys Lys Tyr Pro  Lys Leu Glu
    1295                1300                1305

Ser Glu  Phe Val Tyr Gly Asp  Tyr Lys Val Tyr Asp  Val Arg Lys
    1310                1315                1320

Met Ile  Ala Lys Ser Glu Gln  Glu Ile Gly Lys Ala  Thr Ala Lys
    1325                1330                1335

Tyr Phe  Phe Tyr Ser Asn Ile  Met Asn Phe Phe Lys  Thr Glu Ile
    1340                1345                1350

Thr Leu  Ala Asn Gly Glu Ile  Arg Lys Arg Pro Leu  Ile Glu Thr
    1355                1360                1365

Asn Gly  Glu Thr Gly Glu Ile  Val Trp Asp Lys Gly  Arg Asp Phe
    1370                1375                1380

Ala Thr  Val Arg Lys Val Leu  Ser Met Pro Gln Val  Asn Ile Val
    1385                1390                1395

Lys Lys  Thr Glu Val Gln Thr  Gly Gly Phe Ser Lys  Glu Ser Ile
    1400                1405                1410

Leu Pro  Lys Arg Asn Ser Asp  Lys Leu Ile Ala Arg  Lys Lys Asp
    1415                1420                1425

Trp Asp  Pro Lys Lys Tyr Gly  Gly Phe Asp Ser Pro  Thr Val Ala
    1430                1435                1440

Tyr Ser  Val Leu Val Val Ala  Lys Val Glu Lys Gly  Lys Ser Lys
    1445                1450                1455

Lys Leu  Lys Ser Val Lys Glu  Leu Leu Gly Ile Thr  Ile Met Glu
    1460                1465                1470

Arg Ser  Ser Phe Glu Lys Asn  Pro Ile Asp Phe Leu  Glu Ala Lys
    1475                1480                1485
```

```
Gly Tyr  Lys Glu Val Lys Lys  Asp Leu Ile Ile Lys  Leu Pro Lys
    1490                1495                1500

Tyr Ser  Leu Phe Glu Leu Glu  Asn Gly Arg Lys Arg  Met Leu Ala
    1505                1510                1515

Ser Ala  Gly Glu Leu Gln Lys  Gly Asn Glu Leu Ala  Leu Pro Ser
    1520                1525                1530

Lys Tyr  Val Asn Phe Leu Tyr  Leu Ala Ser His Tyr  Glu Lys Leu
    1535                1540                1545

Lys Gly  Ser Pro Glu Asp Asn  Glu Gln Lys Gln Leu  Phe Val Glu
    1550                1555                1560

Gln His  Lys His Tyr Leu Asp  Glu Ile Ile Glu Gln  Ile Ser Glu
    1565                1570                1575

Phe Ser  Lys Arg Val Ile Leu  Ala Asp Ala Asn Leu  Asp Lys Val
    1580                1585                1590

Leu Ser  Ala Tyr Asn Lys His  Arg Asp Lys Pro Ile  Arg Glu Gln
    1595                1600                1605

Ala Glu  Asn Ile Ile His Leu  Phe Thr Leu Thr Asn  Leu Gly Ala
    1610                1615                1620

Pro Ala  Ala Phe Lys Tyr Phe  Asp Thr Thr Ile Asp  Arg Lys Arg
    1625                1630                1635

Tyr Thr  Ser Thr Lys Glu Val  Leu Asp Ala Thr Leu  Ile His Gln
    1640                1645                1650

Ser Ile  Thr Gly Leu Tyr Glu  Thr Arg Ile Asp Leu  Ser Gln Leu
    1655                1660                1665

Gly Gly  Asp Glu Phe Pro Lys  Lys Lys Arg Lys Val  Gly Gly Gly
    1670                1675                1680

Gly Ser  Pro Lys Lys Lys Arg  Lys Val
    1685                1690
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<210> 49
<211> 1671
<212> PRT
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polypeptide"

<400> 49
Met Val Ser Lys Gly Glu Ala Val Ile Lys Glu Phe Met Arg Phe Lys
1 5 10 15

Val His Met Glu Gly Ser Met Asn Gly His Glu Phe Glu Ile Glu Gly
20 25 30

Glu Gly Glu Gly Arg Pro Tyr Glu Gly Thr Gln Thr Ala Lys Leu Lys
35 40 45

Val Thr Lys Gly Gly Pro Leu Pro Phe Ser Trp Asp Ile Leu Ser Pro
50 55 60

Gln Phe Met Tyr Gly Ser Arg Ala Phe Thr Lys His Pro Ala Asp Ile
65 70 75 80

Pro Asp Tyr Tyr Lys Gln Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg
85 90 95

Val Met Asn Phe Glu Asp Gly Gly Ala Val Thr Val Thr Gln Asp Thr
100 105 110

Ser Leu Glu Asp Gly Thr Leu Ile Tyr Lys Val Lys Leu Arg Gly Thr
115 120 125

Asn Phe Pro Pro Asp Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp
130 135 140

Glu Ala Ser Thr Glu Arg Leu Tyr Pro Glu Asp Gly Val Leu Lys Gly
145 150 155 160

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

```
                165                 170                 175

Asp Phe Lys Thr Thr Tyr Lys Ala Lys Lys Pro Val Gln Met Pro Gly
            180                 185                 190

Ala Tyr Asn Val Asp Arg Lys Leu Asp Ile Thr Ser His Asn Glu Asp
        195                 200                 205

Tyr Thr Val Val Glu Gln Tyr Glu Arg Ser Glu Gly Arg His Ser Thr
    210                 215                 220

Gly Gly Met Asp Glu Leu Tyr Lys Val Asp Ser Gly Gly Ser Ser Gly
225                 230                 235                 240

Gly Ser Ser Gly Ser Glu Thr Pro Gly Thr Ser Glu Ser Ala Thr Pro
                245                 250                 255

Glu Ser Ser Gly Gly Ser Ser Gly Gly Ser Pro Ala Ala Lys Arg Val
            260                 265                 270

Lys Leu Asp Gly Gly Gly Gly Ser Thr Gly Met Asp Lys Lys Tyr Ser
        275                 280                 285

Ile Gly Leu Asp Ile Gly Thr Asn Ser Val Gly Trp Ala Val Ile Thr
    290                 295                 300

Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe Lys Val Leu Gly Asn Thr
305                 310                 315                 320

Asp Arg His Ser Ile Lys Lys Asn Leu Ile Gly Ala Leu Leu Phe Asp
                325                 330                 335

Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu Lys Arg Thr Ala Arg Arg
            340                 345                 350

Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys Tyr Leu Gln Glu Ile Phe
        355                 360                 365

Ser Asn Glu Met Ala Lys Val Asp Asp Ser Phe Phe His Arg Leu Glu
    370                 375                 380
```

```
Glu Ser Phe Leu Val Glu Glu Asp Lys Lys His Glu Arg His Pro Ile
385                 390                 395                 400

Phe Gly Asn Ile Val Asp Glu Val Ala Tyr His Glu Lys Tyr Pro Thr
                405                 410                 415

Ile Tyr His Leu Arg Lys Lys Leu Val Asp Ser Thr Asp Lys Ala Asp
            420                 425                 430

Leu Arg Leu Ile Tyr Leu Ala Leu Ala His Met Ile Lys Phe Arg Gly
        435                 440                 445

His Phe Leu Ile Glu Gly Asp Leu Asn Pro Asp Asn Ser Asp Val Asp
    450                 455                 460

Lys Leu Phe Ile Gln Leu Val Gln Thr Tyr Asn Gln Leu Phe Glu Glu
465                 470                 475                 480

Asn Pro Ile Asn Ala Ser Gly Val Asp Ala Lys Ala Ile Leu Ser Ala
                485                 490                 495

Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala Gln Leu Pro
            500                 505                 510

Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala Leu Ser Leu
        515                 520                 525

Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala Glu Asp Ala
    530                 535                 540

Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu Asp Asn Leu
545                 550                 555                 560

Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu Ala Ala Lys
                565                 570                 575

Asn Leu Ser Asp Ala Ile Leu Leu Ser Asp Ile Leu Arg Val Asn Thr
            580                 585                 590

Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser Met Ile Lys Arg Tyr Asp
```

```
        595                 600                 605

Glu His His Gln Asp Leu Thr Leu Leu Lys Ala Leu Val Arg Gln Gln
    610                 615                 620

Leu Pro Glu Lys Tyr Lys Glu Ile Phe Phe Asp Gln Ser Lys Asn Gly
625                 630                 635                 640

Tyr Ala Gly Tyr Ile Asp Gly Gly Ala Ser Gln Glu Glu Phe Tyr Lys
                645                 650                 655

Phe Ile Lys Pro Ile Leu Glu Lys Met Asp Gly Thr Glu Glu Leu Leu
            660                 665                 670

Val Lys Leu Asn Arg Glu Asp Leu Leu Arg Lys Gln Arg Thr Phe Asp
        675                 680                 685

Asn Gly Ser Ile Pro His Gln Ile His Leu Gly Glu Leu His Ala Ile
    690                 695                 700

Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe Leu Lys Asp Asn Arg Glu
705                 710                 715                 720

Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile Pro Tyr Tyr Val Gly Pro
                725                 730                 735

Leu Ala Arg Gly Asn Ser Arg Phe Ala Trp Met Thr Arg Lys Ser Glu
            740                 745                 750

Glu Thr Ile Thr Pro Trp Asn Phe Glu Glu Val Val Asp Lys Gly Ala
        755                 760                 765

Ser Ala Gln Ser Phe Ile Glu Arg Met Thr Asn Phe Asp Lys Asn Leu
    770                 775                 780

Pro Asn Glu Lys Val Leu Pro Lys His Ser Leu Leu Tyr Glu Tyr Phe
785                 790                 795                 800

Thr Val Tyr Asn Glu Leu Thr Lys Val Lys Tyr Val Thr Glu Gly Met
                805                 810                 815
```

```
Arg Lys Pro Ala Phe Leu Ser Gly Glu Gln Lys Lys Ala Ile Val Asp
            820                 825                 830

Leu Leu Phe Lys Thr Asn Arg Lys Val Thr Val Lys Gln Leu Lys Glu
        835                 840                 845

Asp Tyr Phe Lys Lys Ile Glu Cys Phe Asp Ser Val Glu Ile Ser Gly
    850                 855                 860

Val Glu Asp Arg Phe Asn Ala Ser Leu Gly Thr Tyr His Asp Leu Leu
865                 870                 875                 880

Lys Ile Ile Lys Asp Lys Asp Phe Leu Asp Asn Glu Glu Asn Glu Asp
                885                 890                 895

Ile Leu Glu Asp Ile Val Leu Thr Leu Thr Leu Phe Glu Asp Arg Glu
            900                 905                 910

Met Ile Glu Glu Arg Leu Lys Thr Tyr Ala His Leu Phe Asp Asp Lys
        915                 920                 925

Val Met Lys Gln Leu Lys Arg Arg Arg Tyr Thr Gly Trp Gly Arg Leu
    930                 935                 940

Ser Arg Lys Leu Ile Asn Gly Ile Arg Asp Lys Gln Ser Gly Lys Thr
945                 950                 955                 960

Ile Leu Asp Phe Leu Lys Ser Asp Gly Phe Ala Asn Arg Asn Phe Met
                965                 970                 975

Gln Leu Ile His Asp Asp Ser Leu Thr Phe Lys Glu Asp Ile Gln Lys
            980                 985                 990

Ala Gln Val Ser Gly Gln Gly Asp  Ser Leu His Glu His  Ile Ala Asn
        995                 1000                1005

Leu Ala  Gly Ser Pro Ala Ile  Lys Lys Gly Ile Leu  Gln Thr Val
    1010                1015                1020

Lys Val  Val Asp Glu Leu Val  Lys Val Met Gly Arg  His Lys Pro
```

```
    1025                1030                1035

Glu Asn  Ile Val Ile Glu Met  Ala Arg Glu Asn Gln  Thr Thr Gln
    1040                1045                1050

Lys Gly  Gln Lys Asn Ser Arg  Glu Arg Met Lys Arg  Ile Glu Glu
    1055                1060                1065

Gly Ile  Lys Glu Leu Gly Ser  Gln Ile Leu Lys Glu  His Pro Val
    1070                1075                1080

Glu Asn  Thr Gln Leu Gln Asn  Glu Lys Leu Tyr Leu  Tyr Tyr Leu
    1085                1090                1095

Gln Asn  Gly Arg Asp Met Tyr  Val Asp Gln Glu Leu  Asp Ile Asn
    1100                1105                1110

Arg Leu  Ser Asp Tyr Asp Val  Asp His Ile Val Pro  Gln Ser Phe
    1115                1120                1125

Leu Lys  Asp Asp Ser Ile Asp  Asn Lys Val Leu Thr  Arg Ser Asp
    1130                1135                1140

Lys Asn  Arg Gly Lys Ser Asp  Asn Val Pro Ser Glu  Glu Val Val
    1145                1150                1155

Lys Lys  Met Lys Asn Tyr Trp  Arg Gln Leu Leu Asn  Ala Lys Leu
    1160                1165                1170

Ile Thr  Gln Arg Lys Phe Asp  Asn Leu Thr Lys Ala  Glu Arg Gly
    1175                1180                1185

Gly Leu  Ser Glu Leu Asp Lys  Ala Gly Phe Ile Lys  Arg Gln Leu
    1190                1195                1200

Val Glu  Thr Arg Gln Ile Thr  Lys His Val Ala Gln  Ile Leu Asp
    1205                1210                1215

Ser Arg  Met Asn Thr Lys Tyr  Asp Glu Asn Asp Lys  Leu Ile Arg
    1220                1225                1230
```

```
Glu Val  Lys Val Ile Thr Leu  Lys Ser Lys Leu Val  Ser Asp Phe
    1235                1240                1245

Arg Lys  Asp Phe Gln Phe Tyr  Lys Val Arg Glu Ile  Asn Asn Tyr
    1250                1255                1260

His His  Ala His Asp Ala Tyr  Leu Asn Ala Val Val  Gly Thr Ala
    1265                1270                1275

Leu Ile  Lys Lys Tyr Pro Lys  Leu Glu Ser Glu Phe  Val Tyr Gly
    1280                1285                1290

Asp Tyr  Lys Val Tyr Asp Val  Arg Lys Met Ile Ala  Lys Ser Glu
    1295                1300                1305

Gln Glu  Ile Gly Lys Ala Thr  Ala Lys Tyr Phe Phe  Tyr Ser Asn
    1310                1315                1320

Ile Met  Asn Phe Phe Lys Thr  Glu Ile Thr Leu Ala  Asn Gly Glu
    1325                1330                1335

Ile Arg  Lys Arg Pro Leu Ile  Glu Thr Asn Gly Glu  Thr Gly Glu
    1340                1345                1350

Ile Val  Trp Asp Lys Gly Arg  Asp Phe Ala Thr Val  Arg Lys Val
    1355                1360                1365

Leu Ser  Met Pro Gln Val Asn  Ile Val Lys Lys Thr  Glu Val Gln
    1370                1375                1380

Thr Gly  Gly Phe Ser Lys Glu  Ser Ile Leu Pro Lys  Arg Asn Ser
    1385                1390                1395

Asp Lys  Leu Ile Ala Arg Lys  Lys Asp Trp Asp Pro  Lys Lys Tyr
    1400                1405                1410

Gly Gly  Phe Asp Ser Pro Thr  Val Ala Tyr Ser Val  Leu Val Val
    1415                1420                1425

Ala Lys  Val Glu Lys Gly Lys  Ser Lys Lys Leu Lys  Ser Val Lys
```

```
    1430                1435                1440

Glu Leu  Leu Gly Ile Thr Ile  Met Glu Arg Ser Ser  Phe Glu Lys
    1445                1450                1455

Asn Pro  Ile Asp Phe Leu Glu  Ala Lys Gly Tyr Lys  Glu Val Lys
    1460                1465                1470

Lys Asp  Leu Ile Ile Lys Leu  Pro Lys Tyr Ser Leu  Phe Glu Leu
    1475                1480                1485

Glu Asn  Gly Arg Lys Arg Met  Leu Ala Ser Ala Gly  Glu Leu Gln
    1490                1495                1500

Lys Gly  Asn Glu Leu Ala Leu  Pro Ser Lys Tyr Val  Asn Phe Leu
    1505                1510                1515

Tyr Leu  Ala Ser His Tyr Glu  Lys Leu Lys Gly Ser  Pro Glu Asp
    1520                1525                1530

Asn Glu  Gln Lys Gln Leu Phe  Val Glu Gln His Lys  His Tyr Leu
    1535                1540                1545

Asp Glu  Ile Ile Glu Gln Ile  Ser Glu Phe Ser Lys  Arg Val Ile
    1550                1555                1560

Leu Ala  Asp Ala Asn Leu Asp  Lys Val Leu Ser Ala  Tyr Asn Lys
    1565                1570                1575

His Arg  Asp Lys Pro Ile Arg  Glu Gln Ala Glu Asn  Ile Ile His
    1580                1585                1590

Leu Phe  Thr Leu Thr Asn Leu  Gly Ala Pro Ala Ala  Phe Lys Tyr
    1595                1600                1605

Phe Asp  Thr Thr Ile Asp Arg  Lys Arg Tyr Thr Ser  Thr Lys Glu
    1610                1615                1620

Val Leu  Asp Ala Thr Leu Ile  His Gln Ser Ile Thr  Gly Leu Tyr
    1625                1630                1635
```

Glu Thr Arg Ile Asp Leu Ser Gln Leu Gly Gly Asp Glu Phe Pro
    1640   1645   1650

Lys Lys Lys Arg Lys Val Gly Gly Gly Gly Ser Pro Lys Lys Lys
    1655   1660   1665

Arg Lys Val
    1670

<210> 50
<211> 1678
<212> PRT
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
      polypeptide"

<400> 50
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1   5   10   15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
            20   25   30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35   40   45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50   55   60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65   70   75   80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85   90   95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100   105   110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115   120   125

```
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Lys Leu
        195                 200                 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys Val
225                 230                 235                 240

Asp Ser Gly Gly Ser Ser Gly Gly Ser Ser Gly Ser Glu Thr Pro Gly
                245                 250                 255

Thr Ser Glu Ser Ala Thr Pro Glu Ser Ser Gly Gly Ser Ser Gly Gly
            260                 265                 270

Ser Pro Ala Ala Lys Arg Val Lys Leu Asp Gly Gly Gly Gly Ser Thr
        275                 280                 285

Gly Met Asp Lys Lys Tyr Ser Ile Gly Leu Asp Ile Gly Thr Asn Ser
    290                 295                 300

Val Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys
305                 310                 315                 320

Phe Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu
                325                 330                 335
```

```
Ile Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg
            340                 345                 350

Leu Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile
        355                 360                 365

Cys Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp
    370                 375                 380

Ser Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys
385                 390                 395                 400

Lys His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala
                405                 410                 415

Tyr His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val
            420                 425                 430

Asp Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala
        435                 440                 445

His Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn
    450                 455                 460

Pro Asp Asn Ser Asp Val Asp Lys Leu Phe Ile Gln Leu Val Gln Thr
465                 470                 475                 480

Tyr Asn Gln Leu Phe Glu Glu Asn Pro Ile Asn Ala Ser Gly Val Asp
                485                 490                 495

Ala Lys Ala Ile Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu
            500                 505                 510

Asn Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly
        515                 520                 525

Asn Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn
    530                 535                 540

Phe Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr
545                 550                 555                 560
```

```
Asp Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala
               565                 570                 575

Asp Leu Phe Leu Ala Ala Lys Asn Leu Ser Asp Ala Ile Leu Leu Ser
            580                 585                 590

Asp Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala
        595                 600                 605

Ser Met Ile Lys Arg Tyr Asp Glu His His Gln Asp Leu Thr Leu Leu
    610                 615                 620

Lys Ala Leu Val Arg Gln Gln Leu Pro Glu Lys Tyr Lys Glu Ile Phe
625                 630                 635                 640

Phe Asp Gln Ser Lys Asn Gly Tyr Ala Gly Tyr Ile Asp Gly Gly Ala
               645                 650                 655

Ser Gln Glu Glu Phe Tyr Lys Phe Ile Lys Pro Ile Leu Glu Lys Met
            660                 665                 670

Asp Gly Thr Glu Glu Leu Leu Val Lys Leu Asn Arg Glu Asp Leu Leu
        675                 680                 685

Arg Lys Gln Arg Thr Phe Asp Asn Gly Ser Ile Pro His Gln Ile His
    690                 695                 700

Leu Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro
705                 710                 715                 720

Phe Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg
               725                 730                 735

Ile Pro Tyr Tyr Val Gly Pro Leu Ala Arg Gly Asn Ser Arg Phe Ala
            740                 745                 750

Trp Met Thr Arg Lys Ser Glu Glu Thr Ile Thr Pro Trp Asn Phe Glu
        755                 760                 765
```

```
Glu Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met
    770                 775                 780

Thr Asn Phe Asp Lys Asn Leu Pro Asn Glu Lys Val Leu Pro Lys His
785                 790                 795                 800

Ser Leu Leu Tyr Glu Tyr Phe Thr Val Tyr Asn Glu Leu Thr Lys Val
                805                 810                 815

Lys Tyr Val Thr Glu Gly Met Arg Lys Pro Ala Phe Leu Ser Gly Glu
            820                 825                 830

Gln Lys Lys Ala Ile Val Asp Leu Leu Phe Lys Thr Asn Arg Lys Val
        835                 840                 845

Thr Val Lys Gln Leu Lys Glu Asp Tyr Phe Lys Lys Ile Glu Cys Phe
    850                 855                 860

Asp Ser Val Glu Ile Ser Gly Val Glu Asp Arg Phe Asn Ala Ser Leu
865                 870                 875                 880

Gly Thr Tyr His Asp Leu Leu Lys Ile Ile Lys Asp Lys Asp Phe Leu
                885                 890                 895

Asp Asn Glu Glu Asn Glu Asp Ile Leu Glu Asp Ile Val Leu Thr Leu
            900                 905                 910

Thr Leu Phe Glu Asp Arg Glu Met Ile Glu Glu Arg Leu Lys Thr Tyr
        915                 920                 925

Ala His Leu Phe Asp Asp Lys Val Met Lys Gln Leu Lys Arg Arg Arg
    930                 935                 940

Tyr Thr Gly Trp Gly Arg Leu Ser Arg Lys Leu Ile Asn Gly Ile Arg
945                 950                 955                 960

Asp Lys Gln Ser Gly Lys Thr Ile Leu Asp Phe Leu Lys Ser Asp Gly
                965                 970                 975

Phe Ala Asn Arg Asn Phe Met Gln Leu Ile His Asp Asp Ser Leu Thr
            980                 985                 990
```

```
Phe Lys Glu Asp Ile Gln Lys Ala  Gln Val Ser Gly Gln  Gly Asp Ser
        995                 1000                1005


Leu His  Glu His Ile Ala Asn  Leu Ala Gly Ser Pro  Ala Ile Lys
    1010                1015                1020


Lys Gly  Ile Leu Gln Thr Val  Lys Val Val Asp Glu  Leu Val Lys
    1025                1030                1035


Val Met  Gly Arg His Lys Pro  Glu Asn Ile Val Ile  Glu Met Ala
    1040                1045                1050


Arg Glu  Asn Gln Thr Thr Gln  Lys Gly Gln Lys Asn  Ser Arg Glu
    1055                1060                1065


Arg Met  Lys Arg Ile Glu Glu  Gly Ile Lys Glu Leu  Gly Ser Gln
    1070                1075                1080


Ile Leu  Lys Glu His Pro Val  Glu Asn Thr Gln Leu  Gln Asn Glu
    1085                1090                1095


Lys Leu  Tyr Leu Tyr Tyr Leu  Gln Asn Gly Arg Asp  Met Tyr Val
    1100                1105                1110


Asp Gln  Glu Leu Asp Ile Asn  Arg Leu Ser Asp Tyr  Asp Val Asp
    1115                1120                1125


His Ile  Val Pro Gln Ser Phe  Leu Ala Asp Asp Ser  Ile Asp Asn
    1130                1135                1140


Lys Val  Leu Thr Arg Ser Asp  Lys Asn Arg Gly Lys  Ser Asp Asn
    1145                1150                1155


Val Pro  Ser Glu Glu Val Val  Lys Lys Met Lys Asn  Tyr Trp Arg
    1160                1165                1170


Gln Leu  Leu Asn Ala Lys Leu  Ile Thr Gln Arg Lys  Phe Asp Asn
    1175                1180                1185
```

```
Leu Thr  Lys Ala Glu Arg Gly  Gly Leu Ser Glu Leu  Asp Lys Ala
    1190                 1195                 1200

Gly Phe  Ile Lys Arg Gln Leu  Val Glu Thr Arg Gln  Ile Thr Lys
    1205                 1210                 1215

His Val  Ala Gln Ile Leu Asp  Ser Arg Met Asn Thr  Lys Tyr Asp
    1220                 1225                 1230

Glu Asn  Asp Lys Leu Ile Arg  Glu Val Lys Val Ile  Thr Leu Lys
    1235                 1240                 1245

Ser Lys  Leu Val Ser Asp Phe  Arg Lys Asp Phe Gln  Phe Tyr Lys
    1250                 1255                 1260

Val Arg  Glu Ile Asn Asn Tyr  His His Ala His Asp  Ala Tyr Leu
    1265                 1270                 1275

Asn Ala  Val Val Gly Thr Ala  Leu Ile Lys Lys Tyr  Pro Ala Leu
    1280                 1285                 1290

Glu Ser  Glu Phe Val Tyr Gly  Asp Tyr Lys Val Tyr  Asp Val Arg
    1295                 1300                 1305

Lys Met  Ile Ala Lys Ser Glu  Gln Glu Ile Gly Lys  Ala Thr Ala
    1310                 1315                 1320

Lys Tyr  Phe Phe Tyr Ser Asn  Ile Met Asn Phe Phe  Lys Thr Glu
    1325                 1330                 1335

Ile Thr  Leu Ala Asn Gly Glu  Ile Arg Lys Ala Pro  Leu Ile Glu
    1340                 1345                 1350

Thr Asn  Gly Glu Thr Gly Glu  Ile Val Trp Asp Lys  Gly Arg Asp
    1355                 1360                 1365

Phe Ala  Thr Val Arg Lys Val  Leu Ser Met Pro Gln  Val Asn Ile
    1370                 1375                 1380

Val Lys  Lys Thr Glu Val Gln  Thr Gly Gly Phe Ser  Lys Glu Ser
    1385                 1390                 1395
```

```
Ile Leu  Pro Lys Arg Asn Ser  Asp Lys Leu Ile Ala  Arg Lys Lys
    1400                1405                1410

Asp Trp  Asp Pro Lys Lys Tyr  Gly Gly Phe Asp Ser  Pro Thr Val
    1415                1420                1425

Ala Tyr  Ser Val Leu Val Val  Ala Lys Val Glu Lys  Gly Lys Ser
    1430                1435                1440

Lys Lys  Leu Lys Ser Val Lys  Glu Leu Leu Gly Ile  Thr Ile Met
    1445                1450                1455

Glu Arg  Ser Ser Phe Glu Lys  Asn Pro Ile Asp Phe  Leu Glu Ala
    1460                1465                1470

Lys Gly  Tyr Lys Glu Val Lys  Lys Asp Leu Ile Ile  Lys Leu Pro
    1475                1480                1485

Lys Tyr  Ser Leu Phe Glu Leu  Glu Asn Gly Arg Lys  Arg Met Leu
    1490                1495                1500

Ala Ser  Ala Gly Glu Leu Gln  Lys Gly Asn Glu Leu  Ala Leu Pro
    1505                1510                1515

Ser Lys  Tyr Val Asn Phe Leu  Tyr Leu Ala Ser His  Tyr Glu Lys
    1520                1525                1530

Leu Lys  Gly Ser Pro Glu Asp  Asn Glu Gln Lys Gln  Leu Phe Val
    1535                1540                1545

Glu Gln  His Lys His Tyr Leu  Asp Glu Ile Ile Glu  Gln Ile Ser
    1550                1555                1560

Glu Phe  Ser Lys Arg Val Ile  Leu Ala Asp Ala Asn  Leu Asp Lys
    1565                1570                1575

Val Leu  Ser Ala Tyr Asn Lys  His Arg Asp Lys Pro  Ile Arg Glu
    1580                1585                1590
```

```
Gln Ala  Glu Asn Ile Ile His  Leu Phe Thr Leu Thr  Asn Leu Gly
    1595                 1600                 1605

Ala Pro  Ala Ala Phe Lys Tyr  Phe Asp Thr Thr Ile  Asp Arg Lys
    1610                 1615                 1620

Arg Tyr  Thr Ser Thr Lys Glu  Val Leu Asp Ala Thr  Leu Ile His
    1625                 1630                 1635

Gln Ser  Ile Thr Gly Leu Tyr  Glu Thr Arg Ile Asp  Leu Ser Gln
    1640                 1645                 1650

Leu Gly  Gly Asp Glu Phe Pro  Lys Lys Lys Arg Lys  Val Gly Gly
    1655                 1660                 1665

Gly Gly  Ser Pro Lys Lys Lys  Arg Lys Val
    1670                 1675
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<210> 51
<211> 20
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic oligonucleotide"

<400> 51
gaguccgagc agaagaagaa 20

<210> 52
<211> 20
<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic oligonucleotide"

<400> 52
ggcacugcgg cuggaggugg 20

<210> 53
<211> 20

<212> RNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
oligonucleotide"

<400> 53
ggucacuuuu aacacaccca 20

<210> 54
<211> 55
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (34)..(39)
<223> a, t, g or c

<400> 54
tcgtcggcag cgtcagatgt gtataagaga cagnnnnnnc cccagtggct gctct 55

<210> 55
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (35)..(40)
<223> a, t, g or c

<400> 55
gtctcgtggg ctcggagatg tgtataagag acagnnnnnn ccaggcctcc ccaaagc 57

<210> 56
<211> 59

<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (34)..(39)
<223> a, t, g or c

<400> 56
tcgtcggcag cgtcagatgt gtataagaga cagnnnnnng gaacccaggt agccagaga 59

<210> 57
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (35)..(40)
<223> a, t, g or c

<400> 57
gtctcgtggg ctcggagatg tgtataagag acagnnnnnn ggggtggggt cagacgt 57

<210> 58
<211> 59
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
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<222> (34)..(39)
<223> a, t, g or c

<400> 58
tcgtcggcag cgtcagatgt gtataagaga cagnnnnnng cccattccct ctttagcca 59

<210> 59
<211> 61
<212> DNA
<213> Artificial Sequence

<220>
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<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
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<222> (35)..(40)
<223> a, t, g or c

<400> 59
gtctcgtggg ctcggagatg tgtataagag acagnnnnnn ggagcaggaa agtgaggtta 60

c 61

<210> 60
<211> 61
<212> DNA
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<220>
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<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
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<222> (34)..(39)
<223> a, t, g or c

<400> 60
tcgtcggcag cgtcagatgt gtataagaga cagnnnnnna atggacacat gggtagtcag 60

g 61

<210> 61
<211> 64
<212> DNA
<213> Artificial Sequence

<220>

<221> source
<223> /note="Description of Artificial Sequence: Synthetic
primer"

<220>
<221> modified_base
<222> (35)..(40)
<223> a, t, g or c

<400> 61
gtctcgtggg ctcggagatg tgtataagag acagnnnnnn ggcttatatc caacacttcg 60

tggg 64

<210> 62
<211> 5076
<212> DNA
<213> Artificial Sequence

<220>
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<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 62
atggttagca aaggtgaaga actgtttaca ggtgttgttc cgattctggt tgaactggat 60

ggtgatgtta atggccacaa attttcagtt agcggtgaag gcgaaggtga tgcaacctat 120

ggtaaactga ccctgaaatt tatctgtacc accggcaaac tgccggttcc gtggccgaca 180

ctggttacca cactgaccta tggtgttcag tgttttagcc gttatccgga tcacatgaaa 240

cagcacgatt ttttcaaaag cgcaatgccg gaaggttatg ttcaagaacg taccatcttc 300

ttcaaagatg acggcaacta taaaacccgt gccgaagtta aatttgaagg tgataccctg 360

gtgaatcgca ttgaactgaa aggcatcgat tttaaagagg atggtaatat cctgggccac 420

aaactggaat ataattataa tagccacaac gtgtacatca tggccgacaa acagaaaaat 480

ggcatcaaag tgaacttcaa gatccgccat aatattgaag atggttcagt tcagctggcc 540

gatcattatc agcagaatac cccgattggt gatggtccgg ttctgctgcc ggataatcat 600

tatctgagca cccagagcaa actgagcaaa gatccgaatg aaaaacgtga tcacatggtg 660

ctgctggaat ttgttaccgc agcaggtatt accttaggta tggatgaact gtataaagtc 720

gacgcagaag cagcagcaaa agaagccgct gccaaagaag cggcagcgaa agaggcagcc 780

gcaaaagcac tggaagccga ggctgcggct aaagaggctg ctgcaaaaga agcagccgct 840

aaagaagctg cggctaaggc accggcagca aaacgtgtta aactggacgg tggtggtggt 900

agcaccggta tggacaagaa atacagcatc ggtttggata ttggcacgaa tagcgtgggt 960

tgggccgtta ttaccgacga gtacaaagtg ccgtccaaga aattcaaagt gctgggcaat 1020

accgatcgcc atagcatcaa gaaaaatctg attggcgcac tgctgttcga cagcggtgag 1080

actgccgaag ctacgcgtct gaagcgtacg gcgcgtcgtc gctacacccg ccgtaagaac 1140

cgtatttgct atctgcaaga aatcttcagc aacgaaatgg ccaaagttga tgatagcttt 1200

tttcaccgcc tggaagagag ctttctggtg gaagaggata agaaacacga gcgccatccg 1260

atttttggta acattgtcga tgaagtggca taccatgaga agtacccgac catctaccac 1320

cttcgtaaga aactggtgga cagcaccgat aaagctgatc tgcgtctgat ttacctggcg 1380

ctggcccaca tgattaagtt tcgcggtcat tttctgatcg agggcgatct gaatccggac 1440

aattctgatg ttgacaagct gtttattcaa cttgtacaga cctacaacca gttgttcgaa 1500

gagaacccga tcaatgcgag cggtgttgat gccaaagcaa ttctgagcgc acgcctgagc 1560

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agcgcgtcta tgatcaagcg ctatgacgag caccaccaag atctgaccct gctgaaagct 1920

ctggtgagac aacaattgcc agagaagtat aaagaaattt tctttgacca gagcaaaaac 1980

ggctatgcgg gttacattga cggtggcgcc agccaagaag agttctacaa attcattaag 2040

cctatcctgg agaaaatgga tggcaccgaa gaactgctgg taaagctgaa tcgtgaagat 2100

ctgctgcgca aacagcgcac ttttgataac ggtagcattc cgcaccagat ccatctgggt 2160

gagttgcacg cgattttgcg tcgccaggaa gatttttatc cgttcttgaa agacaaccgt 2220

gagaaaatcg agaaaattct gacgttccgt atcccgtatt atgtcggccc gctggcgcgt 2280

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aaggttacgg tcaaacaact gaaagaggac tatttcaaga aaattgaatg ttttgactcc 2640

gtagagatct ccggtgttga ggaccgtttc aacgcgagcc tgggcaccta ccatgatctg 2700

ctgaaaatta ttaaagacaa agattttctg gacaacgaag agaacgaaga tattctggaa 2760

gatatcgttc tgaccctgac gctgttcgaa gatcgtgaga tgattgagga acgtctgaaa 2820

acctacgcac acttgttcga tgacaaagtt atgaaacagc tgaagcgtcg tcgttacaca 2880

ggttggggcc gtctgagccg taagcttatc aatggtatcc gtgacaaaca gagcggtaag 2940

acgattctgg actttctgaa gtcagatggc ttcgccaatc gcaactttat gcaactgatt 3000

catgacgact ctctgacgtt caaggaagat atccaaaagg cacaggtgag cggtcagggt 3060

gatagcctgc atgagcatat cgcgaacctg gcgggtagcc cggctatcaa aaagggtatc 3120

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aatattgtga tcgagatggc acgtgaaaat cagacgacgc aaaagggtca aaaaaattct 3240

cgtgagcgca tgaaacgtat tgaagagggt atcaaagaat tgggcagcca aattctgaaa 3300

gaacacccgg tcgagaacac ccagctgcaa aacgaaaaac tgtatttata ctatctgcag 3360

aacggtcgtg acatgtacgt ggatcaagaa ctggacatca atcgtttgag cgattacgat 3420

gttgatcata ttgtgcctca gagctttctg aaagacgatt cgatcgacaa caaagtgctg 3480

acccgtagcg acaagaatcg tggtaagagc gataacgtgc cgagcgaaga agtcgttaag 3540

aaaatgaaaa actactggcg tcagctgctg aacgccaagc tgattaccca gcgtaagttc 3600

gataacctga cgaaagccga gcgtggaggc ctgagcgagc tggacaaggc cggctttatc 3660

aagcgtcaac tggtggaaac ccgtcagatc actaaacatg tggcacagat cctggactcc 3720

cgcatgaata cgaaatatga cgagaatgac aagttgatcc gtgaagtcaa agttattacg 3780

ctgaaaagca aactggtgtc cgatttccgt aaagacttcc agttctataa agtccgtgaa 3840

atcaacaact atcatcacgc ccacgatgcg tacttgaacg ctgttgtggg caccgcactg 3900

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cgtaaacggc cgctgattga gactaatggc gaaacgggcg agattgtgtg ggacaagggt 4140

cgcgatttcg ctacggttcg taaggtcctg agcatgccgc aagttaacat tgtcaagaaa 4200

actgaagtgc agacgggtgg ctttagcaaa gaatccatcc tgccgaagcg taatagcgat 4260

aaacttatcg cgcgtaaaaa agactgggac ccaaagaaat atggcggctt tgatagcccg 4320

accgtcgcgt atagcgtgtt agtggtcgcg aaagttgaaa agggcaagag caagaaactg 4380

aagtccgtca aagaacttct gggtatcacc atcatggaac gtagctcctt tgagaagaac 4440

ccgattgact tcttagaggc gaagggttat aaagaagtca aaaaagacct gattatcaag 4500

ctgccgaagt acagcctgtt tgagttggag aatggtcgta agcgcatgct ggcgagcgcg 4560

ggtgagctgc aaaagggcaa cgaactggcg ctgccgtcga aatacgtcaa ttttctgtac 4620

ctggccagcc actacgaaaa gctgaagggt tctccggaag ataacgaaca aaagcaactg 4680

ttcgttgagc aacataaaca ctacttggac gaaatcatcg agcaaattag cgaatttagc 4740

aaacgtgtca tcctggcgga cgcgaatctg gacaaggtcc tgtctgcata caataagcat 4800

cgcgacaaac caattcgtga gcaagcggag aatatcatcc acctgtttac gctgaccaac 4860

ctaggtgcgc cggcggcatt caagtatttc gatacgacca tcgaccgcaa gcgctatacc 4920

agcaccaaag aggtcctgga cgcgaccctg atccaccaga gcattaccgg cttatacgaa 4980

acccgtattg atttgagcca actgggtggc gatgaattcc cgaaaaaaaa gcgcaaagtt 5040

ggtggcggtg gtagcccgaa aaagaaacgt aaagtg 5076

<210> 63
<211> 5013
<212> DNA
<213> Artificial Sequence

<220>
<221> source
<223> /note="Description of Artificial Sequence: Synthetic
polynucleotide"

<400> 63
atggttagca aaggtgaagc cgtgattaaa gaatttatgc gctttaaggt tcacatggaa 60

ggtagcatga atggccatga atttgaaatt gaaggtgaag gcgaaggtcg tccgtatgaa 120

ggcacccaga ccgcaaaact gaaagttacc aaaggtggtc cgctgccgtt tagctgggat 180

attctgagtc cgcagtttat gtatggtagc cgtgcattta ccaaacatcc ggcagatatt 240

ccggattatt acaaacagag ctttccggaa ggttttaaat gggaacgtgt gatgaatttt 300

gaagatggtg gtgcagttac cgttacacag gataccagcc tggaagatgg caccctgatc 360

tataaagtta aactgcgtgg caccaatttt ccgcctgatg gtccggttat gcagaaaaaa 420

acaatgggtt gggaagcaag caccgaacgt ctgtatcctg aagatggcgt tctgaaaggt 480

gatatcaaaa tggcactgcg tctgaaagat ggcggtcgtt atctggcaga tttcaaaacc 540

acctataaag ccaaaaaacc tgttcagatg cctggtgcct ataatgttga tcgtaaactg 600

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