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(54) **Titre : DELIVRANCE, MODIFICATION ET OPTIMISATION DE SYSTEMES, PROCEDES ET COMPOSITIONS POUR CIBLER ET MODELISER DES MALADIES ET DES TROUBLES LIES AUX CELLULES POST-MITOTIQUES**

(54) **Title: DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR TARGETING AND MODELING DISEASES AND DISORDERS OF POST MITOTIC CELLS**

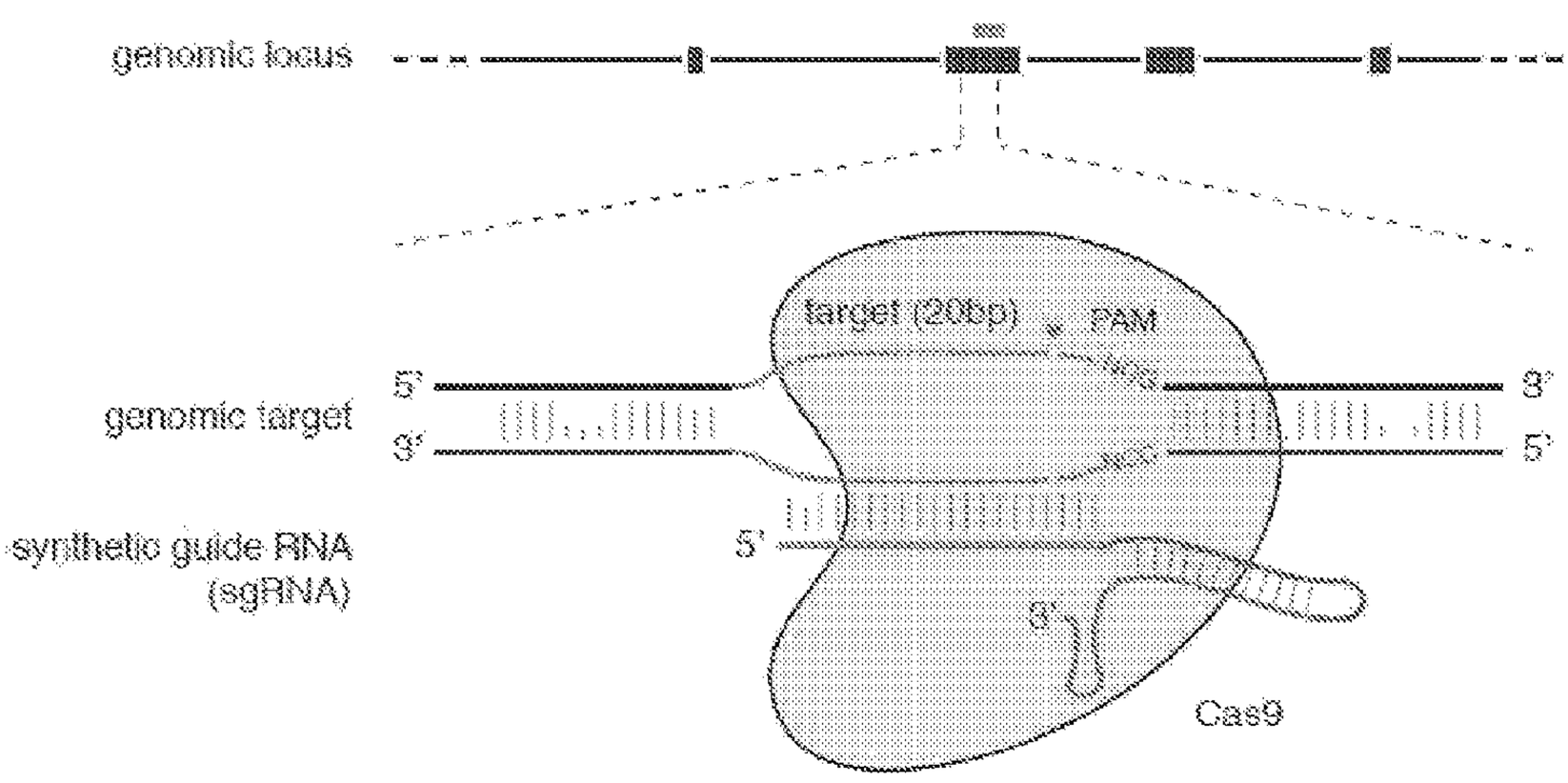


FIG. 1

(57) **Abrégé/Abstract:**
The invention provides for delivery, engineering and optimization of systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are delivery systems and tissues or organ which include post mitotic cells which are targeted as sites for delivery. Also provided are vectors and vector systems some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells to ensure enhanced specificity for target recognition and avoidance of toxicity and to edit or modify a target site in a genomic locus of interest to alter or improve the status of a disease or a condition.

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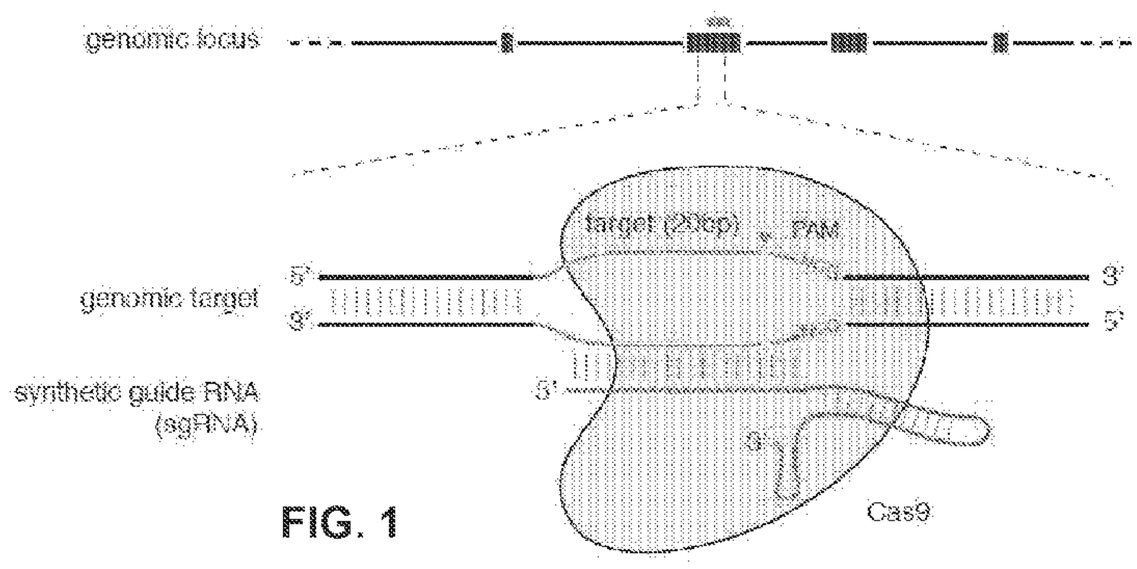


FIG. 1

(57) Abstract: The invention provides for delivery, engineering and optimization of systems, methods, and compositions for manipulation of sequences and/or activities of target sequences. Provided are delivery systems and tissues or organ which include post mitotic cells which are targeted as sites for delivery. Also provided are vectors and vector systems some of which encode one or more components of a CRISPR complex, as well as methods for the design and use of such vectors. Also provided are methods of directing CRISPR complex formation in eukaryotic cells to ensure enhanced specificity for target recognition and avoidance of toxicity and to edit or modify a target site in a genomic locus of interest to alter or improve the status of a disease or a condition.

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CONTENANT LES PAGES 1 À 171

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JUMBO APPLICATIONS/PATENTS

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CONTAINING PAGES 1 TO 171

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DELIVERY, ENGINEERING AND OPTIMIZATION OF SYSTEMS, METHODS AND COMPOSITIONS FOR TARGETING AND MODELING DISEASES AND DISORDERS OF POST MITOTIC CELLS

RELATED APPLICATIONS AND INCORPORATION BY REFERENCE

[0001] Priority is claimed from US provisional patent applications 61/836,123, filed June 17, 2013, 61/847,537, filed July 17, 2013, 61/862,355, filed August 5, 2013, 61/871,301, filed August 28, 2013, 61/915,203, filed December 12, 2013, 61/979,573, filed April 15, 2014, and PCT/US2013/074667, filed December 12, 2013, as to which for purposes of the United States, this application is also a continuation-in-part; and as may be permitted under US law, the US equivalent or National Phase hereto may further claim and claim priority as to PCT/US2013/074667 and applications from which PCT/US2013/074667 claims priority.

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

FIELD OF THE INVENTION

[0003] The present invention generally relates to the delivery, engineering, optimization and therapeutic applications of systems, methods, and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and components thereof. In particular, the present invention relates to aspects related to delivery to post-mitotic cells, including but not limited to the brain or kidney, for gene therapy of conditions in said cells, understanding gene function in said cells and the creation of models comprising said cells.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0004] This invention was made with government support under the NIH Pioneer Award (1DP1MH100706) awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0005] Recent advances in genome sequencing techniques and analysis methods have significantly accelerated the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. Precise genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements, as well as to advance synthetic biology, biotechnological, and medical applications. Although genome-editing techniques such as designer zinc fingers, transcription activator-like effectors (TALEs), or homing meganucleases are available for producing targeted genome perturbations, there remains a need for new genome engineering technologies that are affordable, easy to set up, scalable, and amenable to targeting multiple positions within the eukaryotic genome.

SUMMARY OF THE INVENTION

[0006] The CRISPR-Cas system does not require the generation of customized proteins to target specific sequences but rather a single Cas enzyme can be programmed by a short RNA molecule to recognize a specific DNA target. Adding the CRISPR-Cas system to the repertoire of genome sequencing techniques and analysis methods may significantly simplify the methodology and accelerate the ability to catalog and map genetic factors associated with a diverse range of biological functions and diseases. To utilize the CRISPR-Cas system effectively for genome editing without deleterious effects, it is critical to understand aspects of engineering, optimization and cell-type/tissue/organ specific delivery of these genome engineering tools, which are aspects of the claimed invention.

[0007] There exists a pressing need for alternative and robust systems and techniques for nucleic sequence targeting with a wide array of applications. Aspects of this invention address this need and provide related advantages. An exemplary CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within the target

polynucleotide. The guide sequence is linked to a tracr mate sequence, which in turn hybridizes to a tracr sequence.

[0008] In one aspect, the invention provides methods for using one or more elements of a CRISPR-Cas system. The CRISPR complex of the invention provides an effective means for modifying a target polynucleotide. The CRISPR complex of the invention has a wide variety of utilities including modifying (e.g., deleting, inserting, translocating, inactivating, activating) a target polynucleotide in a multiplicity of cell types in various tissues and organs. As such the CRISPR complex of the invention has a broad spectrum of applications in, e.g., gene or genome editing, gene therapy, drug discovery, drug screening, disease diagnosis, and prognosis. In vivo, in vitro and ex vivo uses are envisaged.

[0009] Aspects of the invention relate to Cas9 enzymes having improved post-mitotic cell targeting specificity in a CRISPR-Cas9 system having guide RNAs having optimal activity, smaller in length than wild-type Cas9 enzymes and nucleic acid molecules coding therefor, and chimeric Cas9 enzymes, as well as methods of improving the target specificity of a Cas9 enzyme or of designing a CRISPR-Cas9 system comprising designing or preparing guide RNAs having optimal activity and/or selecting or preparing a Cas9 enzyme having a smaller size or length than wild-type Cas9 whereby packaging a nucleic acid coding therefor into a delivery vector is more advanced as there is less coding therefor in the delivery vector than for wild-type Cas9, and/or generating chimeric Cas9 enzymes.

[0010] Also provided are uses of the present sequences, vectors, enzymes or systems, in medicine. Also provided are uses of the same in gene or genome editing. This is in relation to post-mitotic cell tissues or cells, whether in or ex vivo,

[0011] In an additional aspect of the invention, a Cas9 enzyme may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may be artificially introduced mutations or gain- or loss-of-function mutations. The mutations may include but are not limited to mutations in one of the catalytic domains (D10 and H840) in the RuvC and HNH catalytic domains, respectively. Further mutations have been characterized. In one aspect of the invention, the transcriptional activation domain may be VP64. In other aspects of the invention, the transcriptional repressor domain may be KRAB or SID4X. Other aspects of the invention relate to the mutated Cas 9 enzyme being fused to domains which include but are not limited to a transcriptional activator,

repressor, a recombinase, a transposase, a histone remodeler, a demethylase, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain or a chemically inducible/controllable domain.

[0012] In a further embodiment, the invention provides for methods to generate mutant tracrRNA and direct repeat sequences or mutant chimeric guide sequences that allow for enhancing performance of these RNAs in cells. Aspects of the invention also provide for selection of said sequences.

[0013] Aspects of the invention also provide for methods of simplifying the cloning and delivery of components of the CRISPR complex. In the preferred embodiment of the invention, a suitable promoter, such as the U6 promoter, is amplified with a DNA oligo and added onto the guide RNA. The resulting PCR product can then be transfected into cells to drive expression of the guide RNA. Aspects of the invention also relate to the guide RNA being transcribed *in vitro* or ordered from a synthesis company and directly transfected.

[0014] In one aspect, the invention provides for methods to improve activity by using a more active polymerase. In a preferred embodiment, the expression of guide RNAs under the control of the T7 promoter is driven by the expression of the T7 polymerase in the cell. In an advantageous embodiment, the cell is a eukaryotic cell. In a preferred embodiment the eukaryotic cell is a human cell. In a more preferred embodiment the human cell is a patient specific cell.

[0015] In one aspect, the invention provides for methods of reducing the toxicity of Cas enzymes. In certain aspects, the Cas enzyme is any Cas9 as described herein, for instance any naturally-occurring bacterial Cas9 as well as any chimaeras, mutants, homologs or orthologs. In a preferred embodiment, the Cas9 is delivered into the cell in the form of mRNA. This allows for the transient expression of the enzyme thereby reducing toxicity. In another preferred embodiment, the invention also provides for methods of expressing Cas9 under the control of an inducible promoter, and the constructs used therein.

[0016] In another aspect, the invention provides for methods of improving the *in vivo* applications of the CRISPR-Cas system. In the preferred embodiment, the Cas enzyme is wildtype Cas9 or any of the modified versions described herein, including any naturally-occurring bacterial Cas9 as well as any chimaeras, mutants, homologs or orthologs. An advantageous aspect of the invention provides for the selection of Cas9 homologs that are easily

packaged into viral vectors for delivery. Cas9 orthologs typically share the general organization of 3-4 RuvC domains and a HNH domain. The 5' most RuvC domain cleaves the non-complementary strand, and the HNH domain cleaves the complementary strand. All notations are in reference to the guide sequence.

[0017] The catalytic residue in the 5' RuvC domain is identified through homology comparison of the Cas9 of interest with other Cas9 orthologs (from S. pyogenes type II CRISPR locus, S. thermophilus CRISPR locus 1, S. thermophilus CRISPR locus 3, and Franciscilla novicida type II CRISPR locus), and the conserved Asp residue (D10) is mutated to alanine to convert Cas9 into a complementary-strand nicking enzyme. Similarly, the conserved His and Asn residues in the HNH domains are mutated to Alanine to convert Cas9 into a non-complementary-strand nicking enzyme. In some embodiments, both sets of mutations may be made, to convert Cas9 into a non-cutting enzyme.

[0018] In some embodiments, the CRISPR enzyme is a type I or III CRISPR enzyme, preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A preferred Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[0019] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCas9, St1Cas9 and so forth. Further examples are provided herein. The skilled person will be able to determine appropriate corresponding residues in Cas9 enzymes other than SpCas9 by comparison of the relevant amino acid sequences. Thus, where a specific amino acid replacement is referred to using the SpCas9 numbering, then, unless the context makes it apparent this is not intended to refer to other Cas9 enzymes, the disclosure is intended to encompass corresponding modifications in other Cas9 enzymes. SaCas9 is particularly advantageous.

[0020] An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known.

[0021] In further embodiments, the invention provides for methods of enhancing the function of Cas9 by generating chimeric Cas9 proteins. Chimeric Cas9 proteins chimeric Cas9s may be new Cas9 containing fragments from more than one naturally occurring Cas9. These methods may comprise fusing N-terminal fragments of one Cas9 homolog with C-terminal fragments of another Cas9 homolog. These methods also allow for the selection of new properties displayed by the chimeric Cas9 proteins.

[0022] It will be appreciated that in the present methods, where the organism is an animal or a plant, the modification may occur *ex vivo* or *in vitro*, for instance in a cell culture and in some instances not *in vivo*. In other embodiments, it may occur *in vivo*.

[0023] In one aspect, the invention provides a method of modifying an organism or a non-human organism by manipulation of a target sequence in a genomic locus of interest comprising: delivering a non-naturally occurring or engineered composition comprising :

A) - I. a CRISPR-Cas system RNA polynucleotide sequence, optionally a chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA,

or

(B) I. polynucleotides comprising:

(a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding a CRISPR enzyme, and

III. a polynucleotide sequence comprising a tracr sequence,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA.

[0024] The following applies equally to all aspects of the invention. The target sequence is most preferably a post-mitotic cell target sequence. Where liver may be mentioned herein, this is will understood to be reference to post-mitotic cells in general, especially kidney or brain cells. The post-mitotic cell may be in or from (i.e. the source of the cells or the cell type) any one of the following organs or may be organoids or *ex vivo* models or collections of cells comprising cells of the:

[0025] Kidney, such as glomerulus cells;

[0026] Digestive System including the stomach, pancreas, duodenum, ileum and/or colon;

[0027] Heart;

[0028] Lung;

[0029] Brain, in particular neurones, and/or CNS in general;

[0030] Eye, including retinal tissue;

[0031] Ear, including the inner ear;

[0032] Skin;

[0033] Muscle;

[0034] Bone; and/or

[0035] Liver in general, although this is excluded in some embodiments as it is also the subject of a separate application.

[0036] Brain and Kidney are particularly preferred. In some embodiments, the cell is a brain cell, such as a neurone. In some embodiments, the cell is a kidney cell.

[0037] Preferred kidney cells in clude any one or more of:

- Kidney glomerulus parietal cell;
- Kidney glomerulus podocyte;
- Kidney proximal tubule brush border cell;
- Loop of Henle thin segment cell;
- Thick ascending limb cell;
- Kidney distal tubule cell;
- Kidney collecting duct cell; and
- Interstitial kidney cells.

[0038] Preferred exaples of kidney targets are provided in the table below uner the section entitled kidney, as well as in Table B. Any one or more of these targets is preferred. Examples 1 and 18 also target Kidney cells (albeit stem cels, which are not post-mitotic cells), but the teaching re delivery may be applicable.

[0039] In some particularly preferred embodiments, manipulation invokes a phenotypic change in the cell.

[0040] In some embodiments, the phenotypic change may be invoked in or maintained in the cell *in vivo*. Either the cell is transfected in vivo or is extracted, transfected ex vivo and then re-inserted (transplanted) back into the same or a different host.

[0041] Expression of the CRISPR enzyme, and optionally the guide sequence, may be under the control of a promoter specific for the post-mitotic cell, for instance comprised within an expression cassette capable of expressing the enzyme and the optional guide in said post-mitotic cell. In other words, the CRISPR enzyme, and optionally the guide sequence, is/are operably linked to said promoter specific for the post-mitotic cell. AAV vector systems are particularly preferred, especially where the post-mitotic cell is a a neurone.

[0042] The promoter for the CRISPR enzyme and the optional promoter for the guide sequence may be the same or different.

The following also applies to any method, use or composition describe herein. The CRISPR-Cas system RNA may be a chimeric RNA (chiRNA). The CRISPR-Cas system may be a multiplexed CRISPR enzyme system further comprising multiple chimeras and/or multiple multiguide sequences and a single tracr sequence. The CRISPR enzyme may be a nuclease directing cleavage of both strands at the location of the target sequence. The CRISPR enzyme

may comprise one or more mutations. The CRISPR enzyme may comprise one or more mutations D10A, E762A, H840A, N854A, N863A or D986A. The one or more mutations may be in a RuvC1 domain of the CRISPR enzyme. The CRISPR enzyme may be a nickase directing cleavage at the location of the target sequence. The nickase may be a double nickase. At least two or more NLS are preferred.

The CRISPR enzyme may be type II, preferably a Cas and most preferably a Cas9. Reference to Cas or Cas9 (for instance in CRISPR-Cas or CRISR Cas9) will be understood to be any Cas, most preferably Cas9 and particularly Sa or Sp Cas9 (encompassing all mutations such as D10A to provided DSB, nickase or dual nickase function).

The CRISPR enzyme may have one or more mutations in a catalytic domain, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain. The functional domain may be a transcriptional activation domain. The transcriptional activation domain may be VP64.

The methods may further comprise minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising

delivering a non-naturally occurring or engineered composition comprising:

I. a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a first guide sequence capable of hybridizing to the first target sequence,

(b) a first tracr mate sequence,

(c) a first tracr sequence,

(d) a second guide sequence capable of hybridizing to the second target sequence,

(e) a second tracr mate sequence, and

(f) a second tracr sequence, and

optionally, wherein a linker sequence is present between the first tracr sequence and the second guide sequence, whereby the first guide sequence and the second guide sequence are in tandem; and

II. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences, wherein (a), (b), (c), (d), (e) and (f) are arranged in a 5' to

3' orientation, wherein the polynucleotide sequence comprises a linker sequence between the first tracr sequence and the second guide sequence, whereby the first guide sequence and the second guide sequence are in tandem, and wherein when transcribed, the first and the second tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively,

or

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and wherein components I and II are located on the same or different vectors of the system, and when transcribed, a first tracr mate sequence hybridizes to a first tracr sequence and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively;

wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence,

wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence,

wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and

wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

[0043] In some embodiments, the second alternative above (B) is preferred. The first alternative (A) is particularly preferred, however. This applies to all aspects of the invention featuring the two alternative CRISPR approaches.

[0044] It will be appreciated that the present application is directed to post-mitotic cells, whether that is an organ per se or a tissue within it or simply one or post-mitotic cells, such as neurones. Neurones and kidney cells are preferred. The post-mitotic cells may be comprised within a vertebrate animal, either a patient (in the sense of an animal in need of CRISPR-directed gene therapy) or a model organism, or may be in cell culture , an organoid or other ex vivo

tissue, such a "liver on a chip" for instance where hepatocytes are seeded and grown on a scaffold. Harvested hepatocytes from un-transplanted organs are also a useful target. With the development of 3-D printing techniques being applied to biology, printed tissues are within grasp and it is entirely feasible that liver cells or tissues printed in this way to create an organoid or onto a chip could also be targeted. Non-liver alternatives are also envisaged, particularly for kidney tissues or other post-mitotic cells/tissues.

[0045] Thus, provided is a model organism comprising post-mitotic cells, such as neurones or kidney cells, to which the present CRISPR-Cas system has been delivered. Similarly, also provided is an *ex vivo* collection of two or more post-mitotic cells, such as neurones or kidney cells, to which the present CRISPR-Cas system has been delivered. Such collections may include post-mitotic organs, organoids, cells populating a scaffold ('kidney on a chip'). Methods of creating such models or collections are also provided.

[0046] In particular, such post-mitotic cells may express, or comprise polynucleotides capable of expressing, a Cas enzyme. As discussed herein, this has the advantage of providing a ready model for interrogating gene function through gene perturbation, including knock down. This is particularly useful in studying conditions of the post-mitotic cells, such as the kidney or brain, such as those listed herein, as well as broader conditions such as obesity.

[0047] Methods of interrogating post-mitotic cell gene function are also provided herein. These typically comprise delivering to post-mitotic cells, either *in* or *ex vivo*, the CRISPR-Cas system. However, if the cells already comprise Cas, whether expressed as a protein or encoded by polynucleotides already comprised within the cells, then only the CRISPR polynucleotide needs to be delivered. The method may include extraction from and, optionally, re-insertion back into the post-mitotic cell. By delivering, it is meant actually physical delivery of the polynucleotides to the nucleus of the cell, but also transfection. Therefore, delivery should also be read as including transfection unless otherwise apparent.

[0048] Also provided is a method of inducing gene perturbation in one or more post-mitotic cells, comprising transducing a first population of cells with a CRISPR-Cas system according to the present invention to thereby alter the genome of the first population of cells to obtain a second population of cells. The method may be *ex vivo* or *in vitro*, for instance in a cell culture or in an *ex vivo* or *in vitro* model (such as an organoid or 'post-mitotic cell on a chip'). Alternatively, the method may be *in vivo*, in which case it may also include isolating the first

population of cells from the subject, and transplanting the second population of cells (back) into the subject. Gene perturbation may be for one or more, or two or more, or three or more, or four or more genes. The gene perturbation may be a reduction in gene function (i.e. activity in the encoded gene product). This may be, for instance, induced through alteration of the genome of the first population of cells to obtain the second population of cells, wherein the second population of cells has a defective genotype, such as a monogenic condition, which is absent in the first population of cells. This may require a corresponding repair template , as discussed herein, to provide the defective sequence or it may be through induction of a DSB. In particular, the gene perturbation is a gene knockdown. In some embodiments, the post-mitotic cell is most preferably a kidney or brain (neuron) cell or a liver cell, such as a primary hepatocyte.

[0049] Alternatively, the gene perturbation may be an increase in gene function (i.e. activity in the encoded gene product). This may be, for instance, induced through alteration of the genome of the first population of cells to obtain the second population of cells, wherein the first population of cells has a defective genotype, such as a monogenic condition, which is absent in (i.e. corrected for in) the second population of cells. This may require a corresponding repair template, as discussed herein, to provide the corrected sequence.

[0050] If multiplexing is used, then a mixture of reduction of one or more genes and increase of one or more genes is envisaged. This may be achieved through provision of one or more of the guides (in the multiplex) and corresponding repair templates may be used to reduce function, whilst one or more of the guides and their corresponding templates may be used to increase function.

[0051] Also provided is a method of interrogating function of one or more genes in one or more post-mitotic cells, comprising determining changes in expression of the one or more genes in the first populations of post-mitotic cells, inducing said gene perturbation in said first population to provide said second population with an altered genome (or genotype), and determining changes in expression of the one or more genes in the second population of post-mitotic cells, thereby interrogating the function of the one or more genes. In some embodiments, the post-mitotic cell is most preferably a kidney or brain (neuron) cell or a liver cell, such as a primary hepatocyte.

[0052] Also provided is a model and a method of creating said model. The model may be an animal comprising a post-mitotic cell (an *in vivo* model) or it may be an *ex vivo* or *in vitro*

model, such as a post-mitotic organoid or 'post-mitotic cell on a chip' or the collection of post-mitotic cells, such as a on a scaffold, as described herein. The post-mitotic cells of either model will preferably be transfected with Cas9. Accordingly, there is specifically provided a model comprising one or more post-mitotic cells comprising the CRISPR enzyme, preferably a Cas9 such as Sa or SpCas9. The model cells may have been transfected or transduced with the second regulatory element provided herein, which is second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at list one or more nuclear localization sequences (NLSs). The model may be, as described above, an *in vivo* model or it may be an *ex vivo* or *in vitro* model. Such a model allows rapid interrogation of function of one or more genes, as only the CRISPR-Cas system polynucleotide sequence (comprising one or more guide sequences targeting said one or more genes) needs to be delivered to perturb the function of said gene. In other words, methods of interrogating gene function in such models may comprise only delivery of the CRISPR-Cas system polynucleotide sequence (comprising the one or more guide sequences), the Cas (CRISPR enzyme) having already been provided in the cell(s) of the model. Methods of creating such models are also provided, comprising transducing or transfecting one or more post-mitotic cells in a first population of post-mitotic cells with a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at list one or more nuclear localization sequences (NLSs) as described herein to thereby provide one or more second population of post-mitotic cells comprising or expressing the CRISPR enzyme. In some embodiments, the post-mitotic cell is most preferably a kidney or brain (neuron) cell or a liver cell, such as a primary hepatocyte.

[0053] Methods of creating gene perturbed models, in particular gene knock down models, are also provided. These methods may typically comprise inducing gene perturbation in one or more genes, as described herein, in a first population of cells to thereby provide a second population of cells with an altered genome (or genotype). The second population of cells may then be seeded into a scaffold or onto a chip, for instance, to thereby provide an *ex vivo* or *in vitro* model. Alternatively, the second population of may be comprised within an *in vivo* animal.

[0054] Methods of gene therapy are also envisaged. For instance, correction of one or more deficient genotypes (for example single point mutations) is achievable through the use of the present CRISPR-Cas system in the post-mitotic cells discussed herein (including the models).

Monogenic conditions associated with the post-mitotic are particularly preferred and are exemplified herein, see Example 36 where the CRISPR-Cas9 system target was ApoB, a lipid metabolism gene, was effective at inducing a phenotypic change in vivo. Example 38 is also instructive in relation to phenotypic behaviour changes seen in vivo in the brain of mice transduced with the present system. Compositions for use in gene therapy are also provided.

[0055] Although various Cas enzymes are envisaged, Cas9 is particularly preferred and we have shown particular efficacy in the liver for SaCa9. Tracr sequence from Sa is also preferred if the Cas enzyme is an Sa Cas enzyme. A suitable PAM in such circumstance is NNGRR.

[0056] Although one guide may be used, so-called multiplexing with two, three, four or more guides, is particularly useful in interrogation of gene function and model creation (to provide multiple gene knock downs), but also in gene therapy where multiple defective genotypes are to be corrected (either multiple errors in a single gene or, more likely, multiple errors spread across several genes). Alternatively, multiplexing with two guides is useful in a dual nickase approach to reduce off-target effects or simply selection of multiple targets within one gene to ensure Cas recruitment. Triple and quadruple guides are preferred. Reference to gene herein is made interchangeably with genomic locus.

[0057] The intron approach described here is also useful in this regard, where the guide is positioned within the Cas intron.

[0058]

[0059] Preferred means of delivery include the methods described by Kanasty below, such as LNP, especially where only the guide is to be delivered or it is to be delivered alone. However, viral vectors including lentiviral and AAV are generally preferred for the liver as they have been successful to date. Of these, AAV is preferred and especially serotype 8, with AAV2/8 shown to be effective. Some preferred targets, to the extent that they are present in or conditions of the kidney are metabolic disorders, such as any one of: Amyloid neuropathy (TTR, PALB); Amyloidosis (APOA1, APP, AAA, CVAP, AD1, GSN, FGA, LYZ, TTR, PALB); Cirrhosis (KRT18, KRT8, CIRH1A, NAIC, TEX292, KIAA1988); Cystic fibrosis (CFTR, ABCC7, CF, MRP7); Glycogen storage diseases (SLC2A2, GLUT2, G6PC, G6PT, G6PT1, GAA, LAMP2, LAMPB, AGL, GDE, GBE1, GYS2, PYGL, PFKM); Hepatic adenoma, 142330 (TCF1, HNF1A, MODY3), Hepatic failure, early onset, and neurologic disorder (SCOD1, SCO1), Hepatic lipase deficiency (LIPC), Hepatoblastoma, cancer and carcinomas (CTNNB1, PDGFRL, PDGRL, PRLTS, AXIN1, AXIN, CTNNB1, TP53, P53, LFS1, IGF2R, MPRI, MET, CASP8, MCH5; Medullary cystic kidney disease (UMOD, HNFJ, FJHN, MCKD2,

ADMCKD2); Phenylketonuria (PAH, PKU1, QDPR, DHPR, PTS); Polycystic kidney and hepatic disease (FCYT, PKHD1, ARPKD, PKD1, PKD2, PKD4, PKDTS, PRKCSH, G19P1, PCLD, SEC63). Other preffered targets include any one or more of: PCSK9, HMGCR, APOB, LDLR, ANGPTL3, F8, F9/FIX, AAT, FAH, HPD, TAT, ATP7B, UGT1A1, OTC, ARH.

[0060] It will be appreciated that methods of altering expression in the post-mitotic cell do not involve alteration of the germline, which may be excluded on moral grounds. In fact, although transfection of stem cells is envisaged and certainly preferred in some embodiments, neurones or kidney cells are particularly preferred, particularly where they may show or be stimulated to show some regeneration.

[0061] Type II CRISPRS are particularly preferred, especially for use in eukaryotes, as in the present case, where livers are only found in eukaryotes, particularly vertebrate animals, in any case.

[0062] Use of the CRISPR-Cas systems to invoke a phenotypic change is a particular advantage, especially *in vivo*. We have shown this in the present application.

[0063] Where therapeutic applications are envisaged, or for other genome engineering in the post-mitotic cells, then where a correction is required it will be appreciated that following nicking or cleavage of the genomic DNA target, then correction via the HDR pathway is preferred. For gene knockdown, NHEJ is advantageous, however, correction via the HDR pathway is preferred for therapy. In such circumstances, it is preferable to deliver a repair template. This is most preferably ssDNA although RNA via a retroviral vector to provide a corresponding DNA template is also possible. The skilled person can readily put the invention into practice from the herein teachings contributing to the knowledge in the art; and in this regard mention is made that the skilled person from the herein teachings contributing to the knowledge in the art can readily appreciate and implement considerations as to homologous arm length. Mention is made of patent applications and publications including herein inventor Zhang, including those cited herein. The repair template is preferably co-delivered with one or more elements of the CRISPR-Cas system.

[0064] Also provided is a method of altering expression of at least one post-mitotic cell gene product comprising introducing into a eukaryotic liver cell, for example a hepatocyte, containing and expressing a DNA molecule having a target sequence and encoding the gene product, an engineered, non-naturally occurring Clustered Regularly Interspaced Short Palindromic Repeats

(CRISPR)-CRISPR associated (Cas) (CRISPR-Cas) system comprising one or more vectors comprising:

[0065] a) a first regulatory element operable in a eukaryotic cell operably linked to at least one nucleotide sequence encoding a CRISPR-Cas system guide RNA that hybridizes with the target sequence, and

[0066] b) a second regulatory element operable in a eukaryotic cell operably linked to a nucleotide sequence encoding a Type-II Cas9 protein,

[0067] wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNA targets the target sequence and the Cas9 protein cleaves the DNA molecule, whereby expression of the at least one post-mitotic cell gene product is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together.

Reference below to targets will be understood to be post-mitotic cell targets or genes otherwise expressed in the post-mitotic cell unless otherwise apparent

[0068] Any or all of the polynucleotide sequence encoding a CRISPR enzyme, guide sequence, tracr mate sequence or tracr sequence, may be RNA. The polynucleotides encoding the sequence encoding a CRISPR enzyme, the guide sequence, tracr mate sequence or tracr sequence may be RNA and may be delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0069] It will be appreciated that where reference is made to a polynucleotide, which is RNA and is said to 'comprise' a feature such a tracr mate sequence, the RNA sequence includes the feature. Where the polynucleotide is DNA and is said to comprise a feature such a tracr mate sequence, the DNA sequence is or can be transcribed into the RNA including the feature at issue. Where the feature is a protein, such as the CRISPR enzyme, the DNA or RNA sequence referred to is, or can be, translated (and in the case of DNA transcribed first).

[0070] Accordingly, in certain embodiments the invention provides a method of modifying post-mitotic cells of an organism, e.g., mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest comprising delivering a non-naturally occurring or engineered composition comprising a viral or plasmid vector system comprising one or more viral or plasmid vectors operably encoding a composition for expression thereof, wherein the composition comprises: (A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one or more nuclear localization sequences as some embodiments can involve no NLS), wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral or plasmid vector system as described herein.

[0071] Preferably, the vector is a viral vector, such as a lenti- or baculo- or preferably adeno-viral/adeno-associated viral vectors, but other means of delivery are known (such as yeast systems, microvesicles, gene guns/means of attaching vectors to gold nanoparticles) and are provided. In some embodiments, one or more of the viral or plasmid vectors may be delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0072] By manipulation of a target sequence, Applicants also mean the epigenetic manipulation of a target sequence. This may be of the chromatin state of a target sequence, such as by modification of the methylation state of the target sequence (i.e. addition or removal of methylation or methylation patterns or CpG islands), histone modification, increasing or reducing accessibility to the target sequence, or by promoting 3D folding.

[0073] It will be appreciated that where reference is made to a method of modifying an organism or mammal including human or a non-human mammal or organism by manipulation of a target sequence in a genomic locus of interest, this may apply to the organism (or mammal) as a whole or just a single cell or population of cells from that organism (if the organism is multicellular). In the case of humans, for instance, Applicants envisage, *inter alia*, a single cell or a population of cells and these may preferably be modified *ex vivo* and then re-introduced. In this case, a biopsy or other tissue or biological fluid sample may be necessary. Stem cells are also particularly preferred in this regard. But, of course, *in vivo* embodiments are also envisaged.

[0074] In certain embodiments the invention provides a method of treating or inhibiting a condition caused by a defect in a target sequence in a genomic locus of interest in a subject (e.g., mammal or human) or a non-human subject (e.g., mammal) in need thereof comprising modifying the subject or a non-human subject by manipulation of the target sequence and wherein the condition is susceptible to treatment or inhibition by manipulation of the target sequence comprising providing treatment comprising: delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the target sequence is manipulated by the composition when expressed, wherein the composition comprises: (A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises (a) a guide sequence capable of hybridizing to a target

sequence in a eukaryotic cell, (b) a tracr mate sequence, and (c) a tracr sequence, and II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences (or optionally at least one or more nuclear localization sequences as some embodiments can involve no NLS) wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, or (B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising I. a first regulatory element operably linked to (a) a guide sequence capable of hybridizing to a target sequence in a eukaryotic cell, and (b) at least one or more tracr mate sequences, II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and III. a third regulatory element operably linked to a tracr sequence, wherein components I, II and III are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In some embodiments, components I, II and III are located on the same vector. In other embodiments, components I and II are located on the same vector, while component III is located on another vector. In other embodiments, components I and III are located on the same vector, while component II is located on another vector. In other embodiments, components II and III are located on the same vector, while component I is located on another vector. In other embodiments, each of components I, II and III is located on different vectors. The invention also provides a viral (e.g. AAV or lentivirus) vector system as described herein. , and can be part of a vector system as described herein.

[0075] Some methods of the invention can include inducing expression. The organism or subject is a eukaryote (including mammal including human) or a non-human eukaryote or a non-human animal or a non-human mammal, provided it has post-mitotic cell (e.g. the brain or

kidney) and corresponding function. In some embodiments, the organism or subject is a non-human animal, and may be an arthropod, for example, an insect, or may be a nematode. In some methods of the invention the organism or subject is a mammal or a non-human mammal. A non-human mammal may be for example a rodent (preferably a mouse or a rat), an ungulate, or a primate. In some methods of the invention the viral vector is an AAV or a lentivirus, and can be part of a vector system as described herein. In some methods of the invention the CRISPR enzyme is a Cas9. In some methods of the invention the expression of the guide sequence is under the control of the T7 promoter and is driven by the expression of T7 polymerase.

[0076] The invention in some embodiments comprehends a method of delivering a CRISPR enzyme comprising delivering to a cell mRNA encoding the CRISPR enzyme. In some of these methods the CRISPR enzyme is a Cas9.

[0077] The invention also provides methods of preparing the vector systems of the invention, in particular the viral vector systems as described herein. The invention in some embodiments comprehends a method of preparing the AAV of the invention comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV into AAV-infected cells, and supplying AAV rep and/or cap obligatory for replication and packaging of the AAV. In some embodiments the AAV rep and/or cap obligatory for replication and packaging of the AAV are supplied by transfecting the cells with helper plasmid(s) or helper virus(es). In some embodiments the helper virus is a poxvirus, adenovirus, herpesvirus or baculovirus. In some embodiments the poxvirus is a vaccinia virus. In some embodiments the cells are mammalian cells. And in some embodiments the cells are insect cells and the helper virus is baculovirus. In other embodiments, the virus is a lentivirus.

[0078] The invention further comprehends a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) for use in medicine or in therapy. In some embodiments the invention comprehends a composition according to the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) for use in a method according to the invention. In some embodiments the invention provides for the use of a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme) in *ex vivo* gene or genome editing. In certain embodiments the invention comprehends use of a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA

encoding the CRISPR enzyme) in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method according of the invention. The invention comprehends in some embodiments a composition of the invention or a CRISPR enzyme thereof (including or alternatively mRNA encoding the CRISPR enzyme), wherein the target sequence is flanked at its 3' end by a PAM (protospacer adjacent motif) sequence comprising 5'-motif, especially where the Cas9 is (or is derived from) *S. pyogenes* or *S. aureus* Cas9. For example, a suitable PAM is 5'-NRG or 5'-NNGRR (where N is any Nucleotide) for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively, as mentioned below.

[0079] It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9. It may of course, be mutated or otherwise changed from the wild type to suit the intended use, as described herein. The dual nickase D10A mutant or variant is preferred, especially in combination with two overlapping guides directed as opposing sites on differing strands of the same chromosome.

[0080] Apects of the invention comprehend improving the specificity of a CRISPR enzyme, e.g. Cas9, mediated gene targeting and reducing the likelihood of off-target modification by the CRISPR enzyme, e.g. Cas9. The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising delivering a non-naturally occurring or engineered composition comprising :

[0081] I. a first CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the first polynucleotide sequence comprises:

(a) a first guide sequence capable of hybridizing to the first target sequence,

(b) a first tracr mate sequence, and

(c) a first tracr sequence,

[0082] II. a second CRISPR-Cas system chiRNA polynucleotide sequence, wherein the second polynucleotide sequence comprises:

(a) a second guide sequence capable of hybridizing to the second target sequence,

(b) a second tracr mate sequence, and

(c) a second tracr sequence, and

[0083] III. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences and comprising one or more mutations, wherein (a), (b) and (c) are arranged in a 5' to 3' orientation, wherein when transcribed, the first and the second tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

[0084] In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the polynucleotides encoding the sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA and are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun. In certain embodiments of the invention, the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In some embodiments, the polynucleotides may be comprised within a vector system comprising one or more vectors. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the CRISPR enzyme has the D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary

strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme.

[0085] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of the other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs. Most preferably, the overlap is between 5 and -1 base pairs.

[0086] The invention in some embodiments comprehends a method of modifying an organism or a non-human organism by minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising delivering a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

[0087] I. a first regulatory element operably linked to

(a) a first guide sequence capable of hybridizing to the first target sequence, and

(b) at least one or more tracr mate sequences,

[0088] II. a second regulatory element operably linked to

(a) a second guide sequence capable of hybridizing to the second target sequence, and

(b) at least one or more tracr mate sequences,

[0089] III. a third regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and

[0090] IV. a fourth regulatory element operably linked to a tracr sequence,

[0091] wherein components I, II, III and IV are located on the same or different vectors of the system, when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the first and the second guide sequence direct sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively, wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the tracr mate sequence that is hybridized to the

tracr sequence, wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of the other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

[0092] The invention also provides a vector system as described herein. The system may comprise one, two, three or four different vectors. Components I, II, III and IV may thus be located on one, two, three or four different vectors, and all combinations for possible locations of the components are herein envisaged, for example: components I, II, III and IV can be located on the same vector; components I, II, III and IV can each be located on different vectors; components I, II, II I and IV may be located on a total of two or three different vectors, with all combinations of locations envisaged, etc.

[0093] In some methods of the invention any or all of the polynucleotide sequence encoding the CRISPR enzyme, the first and the second guide sequence, the first and the second tracr mate sequence or the first and the second tracr sequence, is/are RNA. In further embodiments of the invention the first and second tracr mate sequence share 100% identity and/or the first and second tracr sequence share 100% identity. In preferred embodiments of the invention the CRISPR enzyme is a Cas9 enzyme, e.g. SpCas9. In an aspect of the invention the CRISPR enzyme comprises one or more mutations in a catalytic domain, wherein the one or more mutations are selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the CRISPR enzyme has the D10A mutation. In preferred embodiments, the first CRISPR enzyme has one or more mutations such that the enzyme is a complementary strand nicking enzyme, and the second CRISPR enzyme has one or more mutations such that the enzyme is a non-complementary strand nicking enzyme. Alternatively the first enzyme may be a non-complementary strand nicking enzyme, and the second enzyme may be a complementary strand nicking enzyme. In a further embodiment of the invention, one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[0094] In preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0095] The invention in some embodiments comprehends a method of modifying a genomic locus of interest by minimizing off-target modifications by introducing into a cell containing and expressing a double stranded DNA molecule encoding a gene product of interest an engineered, non-naturally occurring CRISPR-Cas system comprising a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand of the DNA molecule respectively, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[0096] In preferred methods of the invention the Cas protein nicking each of the first strand and the second strand of the DNA molecule encoding the gene product results in a 5' overhang. In embodiments of the invention the 5' overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5' overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs.

[0097] Embodiments of the invention also comprehend the guide RNAs comprising a guide sequence fused to a tracr mate sequence and a tracr sequence. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[0098] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[0099] The invention also comprehends an engineered, non-naturally occurring CRISPR-Cas system comprising a Cas protein having one or more mutations and two guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule encoding a gene product in a cell, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[00100] In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[00101] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[00102] The invention also comprehends an engineered, non-naturally occurring vector system comprising one or more vectors comprising:

a) a first regulatory element operably linked to each of two CRISPR-Cas system guide RNAs that target a first strand and a second strand respectively of a double stranded DNA molecule encoding a gene product,
b) a second regulatory element operably linked to a Cas protein,
wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNAs target the DNA molecule encoding the gene product and the Cas protein nicks each of the first strand and the second strand of the DNA molecule encoding the gene product, whereby expression of the gene product is altered; and, wherein the Cas protein and the two guide RNAs do not naturally occur together.

[00103] In aspects of the invention the guide RNAs may comprise a guide sequence fused to a tracr mate sequence and a tracr sequence. In an embodiment of the invention the Cas protein is a type II CRISPR-Cas protein. In an aspect of the invention the Cas protein is codon optimized for expression in a eukaryotic cell, preferably a mammalian cell or a human cell. In further embodiments of the invention the Cas protein is a type II CRISPR-Cas protein, e.g. a Cas 9 protein. In a highly preferred embodiment the Cas protein is a Cas9 protein, e.g. SpCas9. In aspects of the invention the Cas protein has one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A and D986A. In a highly preferred embodiment the Cas protein has the D10A mutation.

[00104] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5' overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein. In preferred embodiments of the invention the vectors of the system are viral vectors. In a further embodiment, the vectors of the system are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

[00105] In one aspect, the invention provides a method of modifying a target polynucleotide in a post-mitotic eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence

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

[00106] In one aspect, the invention provides a method of modifying expression of a polynucleotide in a post-mitotic eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the polynucleotide such that said binding results in increased or decreased expression of said polynucleotide; wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. In some embodiments, the method further comprises delivering one or more vectors to said eukaryotic cells, wherein the one or more vectors drive expression of one or more of: the CRISPR enzyme, the guide sequence linked to the tracr mate sequence, and the tracr sequence.

[00107] In one aspect, the invention provides a method of generating a model post-mitotic eukaryotic cell comprising a mutated disease gene. In some embodiments, a disease gene is any gene associated with an increase in the risk of having or developing a disease. In some embodiments, the method comprises (a) introducing one or more vectors into a eukaryotic cell,

wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, and a tracr sequence; and (b) allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said disease gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, thereby generating a model eukaryotic cell comprising a mutated disease gene. In some embodiments, said cleavage comprises cleaving one or two strands at the location of the target sequence by said CRISPR enzyme. In some embodiments, said cleavage results in decreased transcription of a target gene. In some embodiments, the method further comprises repairing said cleaved target polynucleotide by homologous recombination with an exogenous template polynucleotide, wherein said repair results in a mutation comprising an insertion, deletion, or substitution of one or more nucleotides of said target polynucleotide. In some embodiments, said mutation results in one or more amino acid changes in a protein expression from a gene comprising the target sequence.

[00108] In one aspect the invention provides for a method of selecting one or more post-mitotic cell(s) by introducing one or more mutations in a gene in the one or more cell (s), the method comprising: introducing one or more vectors into the cell (s), wherein the one or more vectors drive expression of one or more of: a CRISPR enzyme, a guide sequence linked to a tracr mate sequence, a tracr sequence, and an editing template; wherein the editing template comprises the one or more mutations that abolish CRISPR enzyme cleavage; allowing homologous recombination of the editing template with the target polynucleotide in the cell(s) to be selected; allowing a CRISPR complex to bind to a target polynucleotide to effect cleavage of the target polynucleotide within said gene, wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence within the target polynucleotide, and (2) the tracr mate sequence that is hybridized to the tracr sequence, wherein binding of the CRISPR complex to the target polynucleotide induces cell death, thereby allowing one or more prokaryotic cell(s) in which one or more mutations have been introduced to be selected. In a preferred embodiment, the CRISPR enzyme is Cas9. Aspects of the invention allow for selection of specific cells without requiring a selection marker or a two-step process that may include a counter-selection system.

[00109] In one aspect, the invention provides for methods of modifying a target polynucleotide in a post-mitotic eukaryotic cell. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence.

[00110] In other embodiments, this invention provides a method of modifying expression of a polynucleotide in a post-mitotic eukaryotic cell. The method comprises increasing or decreasing expression of a target polynucleotide by using a CRISPR complex that binds to the polynucleotide.

[00111] Where desired, to effect the modification of the expression in a cell, one or more vectors comprising a tracr sequence, a guide sequence linked to the tracr mate sequence, a sequence encoding a CRISPR enzyme is delivered to a cell. In some methods, the one or more vectors comprises a regulatory element operably linked to an enzyme-coding sequence encoding said CRISPR enzyme comprising a nuclear localization sequence; and a regulatory element operably linked to a tracr mate sequence and one or more insertion sites for inserting a guide sequence upstream of the tracr mate sequence. When expressed, the guide sequence directs sequence-specific binding of a CRISPR complex to a target sequence in a cell. Typically, the CRISPR complex comprises a CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

[00112] In some methods, a target polynucleotide can be inactivated to effect the modification of the expression in a cell. For example, upon the binding of a CRISPR complex to a target sequence in a cell, the target polynucleotide is inactivated such that the sequence is not transcribed, the coded protein is not produced, or the sequence does not function as the wild-type sequence does. For example, a protein or microRNA coding sequence may be inactivated such that the protein is not produced.

[00113] In certain embodiments, the CRISPR enzyme comprises one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A or D986A and/or the one or more mutations is in a RuvC1 or HNH domain of the CRISPR enzyme or is a

mutation as otherwise as discussed herein. In some embodiments, the CRISPR enzyme has one or more mutations in a catalytic domain, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain. In some embodiments, the functional domain is a transcriptional activation domain, preferably VP64. In some embodiments, the functional domain is a transcription repression domain, preferably KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X). In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain is an activation domain, which may be the P65 activation domain.

[00114] In some embodiments, the CRISPR enzyme is a type I or III CRISPR enzyme, but is preferably a type II CRISPR enzyme. This type II CRISPR enzyme may be any Cas enzyme. A Cas enzyme may be identified as Cas9 as this can refer to the general class of enzymes that share homology to the biggest nuclease with multiple nuclease domains from the type II CRISPR system. Most preferably, the Cas9 enzyme is from, or is derived from, spCas9 or saCas9. By derived, Applicants mean that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as described herein.

[00115] It will be appreciated that the terms Cas and CRISPR enzyme are generally used herein interchangeably, unless otherwise apparent. As mentioned above, many of the residue numberings used herein refer to the Cas9 enzyme from the type II CRISPR locus in *Streptococcus pyogenes*. However, it will be appreciated that this invention includes many more Cas9s from other species of microbes, such as SpCas9, SaCa9, St1Cas9 and so forth.

[00116] An example of a codon optimized sequence, in this instance optimized for humans (i.e. being optimized for expression in humans) is provided herein, see the SaCas9 human codon optimized sequence. Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species is known.

[00117] Preferably, delivery is in the form of a vector which may be a viral vector, such as a lenti- or baculo- or preferably adeno-viral/adeno-associated viral vectors, but other means of delivery are known (such as yeast systems, microvesicles, gene guns/means of attaching vectors

to gold nanoparticles) and are provided. A vector may mean not only a viral or yeast system (for instance, where the nucleic acids of interest may be operably linked to and under the control of (in terms of expression, such as to ultimately provide a processed RNA) a promoter), but also direct delivery of nucleic acids into a host cell. While in herein methods the vector may be a viral vector and this is advantageously an AAV, other viral vectors as herein discussed can be employed, such as lentivirus. For example, baculoviruses may be used for expression in insect cells. These insect cells may, in turn be useful for producing large quantities of further vectors, such as AAV or lentivirus vectors adapted for delivery of the present invention. Also envisaged is a method of delivering the present CRISPR enzyme comprising delivering to a cell mRNA encoding the CRISPR enzyme. It will be appreciated that in certain embodiments the CRISPR enzyme is truncated, and/or comprised of less than one thousand amino acids or less than four thousand amino acids, and/or is a nuclease or nickase, and/or is codon-optimized, and/or comprises one or more mutations, and/or comprises a chimeric CRISPR enzyme, and/or the other options as herein discussed. AAV and lentiviral vectors are preferred.

[00118] In certain embodiments, the target sequence is flanked or followed, at its 3' end, by a PAM suitable for the CRISPR enzyme, typically a Cas and in particular a Cas9.

[00119] For example, a suitable PAM is 5'-NRG or 5'-NNGRR for SpCas9 or SaCas9 enzymes (or derived enzymes), respectively.

[00120] It will be appreciated that SpCas9 or SaCas9 are those from or derived from *S. pyogenes* or *S. aureus* Cas9.

[00121] Accordingly, it is an object of the invention to not encompass within the invention any previously known product, process of making the product, or method of using the product such that Applicants reserve the right and hereby disclose a disclaimer of any previously known product, process, or method. It is further noted that the invention does not intend to encompass within the scope of the invention any product, process, or making of the product or method of using the product, which does not meet the written description and enablement requirements of the USPTO (35 U.S.C. §112, first paragraph) or the EPO (Article 83 of the EPC), such that Applicants reserve the right and hereby disclose a disclaimer of any previously described product, process of making the product, or method of using the product.

[00122] It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning

attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[00123] These and other embodiments are disclosed or are obvious from and encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00124] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[00125] Figure 1 shows a schematic model of the CRISPR system. The Cas9 nuclease from Streptococcus pyogenes (yellow) is targeted to genomic DNA by a synthetic guide RNA (sgRNA) consisting of a 20-nt guide sequence (blue) and a scaffold (red). The guide sequence base-pairs with the DNA target (blue), directly upstream of a requisite 5'-NGG protospacer adjacent motif (PAM; magenta), and Cas9 mediates a double-stranded break (DSB) ~3 bp upstream of the PAM (red triangle).

[00126] Figure 2A-F shows an exemplary CRISPR system, a possible mechanism of action, an example adaptation for expression in eukaryotic cells, and results of tests assessing nuclear localization and CRISPR activity.

[00127] Figure 3A-D shows results of an evaluation of SpCas9 specificity for an example target.

[00128] Figure 4A-G show an exemplary vector system and results for its use in directing homologous recombination in eukaryotic cells.

[00129] Figure 5 provides a table of protospacer sequences and summarizes modification efficiency results for protospacer targets designed based on exemplary S. pyogenes and S. thermophilus CRISPR systems with corresponding PAMs against loci in human and mouse genomes. Cells were transfected with Cas9 and either pre-crRNA/tracrRNA or chimeric RNA,

and analyzed 72 hours after transfection. Percent indels are calculated based on Surveyor assay results from indicated cell lines (N=3 for all protospacer targets, errors are S.E.M., N.D. indicates not detectable using the Surveyor assay, and N.T. indicates not tested in this study).

[00130] Figure 6A-C shows a comparison of different tracrRNA transcripts for Cas9-mediated gene targeting.

[00131] Figure 7 shows a schematic of a surveyor nuclease assay for detection of double strand break-induced micro-insertions and –deletions.

[00132] Figure 8A-B shows exemplary bicistronic expression vectors for expression of CRISPR system elements in eukaryotic cells.

[00133] Figure 9A-C shows histograms of distances between adjacent S. pyogenes SF370 locus 1 PAM (NGG) (Figure 9A) and S. thermophilus LMD9 locus 2 PAM (NNAGAAW) (Figure 9B) in the human genome; and distances for each PAM by chromosome (Chr) (Figure 9C).

[00134] Figure 10A-D shows an exemplary CRISPR system, an example adaptation for expression in eukaryotic cells, and results of tests assessing CRISPR activity.

[00135] Figure 11A-C shows exemplary manipulations of a CRISPR system for targeting of genomic loci in mammalian cells.

[00136] Figure 12A-B shows the results of a Northern blot analysis of crRNA processing in mammalian cells.

[00137] Figure 13A-B shows an exemplary selection of protospacers in the human PVALB and mouse Th loci.

[00138] Figure 14 shows example protospacer and corresponding PAM sequence targets of the S. thermophilus CRISPR system in the human EMX1 locus.

[00139] Figure 15 provides a table of sequences for primers and probes used for Surveyor, RFLP, genomic sequencing, and Northern blot assays.

[00140] Figure 16A-C shows exemplary manipulation of a CRISPR system with chimeric RNAs and results of SURVEYOR assays for system activity in eukaryotic cells.

[00141] Figure 17A-B shows a graphical representation of the results of SURVEYOR assays for CRISPR system activity in eukaryotic cells.

[00142] Figure 18 shows an exemplary visualization of some S. pyogenes Cas9 target sites in the human genome using the UCSC genome browser.

[00143] Figure 19A-D shows a circular depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[00144] Figure 20A-F shows the linear depiction of the phylogenetic analysis revealing five families of Cas9s, including three groups of large Cas9s (~1400 amino acids) and two of small Cas9s (~1100 amino acids).

[00145] Figure 21A-D shows genome editing via homologous recombination. (a) Schematic of SpCas9 nickase, with D10A mutation in the RuvC I catalytic domain. (b) Schematic representing homologous recombination (HR) at the human EMX1 locus using either sense or antisense single stranded oligonucleotides as repair templates. Red arrow above indicates sgRNA cleavage site; PCR primers for genotyping (Tables J and K) are indicated as arrows in right panel. (c) Sequence of region modified by HR. d, SURVEYOR assay for wildtype (wt) and nickase (D10A) SpCas9-mediated indels at the EMX1 target 1 locus (n=3). Arrows indicate positions of expected fragment sizes.

[00146] Figure 22A-B shows single vector designs for SpCas9.

[00147] Figure 23 shows a graph representing the length distribution of Cas9 orthologs.

[00148] Figure 24A-M shows sequences where the mutation points are located within the SpCas9 gene.

[00149] Figure 25A shows the Conditional Cas9, Rosa26 targeting vector map.

[00150] Figure 25B shows the Constitutive Cas9, Rosa26 targeting vector map.

[00151] Figure 26 shows a schematic of the important elements in the Constitutive and Conditional Cas9 constructs.

[00152] Figure 27 shows delivery and *in vivo* mouse brain Cas9 expression data.

[00153] Figure 28 shows RNA delivery of Cas9 and chimeric RNA into cells (A) Delivery of a GFP reporter as either DNA or mRNA into Neuro-2A cells. (B) Delivery of Cas9 and chimeric RNA against the Icam2 gene as RNA results in cutting for one of two spacers tested. (C) Delivery of Cas9 and chimeric RNA against the F7 gene as RNA results in cutting for one of two spacers tested.

[00154] Figure 29 shows how DNA double-strand break (DSB) repair promotes gene editing. In the error-prone non-homologous end joining (NHEJ) pathway, the ends of a DSB are processed by endogenous DNA repair machineries and rejoined together, which can result in

random insertion/deletion (indel) mutations at the site of junction. Indel mutations occurring within the coding region of a gene can result in frame-shift and a premature stop codon, leading to gene knockout. Alternatively, a repair template in the form of a plasmid or single-stranded oligodeoxynucleotides (ssODN) can be supplied to leverage the homology-directed repair (HDR) pathway, which allows high fidelity and precise editing.

[00155] Figure 30A-C shows anticipated results for HDR in HEK and HUES9 cells. (a) Either a targeting plasmid or an ssODN (sense or antisense) with homology arms can be used to edit the sequence at a target genomic locus cleaved by Cas9 (red triangle). To assay the efficiency of HDR, we introduced a HindIII site (red bar) into the target locus, which was PCR-amplified with primers that anneal outside of the region of homology. Digestion of the PCR product with HindIII reveals the occurrence of HDR events. (b) ssODNs, oriented in either the sense or the antisense (s or a) direction relative to the locus of interest, can be used in combination with Cas9 to achieve efficient HDR-mediated editing at the target locus. A minimal homology region of 40 bp, and preferably 90 bp, is recommended on either side of the modification (red bar). (c) Example of the effect of ssODNs on HDR in the EMX1 locus is shown using both wild-type Cas9 and Cas9 nickase (D10A). Each ssODN contains homology arms of 90 bp flanking a 12-bp insertion of two restriction sites.

[00156] Figure 31A-C shows the repair strategy for Cystic Fibrosis delta F508 mutation.

[00157] Figure 32A-B (a) shows a schematic of the GAA repeat expansion in FXN intron 1 and (b) shows a schematic of the strategy adopted to excise the GAA expansion region using the CRISPR/Cas system.

[00158] Figure 33 shows a screen for efficient SpCas9 mediated targeting of Tet1-3 and Dnmt1, 3a and 3b gene loci. Surveyor assay on DNA from transfected N2A cells demonstrates efficient DNA cleavage by using different gRNAs.

[00159] Figure 34 shows a strategy of multiplex genome targeting using a 2-vector system in an AAV1/2 delivery system. Tet1-3 and Dnmt1, 3a and 3b gRNA under the control of the U6 promoter. GFP-KASH under the control of the human synapsin promoter. Restriction sides shows simple gRNA replacement strategy by subcloning. HA-tagged SpCas9 flanked by two nuclear localization signals (NLS) is shown. Both vectors are delivered into the brain by AAV1/2 virus in a 1:1 ratio.

[00160] Figure 35 shows verification of multiplex DNMT targeting vector #1 functionality using Surveyor assay. N2A cells were co-transfected with the DNMT targeting vector #1 (+) and the SpCas9 encoding vector for testing SpCas9 mediated cleavage of DNMTs genes family loci. gRNA only (-) is negative control. Cells were harvested for DNA purification and downstream processing 48 h after transfection.

[00161] Figure 36 shows verification of multiplex DNMT targeting vector #2 functionality using Surveyor assay. N2A cells were co-transfected with the DNMT targeting vector #1 (+) and the SpCas9 encoding vector for testing SpCas9 mediated cleavage of DNMTs genes family loci. gRNA only (-) is negative control. Cells were harvested for DNA purification and downstream processing 48 h after transfection.

[00162] Figure 37 shows schematic overview of short promoters and short polyA versions used for HA-SpCas9 expression in vivo. Sizes of the encoding region from L-ITR to R-ITR are shown on the right.

[00163] Figure 38 shows schematic overview of short promoters and short polyA versions used for HA-SaCas9 expression in vivo. Sizes of the encoding region from L-ITR to R-ITR are shown on the right.

[00164] Figure 39 shows expression of SpCas9 and SaCas9 in N2A cells. Representative Western blot of HA-tagged SpCas9 and SaCas9 versions under the control of different short promoters and with or short polyA (spA) sequences. Tubulin is loading control. mCherry (mCh) is a transfection control. Cells were harvested and further processed for Western blotting 48 h after transfection.

[00165] Figure 40 shows screen for efficient SaCas9 mediated targeting of Tet3 gene locus. Surveyor assay on DNA from transfected N2A cells demonstrates efficient DNA cleavage by using different gRNAs with NNGGGT PUM sequence. GFP transfected cells and cells expressing only SaCas9 are controls.

[00166] Figure 41 shows expression of HA-SaCas9 in the mouse brain. Animals were injected into dentate gyri with virus driving expression of HA-SaCas9 under the control of human Synapsin promoter. Animals were sacrificed 2 weeks after surgery. HA tag was detected using rabbit monoclonal antibody C29F4 (Cell Signaling). Cell nuclei stained in blue with DAPI stain.

[00167] Figure 42 shows expression of SpCas9 and SaCas9 in cortical primary neurons in culture 7 days after transduction. Representative Western blot of HA-tagged SpCas9 and SaCas9

versions under the control of different promoters and with bgh or short polyA (spA) sequences. Tubulin is loading control.

[00168] Figure 43 shows LIVE/DEAD stain of primary cortical neurons 7 days after transduction with AAV1 particles carrying SpCas9 with different promoters and multiplex gRNAs constructs (example shown on the last panel for DNMTs). Neurons after AAV transduction were compared with control untransduced neurons. Red nuclei indicate permeabilized, dead cells (second line of panels). Live cells are marked in green color (third line of panels).

[00169] Figure 44 shows LIVE/DEAD stain of primary cortical neurons 7 days after transduction with AAV1 particles carrying SaCas9 with different promoters. Red nuclei indicate permeabilized, dead cells (second line of panels). Live cells are marked in green color (third line of panels).

[00170] Figure 45 shows comparison of morphology of neurons after transduction with AAV1 virus carrying SpCas9 and gRNA multiplexes for TETs and DNMTs genes loci. Neurons without transduction are shown as a control.

[00171] Figure 46 shows verification of multiplex DNMT targeting vector #1 functionality using Surveyor assay in primary cortical neurons. Cells were co-transduced with the DNMT targeting vector #1 and the SpCas9 viruses with different promoters for testing SpCas9 mediated cleavage of DNMTs genes family loci.

[00172] Figure 47 shows in vivo efficiency of SpCas9 cleavage in the brain. Mice were injected with AAV1/2 virus carrying gRNA multiplex targeting DNMT family genes loci together with SpCas9 viruses under control of 2 different promoters: mouse Mecp2 and rat Map1b. Two weeks after injection brain tissue was extracted and nuclei were prepped and sorted using FACS, based on the GFP expression driven by Synapsin promoter from gRNA multiplex construct. After gDNA extraction Surveyor assay was run. + indicates GFP positive nuclei and – control, GFP-negative nuclei from the same animal. Numbers on the gel indicate assessed SpCas9 efficiency.

[00173] Figure 48 shows purification of GFP-KASH labeled cell nuclei from hippocampal neurons. The outer nuclear membrane (ONM) of the cell nuclear membrane is tagged with a fusion of GFP and the KASH protein transmembrane domain. Strong GFP expression in the brain after one week of stereotactic surgery and AAV1/2 injection. Density gradient

centrifugation step to purify cell nuclei from intact brain. Purified nuclei are shown. Chromatin stain by Vybrant® DyeCycle™ Ruby Stain is shown in red, GFP labeled nuclei are green. Representative FACS profile of GFP+ and GFP- cell nuclei (Magenta: Vybrant® DyeCycle™ Ruby Stain, Green: GFP).

[00174] Figure 49 shows efficiency of SpCas9 cleavage in the mouse brain. Mice were injected with AAV1/2 virus carrying gRNA multiplex targeting TET family genes loci together with SpCas9 viruses under control of 2 different promoters: mouse Mecp2 and rat Map1b. Three weeks after injection brain tissue was extracted, nuclei were prepped and sorted using FACS, based on the GFP expression driven by Synapsin promoter from gRNA multiplex construct. After gDNA extraction Surveyor assay was run. + indicates GFP positive nuclei and – control, GFP-negative nuclei from the same animal. Numbers on the gel indicate assessed SpCas9 efficiency.

[00175] Figure 50 shows GFP-KASH expression in cortical neurons in culture. Neurons were transduced with AAV1 virus carrying gRNA multiplex constructs targeting TET genes loci. The strongest signal localize around cells nuclei due to KASH domain localization.

[00176] Figure 51 shows (top) a list of spacing (as indicated by the pattern of arrangement for two PAM sequences) between pairs of guide RNAs. Only guide RNA pairs satisfying patterns 1, 2, 3, 4 exhibited indels when used with SpCas9(D10A) nickase. (bottom) Gel images showing that combination of SpCas9(D10A) with pairs of guide RNA satisfying patterns 1, 2, 3, 4 led to the formation of indels in the target site.

[00177] Figure 52 shows a list of U6 reverse primer sequences used to generate U6-guide RNA expression casssettes. Each primer needs to be paired with the U6 forward primer "gcactgagggcctatttcccatgattc" to generate amplicons containing U6 and the desired guide RNA.

[00178] Figure 53 shows a Genomic sequence map from the human Emx1 locus showing the locations of the 24 patterns listed in Figure 33.

[00179] Figure 54 shows on (right) a gel image indicating the formation of indels at the target site when variable 5' overhangs are present after cleavage by the Cas9 nickase targeted by different pairs of guide RNAs. on (left) a table indicating the lane numbers of the gel on the right and various parameters including identifying the guide RNA pairs used and the length of the 5' overhang present following cleavage by the Cas9 nickase.

[00180] Figure 55 shows a Genomic sequence map from the human Emx1 locus showing the locations of the different pairs of guide RNAs that result in the gel patterns of Fig. 54 (right) and which are further described in Example 35.

[00181] Figure 56A shows that guide (target) 1 induced the highest percentage of indels in ApoB.

[00182] Figure 568B shows the results of a Surveyor nuclease gel assay for indel formation efficiency, 4 weeks post-injection.

[00183] Figure 57B shows oil red staining to detect hepatic lipid accumulation phenotype in vivo following AAV-Cas9-sgRNA delivery. The scale bar in each square represents 20 micrometres.

[00184] Figure 58 shows that 21 ntds/base pairs (bp), represented by the grey bars is the optimal spacer length, at least compared to 20 or 22 base pairs (represented by the black and the white bars, respectively) across a range of targets and within two different genes (AAVS1 and EMX1).

[00185] Figure 59 shows whether a guide sequence could be inserted into the Cas9 intronic sequence

[00186] Figure 60 shows that the full-length H1 promoter (grey bar) is still weaker than U6 promoter (black bar), as the U6 shows increased indel percentage formation for each target tested.

[00187] Figure 61 shows that short H1 promoter is weaker than the full-length H1

[00188] Figure 62 shows distance between the 5' ends of two guide sequences in a construct measured in relation to the cleavage efficiency of the D10A SaCAs9 double nickase.

[00189] Figure 63 (Example 38) shows CRISPR-Cas9 system delivery and targeting of *Mecp2* locus in the mouse brain. (**a**) AAV-SpCas9 and AAV-SpGuide(*Mecp2*) expression vectors. The sgRNA vector contains encoding sequence of the GFP-KASH fusion protein for identification of transduced neurons. (**b**) Expression of HA-Cas9 and GFP-KASH in the dorsal dentate gyrus (DG) of mouse hippocampus. Scale bar, 100 μm. (**c**) Quantification of cells efficiently targeted by the dual-vector Cas9-CRISPR system. (**d**) Graphical representation of the mouse *Mecp2* locus showing Cas9 target location; sgRNA indicated in blue. PAM sequence marked in purple. Representative mutation patterns detected by sequencing of *Mecp2* locus were shown below: green - wild-type sequence; red dashes - deleted bases; red bases: insertion or mutations; red

arrowhead indicates CRISPR-Cas9 cutting site. (**e**) SURVEYOR™ assay gel showing modification of the *Mecp2* locus, 2 weeks after AAV delivery in the DG region. (**f**) Western blot analysis of MeCP2 protein expression in the targeted brain region and quantification of MeCP2 protein levels in dorsal DG (*t*-test, $^{**}p<0.001$, n=4 from 3 animals, error bars: s.e.m.). (**g**) Images of the dorsal DG region, 2 weeks after CRISPR-Cas9 targeting of *Mecp2* locus. Scale bar, 150 μm. (**h**) Quantification of MeCP2 positive cells population within all detected cells (DAPI staining) in the targeted brain region in compare to control collateral site (*t*-test, $^{****}p<0.0001$, n=290 and 249 cells from 2 animals, respectively; error bars: s.e.m). (ITR – inverted terminal repeat; HA – hemagglutinin tag; NLS – nuclear localization signal; spA – synthetic polyadenylation signal; U6 – PolIII promoter; sgRNA – single guide RNA; hSyn – human synapsin 1 promoter; GFP- green fluorescent protein; KASH - Klarsicht, ANC1, Syne Homology nuclear transmembrane domain; bGH pA – bovine growth hormone polyadenylatio signal; WPRE – Woodchuck Hepatitis virus posttranscriptional regulatory element).

[00190] Figure 64 (Example 38) shows analysis of gene expression in Cas9-mediated MeCP2 knockdown neurons. (**a**) Strategy for cell nuclei purification of CRISPR-Cas9 targeted cells from the mouse brain. (**b**) Hierarchical clustering of differentially expressed genes (*t*-test, $p<0.01$, n=19 populations of sorted nuclei from 8 animals) detected by RNAseq. Relative log2(TPM+1) expression levels of genes are normalized for each row and displayed in red-blue color scale. Each column represents a population of targeted 100 neuronal nuclei FACS sorted from the isolated, dentate gyrus population of cells, either from control or *Mecp2* sgRNA transduced animals, as indicated.

[00191] Figure 65 (Example 38) shows cell-autonomous defects in cellular response properties of neurons after CRISPR-mediated MeCP2 knockdown. (a) Cartoon showing *in vivo* experiment configuration from mouse visual cortex and visual stimulation parameter. GFP^+ neuron is shown. Scale bar, 20 μm. (b) Cartoon showing recording configuration in layer 2/3 excitatory neurons that receive both contra- and ipsilateral eye specific input. Genome modified GFP^+ cells are in green whereas unmodified cells are in gray. Normalized spike shape shows regular spiking excitatory neurons. (c,d) Average OSI (c) and evoked FR (d) were measured from GFP^+ cells expressing *Mecp2* and control sgRNA, respectively (*t*-test, $^{*}p<0.05$; numbers in graph indicate numbers of recorded cells; n=2-3 animals; error bars: s.e.m).

[00192] Figure 66 (Example 38) shows simultaneous, multiplex gene editing in the mouse brain. (a) Schematic illustration of CRISPR-Cas9 system designed for multiplex genome targeting. (b) Graphical representation of targeted DNMT mouse loci. Guide RNAs are indicated in blue. PAM sequences are marked in purple. (c) SURVEYOR™ assay gel showing modification of DNMTs loci in FACS sorted GFP-KASH positive cells, 4 weeks after AAV delivery in the DG region. (d) Deep sequencing-based analysis of DNMTs loci modification in single cells, showing co-occurrence of modification in multiple loci. (e) Western blot analysis for Dnmt3a and Dnmt1 proteins after in vivo delivery of CRISPR-Cas9 system targeting DNMT family genes (top). Western blot quantification of Dnmt3a and Dnmt1 protein levels in DG after in vivo CRISPR-Cas9 targeting (bottom; t-test, **p<0.001, *p<0.05, Dnmt3a: n=7; Dnmt1: n=5 from 5 animals; error bars: s.e.m). (f) Contextual learning deficits, 8 weeks after targeting of DNMT genes using SpCas9 in the DG region of hippocampus, tested in training and altered context (t-test, ***p<0.0001, n=18 animals, 2 independent experiments; error bars: s.e.m).

[00193] Figure 67 (Example 38) chows cloning and expression of HA-tagged SpCas9 (HA-SpCas9) for AAV packaging. (a) Schematic overview of different cloning strategies to minimize SpCas9 expression cassette size using short rat *Map1b* promotor (pMap1b), a truncated version of the mouse *Mecp2* promoter (pMecp2) and a short polyA motif (spA). (**b**) Western blot analysis of primary cortical neuron culture expressing HA-SpCas9 using different SpCas9 expression cassettes. (**c**) *Mecp2* promoter drives HA-SpCas9 (red) expression in neurons (Map1b, NeuN; arrows) but not in astroglia (GFAP, arrowheads). Co-expressioin of HA-SpCas9 with GFP-KASH is shown (bottom). Nuclei were labeled with DAPI (blue). Scale bars, 20 μm. (**d**) Schematic overview of GFP-labeling. Enhanced green fluorescent protein (GFP) fused to the nuclear transmembrane KASH domain and integration of GFP-KASH to the outer nuclear membrane is illustrated. (**e**) Co-infection efficiency calculation, showing populations of cell expressing both HA-SpCas9 and GFP-KASH (n=973 neurons from 3 cultures; error bars: s.e.m). (**f**) Cells were stained with LIFE/DEAD® kit 7 days after virus delivery. Quantification of $DAPI^+$ and dead ($DEAD^+$) cells (control n=518 $DAPI^+$ nuclei; SpCas9/GFP-KASH n=1003 $DAPI^+$ nuclei from 2 cultures; error bars: s.e.m). (ITR – inverted terminal repeat; HA – hemagglutinin tag; NLS – nuclear localization signal; spA – synthetic polyadenylation signal; U6 – PolIII promoter; sgRNA – single guide RNA; hSyn – human synapsin 1 promoter; GFP- green fluorescent protein; KASH - Klarsicht, ANC1, Syne Homology nuclear transmembrane domain;

bGH pA – bovine growth hormone polyadenylation signal; WPRE – Woodchuck Hepatitis virus posttranscriptional regulatory element).

[00194] Figure 68 (Example 38) shows targeting of *Mecp2* in Neuro-2a cells. (a) *Mecp2* targeting sequences and corresponding protospacer adjacent motifs (PAM). (b) Evaluation of 6 *Mecp2* sgRNAs co-transfected with SpCas9 into Neuro-2a cells. Locus modification efficiencies were analyzed 48 h after transfection using SURVEYOR™ assay.

[00195] Figure 69 (Example 38) shows CRISPR-SpCas9 targeting of *Mecp2* in primary cortical neurons. (a) Immunofluorescent staining of MeCP2 (red) in cultured neurons 7 days after AAV-CRISPR transduction (green, GFP-KASH). Nuclei were labeled with DAPI (blue). Scale bar, 20 μm. (**b**) Evaluation of *Mecp2* locus targeting using SpCas9 or dSpCas9, together with *Mecp2* sgRNA or control (targeting bacterial *lacZ* gene) sgRNA, using SURVEYOR™ assay gel. (**c**) Quantification of MeCP2 positive nuclei in targeted population of neurons (GFP^+). (**d**) Western blot of MeCP2 protein levels after CRISPR-SpCas9 targeting of *Mecp2* locus and quantification of MeCP2 protein levels (*t*-test, $^{**}p<0.001$, n=5 from 3 cultures, error bars: s.e.m).

[00196] Figure 70 (Example 38) shows morphological changes in dendritic tree of neurons after SpCas9-mediated MeCP2 knockdown *in vitro*. (a) Reduced complexity of dendritic tree in neurons after CRISPR-SpCas9 targeting of *Mecp2* locus. Scale bar, 20 μm. (b) Changes in dendritic spines morphology in neurons targeted with SpCas9 and *Mecp2* sgRNA. Scale bar, 10 μm. Morphology of cells was visualized with co-transfection with mCherry construct. Cells for morphology analysis were chosen based on the result of Mecp2 staining. (c) Dendritic tree morphology assessed with number of dendritic ends and (d) Sholl analysis (*t*-test, $^{***}p<0.0001$, n=40 from 2 cultures). (e) Spine density quantification (*t*-test, $^{***}p<0.0001$, n=40 from 2 cultures, error bars: s.e.m).

[00197] Figure 71 (Example 38) shows RNAseq of neuronal nuclei from control animals and SpCas9-mediated Mecp2 knockdown. Box plot presenting the number of detected genes across the RNA-seq libraries (19 libraries each of 100 nuclei taken from control sgRNA or *Mecp2* sgRNA transduced nuclei; n=4 animals/group) per quantile of expression level. All genes are divided to 10 quantiles by their mean log2(TPM+1) expression level, then for each quantile the number of genes that are detected (log2(TPM+1)>2) was counted in each sample. The three

target sequences shown are SEQ ID NO: ____ , SEQ ID NO: ____ and SEQ ID NO: ____, for Dnmt3a, Dnmt1 and Dnmt3b, respectively.

[00198] Figure 72 (Example 38) shows multiplex genome targeting of DNMT family members *in vitro*. (**a**) *Dnmt3a*, *Dnmt1* and *Dnmt3b* targeting sequences and corresponding protospacer adjacent motifs (PAM). (**b**) SURVEYOR™ nuclease assay analysis of Neuro-2a cells 48 hours after transfection with SpCas9 and DNMT 3xsgRNA vector targeting *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Efficient genome editing of all three targeted genes is shown.

[00199] Figure 73 (Example 38) shows next generation sequencing of targeted *Dnmt3a*, *Dnmt1* and *Dnmt3b* loci. Examples of sequencing results of mutated *Dnmt3a* (**a**), *Dnmt1* (**b**) and *Dnmt3b* (**c**) loci after *in vivo* delivery of SpCas9 and DNMT 3xsgRNA into the mouse dentate gyrus. Green: wild-type sequence, red dashes: deleted bases, red bases: insertion or mutations. Red arrowheads indicate CRISPR-SpCas9 cutting site. The full sequences used in this figure are provide as SEQ ID NO: , SEQ ID NO: , and SEQ ID NO: for the Dnmt3a, the Dnmt1 and the Dnmt3b loci, respectively. They are:

[00200] SEQ ID NO: (Dnmt3a):

CCT CCG TGT CAG CGA CCC ATG CCA A

[00201] SEQ ID NO: (Dnmt1):

CCA GCG TCG AAC AGC TCC AGC CCG

[00202] SEQ ID NO: (Dnmt3b)

AGA GGG TGC CAG CGG GTA TAT GAG G

[00203] Figure 74 shows SaCas9 protein sequences are codon optimized ("reopt") and have their ubiquitination signals removed ("reopt(Ub)") for enhanced expression. Protein blots against FLAG- and HA-tagged SaCas9 show approximately 2-fold increased expression of optimized SaCas9 (reopt, #2-4) relative to the original constructs (#0, 5, and 6), and similar level as SpCas9 (SpCas9 330, top bar left panel; SpCas9 414, top bar right panel). The addition of 3xHA tagging (right panel #6) improves detection signal over the 1xHA tag (right panel #5) by ~2 fold.

[00204] Figure 75 shows indel efficiency using sgRNAs transcribed by U6 promoter as-is (grey, left hand bar for each number of ntds) or appending a "G" (blue, right hand bar for each number of ntds and with a thicker border) to 5'-most position of sgRNA for SaCas9. Total sgRNA spacer lengths (including G) are indicated on the x-axis. Graph represents with aggregated data from 5 sgRNAs.

[00205] Figure 76 shows optimization of sgRNA spacer length (x axis). Graphs show indel formation with different lengths of sgRNA spacer in HEK (left) and Hepa (right) cells.

[00206]

[00207] The figures herein are for illustrative purposes only and are not necessarily drawn to scale.

DETAILED DESCRIPTION OF THE INVENTION

[00208] With respect to general information on CRISPR-Cas Systems: Reference is made to US provisional patent applications 61/758,468; 61/802,174; 61/806,375; 61/814,263; 61/819,803 and 61/828,130, filed on January 30, 2013; March 15, 2013; March 28, 2013; April 20, 2013; May 6, 2013 and May 28, 2013 respectively. Reference is also made to US provisional patent application 61/836,123, filed on June 17, 2013. Reference is also made to US provisional patent applications 61/736,527 and 61/748,427, filed on December 12, 2012 and January 2, 2013, respectively. Reference is also made to US provisional patent application 61/791,409, filed on March 15, 2013. Reference is also made to US provisional patent application 61/799,800, filed March 15, 2013. Reference is also made to US provisional patent applications 61/835,931, 61/835,936, 61/836,127, 61/836, 101, 61/836,080 and 61/835,973, each filed June 17, 2013. Further reference is made to US provisional patent applications 61/862,468 and 61/862,355, filed on August 5, 2013; 61/871,301 filed on August 28, 2013; 61/960,777 filed on September 25, 2013 and 61/961, 980 filed on October 28, 2013. Each of these applications, and all documents cited therein or during their prosecution ("appln cited documents") and all documents cited or referenced in the appln cited documents, together with any instructions, descriptions, product specifications, and product sheets for any products mentioned therein or in any document therein and incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention. All documents (e.g., these applications and the appln cited documents) are incorporated herein by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

[00209] Also with respect to general information on CRISPR-Cas Systems, mention is made of:

- *Multiplex genome engineering using CRISPR/Cas systems*. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., & Zhang, F. *Science* Feb 15;339(6121):819-23 (2013);
- *RNA-guided editing of bacterial genomes using CRISPR-Cas systems*. Jiang W., Bikard D., Cox D., Zhang F, Marraffini LA. Nat Biotechnol Mar;31(3):233-9 (2013);
- *One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering*. Wang H., Yang H., Shivalila CS., Dawlaty MM., Cheng AW., Zhang F., Jaenisch R. Cell May 9;153(4):910-8 (2013);
- *Optical control of mammalian endogenous transcription and epigenetic states*. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F. Nature. 2013 Aug 22;500(7463):472-6. doi: 10.1038/Nature12466. Epub 2013 Aug 23;
- *Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity*. Ran, FA., Hsu, PD., Lin, CY., Gootenberg, JS., Konermann, S., Trevino, AE., Scott, DA., Inoue, A., Matoba, S., Zhang, Y., & Zhang, F. *Cell* Aug 28. pii: S0092-8674(13)01015-5. (2013);
- *DNA targeting specificity of RNA-guided Cas9 nucleases*. Hsu, P., Scott, D., Weinstein, J., Ran, FA., Konermann, S., Agarwala, V., Li, Y., Fine, E., Wu, X., Shalem, O., Cradick, TJ., Marraffini, LA., Bao, G., & Zhang, F. *Nat Biotechnol* doi:10.1038/nbt.2647 (2013);
- *Genome engineering using the CRISPR-Cas9 system*. Ran, FA., Hsu, PD., Wright, J., Agarwala, V., Scott, DA., Zhang, F. Nature Protocols Nov;8(11):2281-308. (2013);
- *Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells*. Shalem, O., Sanjana, NE., Hartenian, E., Shi, X., Scott, DA., Mikkelson, T., Heckl, D., Ebert, BL., Root, DE., Doench, JG., Zhang, F. *Science* Dec 12. (2013). [Epub ahead of print];
- *Crystal structure of cas9 in complex with guide RNA and target DNA*. Nishimasu, H., Ran, FA., Hsu, PD., Konermann, S., Shehata, SI., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. *Cell* Feb 27. (2014). 156(5):935-49;
- *Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells*. Wu X., Scott DA., Kriz AJ., Chiu AC., Hsu PD., Dadon DB., Cheng AW., Trevino AE., Konermann S., Chen S., Jaenisch R., Zhang F., Sharp PA. *Nat Biotechnol*. (2014) Apr 20. doi: 10.1038/nbt.2889, and

- *Development and Applications of CRISPR-Cas9 for Genome Engineering*, Hsu et al, Cell 157, 1262-1278 (June 5, 2014) (Hsu 2014),

each of which is incorporated herein by reference, and discussed briefly below:

- Cong *et al.* engineered type II CRISPR/Cas systems for use in eukaryotic cells based on both *Streptococcus thermophilus* Cas9 and also *Streptoccocus pyogenes* Cas9 and demonstrated that Cas9 nucleases can be directed by short RNAs to induce precise cleavage of DNA in human and mouse cells. Their study further showed that Cas9 as converted into a nicking enzyme can be used to facilitate homology-directed repair in eukaryotic cells with minimal mutagenic activity. Additionally, their study demonstrated that multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several at endogenous genomic loci sites within the mammalian genome, demonstrating easy programmability and wide applicability of the RNA-guided nuclease technology. This ability to use RNA to program sequence specific DNA cleavage in cells defined a new class of genome engineering tools. These studies further showed that other CRISPR loci are likely to be transplantable into mammalian cells and can also mediate mammalian genome cleavage. Importantly, it can be envisaged that several aspects of the CRISPR/Cas system can be further improved to increase its efficiency and versatility.
- Jiang *et al.* used the clustered, regularly interspaced, short palindromic repeats (CRISPR)–associated Cas9 endonuclease complexed with dual-RNAs to introduce precise mutations in the genomes of *Streptococcus pneumoniae* and *Escherichia coli*. The approach relied on dual-RNA:Cas9-directed cleavage at the targeted genomic site to kill unmutated cells and circumvents the need for selectable markers or counter-selection systems. The study reported reprogramming dual-RNA:Cas9 specificity by changing the sequence of short CRISPR RNA (crRNA) to make single- and multinucleotide changes carried on editing templates. The study showed that simultaneous use of two crRNAs enabled multiplex mutagenesis. Furthermore, when the approach was used in combination with recombineering, in *S. pneumoniae*, nearly 100% of cells that were recovered using the described approach contained the desired mutation, and in *E. coli*, 65% that were recovered contained the mutation.

- Konermann *et al.* addressed the need in the art for versatile and robust technologies that enable optical and chemical modulation of DNA-binding domains based CRISPR Cas9 enzyme and also Transcriptional Activator Like Effectors
- As discussed in the present specification, the Cas9 nuclease from the microbial CRISPR-Cas system is targeted to specific genomic loci by a 20 nt guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. To address this, Ran *et al.* described an approach that combined a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. The authors demonstrated that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.
- Hsu *et al.* characterized SpCas9 targeting specificity in human cells to inform the selection of target sites and avoid off-target effects. The study evaluated >700 guide RNA variants and SpCas9-induced indel mutation levels at >100 predicted genomic off-target loci in 293T and 293FT cells. The authors that SpCas9 tolerates mismatches between guide RNA and target DNA at different positions in a sequence-dependent manner, sensitive to the number, position and distribution of mismatches. The authors further showed that SpCas9-mediated cleavage is unaffected by DNA methylation and that the dosage of SpCas9 and sgRNA can be titrated to minimize off-target modification. Additionally, to facilitate mammalian genome engineering applications, the authors reported providing a web-based software tool to guide the selection and validation of target sequences as well as off-target analyses.
- Ran *et al.* described a set of tools for Cas9-mediated genome editing *via* non-homologous end joining (NHEJ) or homology-directed repair (HDR) in mammalian cells, as well as generation of modified cell lines for downstream functional studies. To minimize off-target cleavage, the authors further described a double-nicking strategy using the Cas9 nickase mutant with paired guide RNAs. The protocol provided by the authors

experimentally derived guidelines for the selection of target sites, evaluation of cleavage efficiency and analysis of off-target activity. The studies showed that beginning with target design, gene modifications can be achieved within as little as 1–2 weeks, and modified clonal cell lines can be derived within 2–3 weeks.

- Shalem *et al.* described a new way to interrogate gene function on a genome-wide scale. Their studies showed that delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library targeted 18,080 genes with 64,751 unique guide sequences enabled both negative and positive selection screening in human cells. First, the authors showed use of the GeCKO library to identify genes essential for cell viability in cancer and pluripotent stem cells. Next, in a melanoma model, the authors screened for genes whose loss is involved in resistance to vemurafenib, a therapeutic that inhibits mutant protein kinase BRAF. Their studies showed that the highest-ranking candidates included previously validated genes NF1 and MED12 as well as novel hits NF2, CUL3, TADA2B, and TADA1. The authors observed a high level of consistency between independent guide RNAs targeting the same gene and a high rate of hit confirmation, and thus demonstrated the promise of genome-scale screening with Cas9.
- Nishimasu *et al.* reported the crystal structure of *Streptococcus pyogenes* Cas9 in complex with sgRNA and its target DNA at 2.5 A° resolution. The structure revealed a bilobed architecture composed of target recognition and nuclease lobes, accommodating the sgRNA:DNA heteroduplex in a positively charged groove at their interface. Whereas the recognition lobe is essential for binding sgRNA and DNA, the nuclease lobe contains the HNH and RuvC nuclease domains, which are properly positioned for cleavage of the complementary and non-complementary strands of the target DNA, respectively. The nuclease lobe also contains a carboxyl-terminal domain responsible for the interaction with the protospacer adjacent motif (PAM). This high-resolution structure and accompanying functional analyses have revealed the molecular mechanism of RNA-guided DNA targeting by Cas9, thus paving the way for the rational design of new, versatile genome-editing technologies.
- Wu *et al.* mapped genome-wide binding sites of a catalytically inactive Cas9 (dCas9) from *Streptococcus pyogenes* loaded with single guide RNAs (sgRNAs) in mouse embryonic stem cells (mESCs). The authors showed that each of the four sgRNAs tested

targets dCas9 to between tens and thousands of genomic sites, frequently characterized by a 5-nucleotide seed region in the sgRNA and an NGG protospacer adjacent motif (PAM). Chromatin inaccessibility decreases dCas9 binding to other sites with matching seed sequences; thus 70% of off-target sites are associated with genes. The authors showed that targeted sequencing of 295 dCas9 binding sites in mESCs transfected with catalytically active Cas9 identified only one site mutated above background levels. The authors proposed a two-state model for Cas9 binding and cleavage, in which a seed match triggers binding but extensive pairing with target DNA is required for cleavage.

- Hsu 2014 is a review article that discusses generally CRISPR-Cas9 history from yogurt to genome editing, including genetic screening of cells, that is in the information, data and findings of the applications in the lineage of this specification filed prior to June 5, 2014. The general teachings of Hsu 2014 do not involve the specific models, animals of the instant specification.

[00210] The invention relates to the engineering and optimization of systems, methods and compositions used for the control of gene expression involving sequence targeting, such as genome perturbation or gene-editing, that relate to the CRISPR-Cas system and components thereof. In advantageous embodiments, the Cas enzyme is Cas9.

[00211] The CRISPRS-Cas polynucleotide sequence is generally referred to herein as the guide, or even as guide RNA (sgRNA), although it will be appreciated that this terminology was not as commonplace previously. Furthermore, reference is made herein to a CRISPR-Cas9 system, although it will be appreciated that this is a broad reference to any Cas, provided it has a nuclease function either to induce a DSB, a nick or a double nick, although Cas9 is preferred and SaCas9 is particularly preferred.

[00212] Some of the key points in the present liver data are summarised below an flow through to post-mitotic cells in general, as liver cells are typically post-mitotic:

[00213] **AAV2/8**

[00214] Preferred delivery for the CRISPR-Cas system is through a viral vector. This vector may be a lentiviral vector or an AAV vector, as discussed at some length herein. Whet we have particularly showed is that AAV is a preferred example of a viral vector. Within that, we gone on to show that AAV8 and in particular AAV2/8 (AAV8 packaged with AAV2 packaging signal ITR) is useful in delivery to the liver, especially in vivo.

[00215] Phenotypic Changes seen In Vivo

[00216] As discussed elsewhere, we have been able to show, in vivo, that phenotypic change can be detected. This is a significant step forward as a deficiency often levelled at RNAi is that no lasting effect is seen. With the present invention, phenotypic change can be seen in the liver for the first time. A preferred arrangement to achieve this is to use that in Example 36. Important elements of this are preferred alone or in combination, namely:

- Sa Cas9;
- Use of a chimeric guide RNA comprising the guide, tracr sequence and tracr mate;
- For the tracr sequence, Sa tracr is preferable to recruit the Sa Cas9;
- AAV8 or more preferably AAV2/8;
- For experimental purposes, Rosa26 is a useful negative control;
- Although use of the CMV promoter in an AAV vector is helpful, use of a liver-specific promoter (for liver targeting) such as TBG is particularly effective;
- The target or targets may be wide-ranging as CRISPR has been shown to have broad applicability across targets, once they guides are successfully delivered and the Css9 enzymes are suitably expressed. However, preferred targets in the liver (against which the guides may be designed) nevertheless include one or more of: PCSK9; Hmgcr; SERPINA1; ApoB; and.or LDL.

[00217] Accordingly, in some embodiments it is particularly preferred that the Cas enzyme is an Sa Cas9. Preferably, the CRISPRS-Cas polynucleotide sequence is chimeric and preferably includes an Sa tracr where the Cas9 is an Sa Cas9. A viral vector may be used which is preferably AAV2/8. Furthermore, a liver-specific promoter is ideal and a preferred example is TBG. All of these may be used in combination to provide a chimeric CRISPRS-Cas polynucleotide sequence including an Sa tracr, wherein the Cas9 is an SaCas9, and the vector is AAV2/8, with at least the Cas9 under the control of a liver-specific such as TBG. Any of the above targets may be sued with this system, in particular ApoB due to its importance in obesity.

[00218] Yin and Anderson's later Nature Biotech Paper (NBT 2884, referenced herein) provides further support for the in vivo phenotypic changes that we have already shown.

[00219] Additional data that we provide in then adds further support by demonstrating efficient in vivo editing of somatic liver tissue via Cas9. Moreover, delivery via AAV2/8 and the use of an SaCas9 again show the usefulness of this particular approach in vivo. The preferred ApoB was again targeted.

[00220] Later examples 36 and 37 show excellent in vivo data for effecicacy in inducing a phenotypic change in vivo: specifically ApoB, a lipid metabolism gene, whilst Example 38 shows the applicability of the technique to post-mitotic cells, of which liver is an important example. Example 39 shows that multiple epitope tags are preferable for detection purposes.

[00221] Although viral vectors are preferred, in some embodiments, the use of cell penetrating peptides is a viable alternative and so is also preferred.

[00222] Example 36 showed that both genotypic and, crucially, phenotypic changes are seen with CRISPR-Cas systems. Not only that, but the CRISPR-Cas9 system was effective at inducing a phenotypic change *in vivo*. Specifically, the target was ApoB, a lipid metabolism gene. What is so encouraging is that ApoB can be said to be the "gold-standard" in liver delivery, and is widely used in mouse models of obesity. Liver is a preferred post-mitotic cell in some embodiments, although it may also be excluced in others. Either way, this work provides proof of principle that a phenotypic change is seen, even *in vivo*, and this is equally applicable to other post-mitotic cells. Indeed, Example 38 provides further proof of this in a separate tissue, brain, with post-mitotic neurones. Delivery in Example 36 was via intravenous injection. An AAV vector was used, as well as a Liver-specific promoter (TBG) for Cas9. Delivery through expression from a viral vector as seen here is an improvement over Anderson/Yin's (NBT 2884) use of hydrodynamic delivery as the delivery method, because hydrodynamic delivery requires several mls of fluid to be injected which is stressful on the murine body and can be fatal. Hydrodynamic delivery is best suited for delivery of plasmid (naked) DNA, whereas we have shown that packaging the guide and Cas9 sequences within a viral delivery vector is preferable in terms of greatly increased efficiency. Indeed, only relatively small volumes need to be introduced, and this can be done intravenously (i.v.), which is likely to be much more acceptable therapeutically. What was particularly encouraging was that not only was a genotypic change seen in a "gold-standard" gene for liver such as ApoB, but phenotypic changes were also recorded. Previous work with PCSK9 had shown genotypic, but not phenotypic changes, so the phenotypic changes seen with ApoB validate the plausibility of CRISPR delivery to, and its ability to effect phenotypic change in, the Liver. This is in combination with the more therapeutically acceptable means of delivery (i.v. compared to hydrodynamic delivery). As such, viral delivery of CRISPR-Cas9 system (guide and Cas9) is preferred, especially intravenously).

[00223] Potential targets include: PCSK9, HMGCR, APOB, LDLR, ANGPTL3, F8, F9/FIX, AAT, FAH, HPD, TAT, ATP7B, UGT1A1, OTC, ARH.

[00224] Accordingly, provided are methods of inducing a phenotypic change *in vivo* comprising administering the CRISPR-Cas9 system to the target cells, for instance the liver. Suitable delivery routes are described herein but i.v. injection is preferred in some embodiments. Viral vectors are preferred, particularly AAV, in particular AAV serotype 2/8.

[00225] Also provided is a CRISPR-Cas9 system comprising one or more guides targeting lipid metabolism genes, for instance ApoB. Methods of treating obesity, comprising administering said CRISPR-Cas9 system, are also envisaged. A mouse model comprising one or more liver gene knock down(s), especially of lipid metabolism gene(s), for instance including ApoB, are preferred.

[00226] Liver specific promoters for the Cas9 will be apparent but may include those mentioned herein. A preferred example is TBG.

[00227] As shown in Example 37, the guide may be 18-23 nucleotides in length. It may be 18-22, or 19-22, or 18-21, 20-22, but is preferably 22 and most preferably 21 nucleotides in length.

[00228] Also provided is proof of principle of successful packaging of a guide sequence into a SaCas9 intron. Accordingly, the CRISPR-Cas9 systems, wherein one or more guide sequences are packaged (positioned or inserted) into a Cas9 intron, are preferred.

[00229] The H1 promoter can be used and may be preferable in some circumstances.

[00230] Expanding on the work by Ran (Cell, 154, 21 Aug 2013), the degree of overlap in the dual guide approach using a D10A Double-Nickase was investigated. Optimal results were shown between -5 and +1 bp (5' to 5'). Accordingly, it is prefer to use a dual guide approach to minimise off target effects. These preferably overlap, or come close to overlapping, at their 5' ends, on different stands of DNA at the genomic target . Preferably, the overlap is in the range of -5 to +1 bp. In these instances, it will be appreciated that the Cas9 is a double nickase, such as the preferred D10A variant.

[00231] Multiple or repeat epitope Tags are preferred for the Cas9. In particular, a triple epitope tag was shown in Example 39 to improve detection. The tag is preferably a repeat, more preferably a triple repeat. HA is a preferred Cas9 epitope tag. A triple HA epitope tag is, therefore, preferred in some embodiments.

[00232] Example 38 presents the following specific points. It provides:

- a first demonstration of successful AAV-mediated Cas9 delivery in vivo as well as efficient genome modification in post-mitotic neurons;
- for the development of a nuclear tagging technique which enables easy isolation of neuronal nuclei from Cas9 and sgRNA-expressing cells;
- a demonstration of applications toward RNAseq analysis of neuronal transcriptome;
- how electrophysiological studies and other techniques can be integrated with Cas9-mediated genome perturbation to determine phenotypic changes; and
- a demonstration of multiplex targeting and the ability to study gene function on rodent behavior using Cas9-mediated genome editing.

[00233] Based on this, it can be seen that Example 38 provides further proof of concept in two main areas:

- in the understanding and testing of gene function, including the creation and testing of models; and
- in gene therapy.

[00234] An additional aspect, discussed further below, is in relation to a method for Nuclear Tagging.

[00235] It will be appreciated that reference to CRISPR-Cas9 systems herein is a short-hand for referring to the Cas9 enzymes provided herein in combination with the guides or guides used to target one or more genomic sequences. Reference to guide(s) includes sgRNA, as well as the chimeric polynucleotide sequences described herein which comprises the guide sequences capabale of hybridising to target sequences in the genome of the subject, a tracr mate sequence and a tracr sequence.

[00236] The data essentially shows phenotypic changes resulting from gene knock down using two separate CRISPR-Cas9 systems according to the invention (guide RNA in combination with a Cas9 enzyme), in this case to successfully perturb gene function. The chosen tissue was brain tissue, but the results provide proof of principle for a wide range of post-mitotic tissues. This is an important distinction, because previous work has focussed on dividing cells (i.e. pre-mitotic).

[00237] In other words, whereas SpCas9 has been broadly used to engineer dividing cells, we demonstrate that SpCas9 can also be used to engineer the genome of postmitotic neurons. This is done with high efficiency via NHEJ-mediated indel generation to create knock downs, but therapeutic uses involving correction via the HDR mechanism (upon provision of a repair template) are also envisaged. Both are dependent on successful delivery and functional expression of the Cas9 and RNA guide or guides, which is shown here.

[00238] The fact that genotypic changes induced by the CRISPR-Cas9 systems then results in a phenotypic change is also important for both of the above areas (gene function and gene therapy).

[00239] The first CRISPR-Cas9 system employed guide sequences directed at (targeting) *Mecp2*. A dual vector CRISPR-Cas9 system, with one vector comprising the guide and one comprising the Cas9, was successfully employed showing further proof of principle for such dual vector systems. The dual vector CRISPR-Cas9 system was successfully delivered, via stereotactical injection, to two separate locations in the brain, specifically the Hippocampal dentate gyrus and the visual cortex. In both cases, gene perturbation of the same gene, *Mecp2*, was seen indicating that the dual vector system was successfully delivered and acted as expected, with transcription and functional activity in the Cas9 enzyme (in this case an SpCas9), and successful recruitment of the Cas9 to the genomic target sequence by the guide sequences.

[00240] AAV-mediated in vivo delivery of SpCas9 and sgRNA provides a rapid and powerful technology for achieving precise genomic perturbations within intact neural circuits. AS such, the vector used was an AAV vector, adding further evidence for their use in general and in dual vector CRISPR-Cas9 systems in particular, especially in post-mitotic cells and tissues, and in particular in the brain.

[00241] It will of course be appreciated that the choice of promoter is important in achieving expression from the CRISPR-Cas9 system, in particular the Cas9 or both guide(s) and Cas9. Suitable examples for cell and cell lifecycle stage specificity can be determined from the literature. Nevertheless, we provide some non-limiting examples: TBG, a liver-specific promoter and is used here to drive expression of SaCas9; the H1 promoter; a truncated H1 promoter; the U6 promoter. Also, as guides do not necessarily need a specific promoter, one or more guides could similarly packaged into a/the Cas9 intron

[00242] The second CRISPR-Cas9 system used included a multiplex approach. One key advantage of the SpCas9 system is its ability to facilitate multiplex genome editing. This second system successfully targeted three or more genes from the same family (in this case, *Dmnt1, 3a* and *3b*) by including suitable guides and resulted in stable knockouts of multiple genes. This has broad implications for probing the function of not only individual genes, but also whole gene families, in the tissues of living animals. This is particularly important for tissues such as the brain where this has not been possible before, or could only be achieved through long years of classical genetics. We have shown that single or multiple gene perturbation (even complete knock down) can occur in post-mitotic cells in a normal animal. However, this could equally apply to a model organism (for instance one *already* carrying a gene mutation or perturbation or comprising altered expression of some kind) or a transgenic organism, lending a quick alternative to existing methods of producing model organisms and using model organisms to understand gene function. Further guides (and/or whole CRISPR-Cas9 systems) could be employed to make later rounds of gene perturbations and/or reinstatements (restoring gene function for instance by correction of the perturbed gene through provision, for instance, of a repair template, such as ssDNA suitable for HDR) within the same organism.

[00243] In fact, in general, SpCas9-mediated targeting of single or multiple genes can recapitulate morphological, electrophysiological, and behavioral phenotypes observed using classical, more time-consuming genetic mouse models.

[00244] Alternatively to knocking down whole gene families or related genes, the data here also provides proof of principle that simultaneous knock down or three or more unrelated genes is equally feasible. This is applicable across all tissues, but is particularly strongly presented in respect of post-mitotic tissues, especially the brain.

[00245] Another useful aspect of the work is that it showed that a combined, or integrated, approach could be taken to studying gene function, employing CRISPR to effect a genotypic change and then using classical tools such as electrophysiology (particularly relevant to brain and CNS tissue), biochemical, sequencing, electrophysiological, and/or behavioral readouts to establish what, if any, phenotypic changes result from the genotypic change induced by the CRISPR-Cas9 system. For example in the brain, this allows us to study the function of individual as wells as groups of genes in neural processes and their roles in brain disorders *in vivo*.

[00246] The successful perturbation of genes in this work is equally applicable to correction or reinstatement of gene function, i.e. the use of CRISPR-Cas9 systems in gene therapy. This is particularly in relation to targeting post-mitotic cells, especially the brain.

[00247] In general, the use of CRISPR-Cas9 systems show improvements over existing techniques such as Zn fingers, which take a long time to design and produce and cannot multiplex and shRNA, which has too many off-target effects whereas CRISPR off-target effects can be minimised by using double-nicakse approaches.

[00248] **Targeting of Tissues**

[00249] The work herein supports the use of CRISPR-Cas9 systems to target genes in post-mitotic cells through delivery of the CRISPR-Cas9 system to the appropriate location (i.e. to cells within the organs or tissues of interest). Preferred tissues are within the following organs:

- Kidney;
- Digestive System including the stomach, pancreas, duodenum, ileum and/or colon;
- Heart;
- Lung;
- Brain, in particular neurones, and/or CNS in general;
- Eye, including retinal tissue;
- Ear, including the inner ear;
- Skin;
- Muscle;
- Bone; and/or
- Liver in general.

[00250] It will be appreciated that many of the above may comprise pre-mitotic cells, but that this aspect of the invention is directed to post-mitotic cells or tissues within those organs.

[00251] It is preferred that the organ is the kidney or the brain. Within the brain, the data specifically shows delivery to the Hippocampal dentate gyrus and the visual cortex, which are preferred tissues, although other tissues including any one or more of the following: primary motor cortex, primary auditoty cortex, primary somatosensory cortex, cerebellum, main olfactory bulb, prefrontal cortex, endopiriform nucleus, amygdala, substantia nigra, striatum, pallidum,

thalamus, hypothalamus, Parabranchial nucleus, superior olivary complex, cochlear nuclei, mammillary nuclei, are also preferred in some embodiments.

[00252] Cells from the brain, and neurons in particular, are especially preferred.

[00253] The choice of promoter to drive expression of the CRISPR-Cas9 system, especially the Cas9 is important, as mentioned above. To be considered when selecting a promoter are the cell cycle stage (early/late) and the cell type as promoters will be specific for one of more cell types and cell-cycle stages. Suitable promoters may include any one or more of the following, in some embodiments:

Cell Type	**Promoter**
Excitatory neurons	CamkII
Fast spiking interneurons	Parvalbumin
All interneurons	vGAT
Dopaminoceptive neurons	DR1
Dopaminoceptive neurons	DR2
Astroglia	GFAP
Activated neurons	Arc

[00254] The dual vector CRISPR-Cas9 system used in targeting the brain, in particular the Hippocampal dentate gyrus, packaged SpCas9 and sgRNA expression cassettes on two separate viral vectors. Cas9s, in particular SpCAs9s, are therefore preferably delivered by adenoviral vectors, especially AAV (i.e. as AAV-SpCas9). Guides are preferably delivered as sgRNA expression cassettes by adenoviral vectors, especially AAV (i.e. as AAV-SpGuide). A preferred route for this tissue (the Hippocampal dentate gyrus) and for the brain in general is stereotactical injection.

[00255] **Understanding And Testing Of Gene Function, And The Creation And Use Of Models To So Do**

[00256] Conditions which could be looked at include Huntingdon's, but essentially include any condition found in post-mitotic cells and especially those that may benefit from being studied *in vivo* or lack a useful model.

[00257] As mentioned above, CRISPR-Cas9 systems can be used to interrogate the function of one or more genes in post-mitotic cells. This may be achieved through delivery and expression of the CRISPR-Cas9 system to the post-mitotic cell, wherein the guide(s) of the CRISPR-Cas9 system are designed to recruit the Cas9 to the genomic target or targets of interest. Equally, where the Cas9 is already comprised within the post-mitotic cell, protein (transcribed) form, then delivery of the guides to the post-mitotic cell will suffice. Where the Cas9 is already comprised within the post-mitotic cell, in polynucleotide (untranscribed), then delivery of the guides to the post-mitotic cell as well as induction of transcription of the Cas9 polynucleotide will be necessary. Having the Cas9 under the control of an inducible or repressible promoter, such as the tet (tetracycline) on-off system may be advantageous here.

[00258] One aspect that is particularly promising is the integration of CRISPR techniques with phenotypic assays to determine the phenotypic changes, if any, resulting from gene perturbations, especially knock downs. For instance, Example 40 shows what can be achieved with targeted genomic perturbations coupled with quantitative readouts to provide insights into the biological function of specific genomic elements. In particular, Cas9-mediated in vivo genome editing in the brain can also be coupled with electrophysiological recording to study the effect of genomic perturbation on specific cell types or circuit components. In a broader sense, use of the CRISPR-Cas9 systems (to provide Cas9-mediated genomic perturbations) can be combined with biochemical, sequencing, electrophysiological, and behavioral analysis to study the function of the targeted genomic element.

[00259] Thus in one aspect, there is provided: a method of interrogating the function of one or more genes in a post-mitotic cell, comprising: inducing a deficient genotype or gene knock down as described below; and determining changes in expression of the one or more genes in the condition thereby interrogating the function of the one or more genes.

[00260] Optionally, the method may also include: transplanting the second population of cells into the subject thereby inducing the condition associated with the deficient genotype or gene knock down. This may be prior to the determining step.

[00261] The following applies broadly to appropriate aspects of the invention. The cell may be in a subject, such as a human, animal or model organism, so that gene function is interrogated *in vivo*. However, it is also envisaged that the cell may be ex vivo, for instance in a cell culture or in a model organ or organoid. In some embodiments, the method may include isolation a first

population of cells from the subject, optionally culturing them and transducing them with one or more CRISPR-Cas9 systems. Further optional culturing may follow. Transplantation of the transduced cells back into the subject may then occur.

[00262] The cell may be from any of the tissues or organs described herein. The brain is one preferred example, providing for said method of interrogating the function of one or more genes, wherein the post-mitotic cell is a brain cell, for instance a neurone. Particularly in vivo, this allows for the interrogation of gene function on animal behaviour. The animal is preferably a mammal, for instance a rodent. Given the complexity of the nervous system, which consists of intricate networks of heterogeneous cell types, being able to efficiently edit the genome of neurons in vivo enables direct testing of gene function in relevant cell types embedded in native contexts. This is supported by our data where knockout mice showed impaired memory consolidation when tested under trained context conditions Results herein demonstrate that CRIPSR-Cas9-mediated knockout of DNMT family members in dentate gyrus neurons is sufficient to probe the function of genes in behavioral tasks

[00263] This shows the versatility of Cas9s in facilitating targeted gene knockout in the mammalian brain in vivo, for studying genes functions and, in particular, for dissection of neuronal circuits. Introducing stable knockouts of multiple genes in the brain of living animals will have potentially far-reaching applications, such as causal interrogation of multigenic mechanisms in physiological and neuropathological conditions.

[00264] The specifics of this work are that Applicants chose the mouse Mecp2 promoter (235 bp, pMecp2)7 and a minimal polyadenylation signal (48 bp, spA) based on their ability to achieve sufficient levels of SpCas9 expression in cultured primary mouse cortical neurons. Mecp2 gene, plays a principal role in Rett syndrome, a type of autism spectrum disorder. To target Mecp2, we first designed several sgRNAs targeting exon 3 of the mouse Mecp2 gene and evaluated their efficacy using Neuro-2a cells. The most efficient sgRNA was identified using the SURVEYOR nuclease assay. The delivery was via stereotactical injection of a mixture (1:1 ratio) of high titer AAV-SpCas9 and AAV-SpGuide. We also successfully tested the possibility of multiplex genome editing in the brain Applicants designed a multiplex sgRNA expression vector consisting of three sgRNAs in tandem, along with GFP-KASH for nuclei labelling.

[00265] Thus, also provided are methods of inducing conditions characterised by one or more gene knockdowns in a post-mitotic cell. Examples of such conditions are numerous, but may

include Rett syndrome, as exemplified. Suitable promoters will be apparent, and the Mecp2 promoter is ideal for Rett syndrome. One way to select a promoter to drive expression of the CRISPR-Cas9 system, in particular the Cas9, is to select the promoter for the gene of interest.

[00266] Thus in one aspect, there is provided: a method of inducing conditions characterised by one or more deficient genes (or genotyoes) or gene knockdowns in a post-mitotic cell, comprising:

transducing a first population of cells with a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) one, two, thee, four or more guide sequences capable of hybridizing to three or more target sequences in genome of the subject,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at list one or more nuclear localization sequences (NLSs), wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence direct sequence-specific binding of CRISPR complexes to the target sequence,

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

wherein the CRISPR enzyme alters the genome of the first population of cells to obtain a second population of cells bearing the one or more deficient genes or knocked down genes.

[00267] Optionally, the method may also include: isolating a first population of cells from the subject.

[00268] Optionally, the method may also include: transplanting the second population of cells into the subject thereby inducing the proliferative condition.

[00269] This may involve inducing a non-functional (which include partially non-functional) genotype into the target cell, to thereby provide a model for study (including future restoration of the functional genotype).

[00270] CRISPR-Cas9 systems can also be used to facilitate the study of gene functions in cellular assays by enabling targeted knockout in post-mitotic neurons.

[00271] Methods for delivering nucleotides to neuronal cells are well known and reviewed in The Journal of Neuroscience, by Karra and Dahm (5 May 2010, 30(18): 6171-6177; doi: 10.1523/JNEUROSCI.0183-10.2010). Examples include electrical transfection methods (such as electroporation, nucleofection, and single-cell electroporation); chemical transfection methods (such as Ca2+ phosphate co/precipitation and lipofection); viral delivery (such as Adenoviral, Adeno-Associated Virus (AAV), Lentiviral and Herpes Simplex Virus); and physical transfection methods (such as microinjection and biolistics (DNA-coated gold particles). All of these can be used for delivery of the CRISPR-Cas9 system, but lipofection or viral methods are preferred, especially AAV or Lentiviral.

[00272] **Models**

[00273] Models are provided with single or multiple genes knocked down. An example would be a rodent model for Rett syndrome, a Mecp2 knock down. Others include Dmnt family knock downs, specifically Dmnt1, 3a and 3b knock downs. As such, models studying neurological conditions are provided. All that needs to be done is to identify the target genes of interest, design suitable guide(s) and include these in a suitable CRISPR-Cas9 system and deliver it to the post-mitotic cell(s) whether in vivo or ex vivo, as required. For instance, the models may have altered dendritic tree morphology and/or spine density are provided.

[00274] As mentioned above, models tissues are also provided, such as oraganoids or "Liver on a chip" or non-liver equivalents thereof such as ear, kidney and brain tissues, for instance on a chip or supported in a scaffold. Animal models and model tissues are preferred. These may be already transformed with Cas9 so that they comprise Cas9 in nucleotide or protein form, as mentioned above. These have the advantage that Cas9 does not need to be delivered alongside the guide(s) and this in turn may allow for a much greater degree of (guide) multiplexing to be

accommodated within the delivery vectors. Again, use of inducible or repressible systems such as tet-on or tet-off, may be advantageous here.

[00275] All of these models are obtainable using the CRISPR-Cas9 system as described above. Due to the versatility of the CRISPR-Cas9 system, the range of possible models, whether human, rodent, mammalian or otherwise is hugely diverse and this can be established by simple selection of appropriates guide(s). Methods of creating such models are also provided comprising

[00276] **Gene Therapy**

[00277] The data in Example 40 focuses on gene perturbation, primarily knock down. Gene knock down is likely to be only a small, if important, part of the total quorum of possible applications of CRISPR-Cas9 systems to gene therapy. As already shown in the Yin and Anderson paper (Nature Biotech 2884 published online 30 March 2014), a functional phenotype can be restored following correction of a deficient mutation in hereditary tyrosinemia type I (HTI), an otherwise fatal condition caused by mutation of fumarylacetoacetate hydrolase (FAH) (G to A in the last nucleotide in exon 8) which causes skipping of exon 8 during splicing and results in the formation of a truncated, unstable FAH protein, leading to accumulation of toxic metabolites. Correction of the A mutation back to the wild-type G geneotype resulted in a restored phenotype.

[00278] As such, the approaches taken herein demonstrate that the instant invention can plausibly be applied to gene therapy. In particular, the dual vector approach, the nuclear tagging approach, the specifics of the brain delivery (the form of injection, the promoters and/or viral vectors used), as well as the multiplexing (use of multiple guides for multiple targets either within the same or within different genes) could equally be applied to correctional gene therapy (i.e. where a deficient genotype is corrected) as to the exemplified gene knock down. The main difference between correctional gene therapy and gene knock down is that in order to correct a deficient genotype, such as a point mutation (for instance in Cystic Fibrosis, see ref Schwank *et al*, Cell Stem Cell 13, 653-658 5 Dec 2013), it is advantageous to provide a repair template to stimulate the HDR mechanism and ideally provide a suitable Cas9 nickase as well.

[00279] Accordingly, the present vectors preferably target post-mitotic cells. Where the guide or guides target a deficient genotype, are preferably also provided with a repair template, for instance ssDNA corresponding to the corrected sequence (a genotype providing functional

phenotype). Repair templates are described herein. The Cas9 may be provided in the same or a different vector from the guide or guides. The vectors are preferably viral vectors, more preferably adenoviral vectors and most preferably AAV vectors. Delivery to the cells is preferably by intravenous injection or by stereotactic injection, as appropriate. The selection of the promoter may be important and advantageous examples are provided herein.

[00280] Methods of treating genetic diseases or conditions caused by, or associated with, a deficient genotype in post-mitotic cells are provided, comprising delivery of the CRISPR-Cas9 system to the appropriate cell. A deficient genotype may be the non-wild type genotype. In particular, single point mutations and/or monogenic disorders are especially suited to treatment using CRISPR-Cas9 systems. Where multiple genes require editing or correcting, then a multiplex approach may be used to target them all simultaneously. Alternatively, two or more rounds of different CRISPR-Cas9 systems could be envisaged. Preferably, the wild-type genotype is corrected for. It does not necessarily have to be the most common genotype, provided that function is restored or improved in the phenotype.

[00281] An example of a restored phenotype is the restoration of hearing to restore VGLUT3 function and hence hearing in the inner ear of mice (Omar Akil, Rebecca P. Seal, Kevin Burke, Chuansong Wang, Aurash Alemi, Matthew During, Robert H. Edwards, Lawrence R. Lustig. Restoration of Hearing in the VGLUT3 Knockout Mouse Using Virally Mediated Gene Therapy.*Neuron*, 2012; 75 (2): 283 DOI: 10.1016/j.neuron.2012.05.019). This was using AAV-mediated delivery of VGLUT3 itself, but it is entirely plausible that CRISPR-Cas9 system could also be used, preferably also using AAV vectors, to target the cells of the inner ear and correct the non-functional VGLUT3 genotype, with similar phenotypic consequences, namely restoration of hearing. As such, delivery of the CRISPR-Cas9 system to the inner ear, preferably using AAV vectors, is preferred, thus treating hearing loss. Indeed, restoration of gene function in sensory organs such as the eye, including the retina, nose and ear (particularly the inner ear) is preferred.

[00282] A relatively recent overview, which includes a discussion of disorders in post-mitotic tissues (eye, ear and beyond) is Kaufmann *et al* (EMBO Mol Med (2013(, 5, p1642-1661). This confirms the usefulness of AAV in the correction of monogenic disorders in post-mitotic tissues. It states that "in combination with other characteristics such as low inflammatory activity, they have shown to have an excellent safety profile and are therefore highly attractive tools for *in*

vivo gene therapy. Indeed, Glybera® is a recombinant AAV for direct intramuscular injection…" The paper, with citations, reviews gene therapy in the retina, central nervous system, liver, skeletal and cardiac muscle as target tissues. And, with citations, indicates that "initial studies exploited the prototype AAV serotype 2 vector, the portfolio of AAV vectors has recently been expanded to include additional serotypes and even engineered capsids." Kaufmann and the documents cited in Kaufmann are hereby incorporated herein by reference.

[00283] **RNAseq Analysis of the Transcriptome**

[00284] The combination of SpCas9-mediated genome perturbation and population level RNAseq analysis provides a way to characterize transcriptional regulation and suggest genes that may be important to specific functions or disease processes in the cells under consideration. In particular, the cells are from the brain, in particular neurones. Fast-acting techniques such as a CRISPR-Cas9 system are advantageous in studying the transcriptome, which is, by its nature, transient. As such, the use of CRISPR-Cas9 systems according to the present invention in analysis of the transcriptome (RNAseq) are provided.

[00285] **Nuclear Tagging Method**

[00286] To facilitate immunofluorescence identification of SpCas9-expressing neurons, we tagged SpCas9 with a HA-epitope tag (derived from human influenza hemagluttinin, a general epitope tag widely used in expression vectors).

[00287] For the AAV-SpGuide vector, we packaged an U6-sgRNA expression cassette as well as the green fluorescent protein (GFP)-fused with the KASH nuclear trans-membrane domain driven by the human Synapsin I promoter. The GFP-KASH fusion protein directs GFP to the outer nuclear membrane and enables fluorescence-based identification and purification of intact neuronal nuclei transduced by AAV-SpGuide.

[00288] Accordingly, the vectors of the present invention are preferably adapted in a similar fashion. Thus, the vectors are provided wherein the Cas9 is tagged with an epitope tag, such as the HA-epitope tag. The Cas9 may be any of the Cas9s described herein, for instance Sp or SaCas9 and may be any variant (such as D10A double nickase etc), provide that it is or can be tagged appropriately.

[00289] The vectors of the present invention may also be adapted so that the guide RNA is packaged within an expression cassette, which comprises:

- a reporter protein; and

- optionally, a suitable promoter for the guide RNA, such as U6;

[00290] wherein the reporter protein is fused with a nuclear trans-membrane domain operably linked to a suitable promoter therefor.

[00291] The reporter protein is preferably a fluorescent protein, for instance one of green, red or yellow fluorescent proteins (GFP, RFP, YFP) and so forth.

[00292] Examples of nuclear trans-membrane domains include KASH-like domains, Sun2 domains, LEM domains. In some preferred embodiments, the nuclear trans-membrane domain is the KASH nuclear trans-membrane. Preferably, the promoter for the trans-membrane domain is the human Synapsin I promoter; see also documents cited herein.

[00293] This tagging approach may be used within single or dual vector systems, but preferably within dual vector systems as space is limited in single vector systems and the need for separate tags lessened as well.

[00294] Furthermore, each aspect of this tagging technique can be used independently of the other, so that epitope tagging of the Cas9 can be used alone, or the reporter/fluorescent protein cassette approach can be used alone, or more preferably both can be used together.

[00295] Kanasty and Anderson (Nature Materials, Vol 12 Nov 2013) is a useful review, initially submitted on 11 March 2013 and published online on 23 October 2013 of delivery of RNAi. Due to the similarities between RNAi and CRISPR guide sequences, the teaching of this and other art in respect of RNAi is informative for the mechanisms of delivering the guides in our CRISPR-Cas9 system. Some of the techniques described are also be suitable for delivery of the Cas9 as well. In some instance is may be useful to deliver the guides of our CRISPR-Cas9 system separately from the Cas9. This may be as part of a dual-vector delivery system, where the vectors are considered in the broadest light as simply any means of delivery, rather than specifically viral vectors. It is envisaged that the Cas9 may be delivered via a viral vector and that guides specific to genomic targets are delivered separately. As discussed herein, the guides could be delivered via the same vector types as the Cas9, for example a dual-vector system where the Cas9 is delivered in an AAV vector and the guide(s) are delivered in a separate AAV vector. This can be done substantially contemporaneously (i.e. co-delivery), but it could also be done at separate points in time, separated even by weeks or months. For example, if a first round of CRISPR-Cas9 systems have been delivered, but then it is subsequently required to provide further guides, then the original Cas9 which is hopefully still functional in the target cells may be

re-used. If the Cas9 is under the control of an inducible promoter, then induction of transcription of new CAs9 in the target cells is preferred. Equally, if a CAs9-expressing model provided for herein is used, then only delivery of guide(s) is necessary. Accordingly, where delivery of guide(s) is required separately from Cas9, then it may be delivered in much the same way as RNAi. As such, the review by Kanasty is helpful in pointing out a number of known approaches that are suitable, with particular focus on the liver, although the means of delivery are generally appropriate for a broad range of cells. Examples include:

- "Liposomal delivery system, as well as siRNA conjugated to lipophilic molecules, interact with serum lipoproteins and subsequently gain entry into hepatocytes that take up those lipoproteins;"
- PEGylation;
- Conjugates such as:
    a. Dynamic Polyconjugates (DPCs, 10nm nanoparticles), which have been shown to deliver RNAi to successfully supress ApoB (thereby crossing over with our work on targeting ApoB via a CRISPR-Cas9 system); and
    b. Triantennary GalNAc conjugates
    c. are "both highly effective" especially GalNAc;
- Other nanoparticles include:
    d. Cyclodextrin Polymer nanoparticles (CDP), including additional formulation components such as adamantine-PEG (AD-PEG) and adamantine-PEG-transferrin (AD-PEG-Tf) ;
    e. Lipid Nanoparticles (LNP), including cationic or ionisable lipids, shielding lipids, cholesterol and endogenous or exogenous targeting ligands. An example of an endogenous targeting ligand is Retinol Binding protein (RBP) useful for targeting hepatic and pancreatic stellate cells, which express the RBP receptor. An example of an exogenous targeting ligand is GalNac, which also targets the liver via the asialoglycoprotein receptor on hepatocytes. A combined approach is seen in Anlylams ALN-VSP;
- "Fenestrations in the liver endothelium allow molecules 100-200 nm in diameter to diffuse out of the bloodstream and gain access to the hepatocytes and other liver cells";

- Ligands such as GalNAc are suitable for delivery to non-parenchymal liver cells expressing the mannose receptor, and to hepatocytes where conjugation of suitable siRNA to a GalNAc ligand has been shown to successfully supress PCSK9; and
- Oligonucleotide nanoparticles (ONPs) composed of composed of complimentary DNA fragments designed to hybridise into a pre-defined 3D structure. Using suitable 3' overhand sequences, 6 siRNA strands could be attached to each particle, even at a specified position. The hydrodynamic diameter was about 29nm.

[00296] These approaches are preferred in some embodiments for delivery of at least the guides for a CRISPR-Cas9 system. Especially preferred are Dynamic Polyconjugates or the use of an endogenous targeting ligands such as Retinol Binding protein or exogenous targeting ligands such as GalNac.

[00297] Particle delivery systems and/or formulations:

[00298] Several types of particle delivery systems and/or formulations are known to be useful in a diverse spectrum of biomedical applications. In general, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Particles are further classified according to diameter Coarse particles cover a range between 2,500 and 10,000 nanometers. Fine particles are sized between 100 and 2,500 nanometers. Ultrafine particles, or nanoparticles, are generally between 1 and 100 nanometers in size. The basis of the 100-nm limit is the fact that novel properties that differentiate particles from the bulk material typically develop at a critical length scale of under 100 nm.

[00299] As used herein, a particle delivery system/formulation is defined as any biological delivery system/formulation which includes a particle in accordance with the present invention. A particle in accordance with the present invention is any entity having a greatest dimension (e.g. diameter) of less than 100 microns (μm). In some embodiments, inventive particles have a greatest dimension of less than 10 μm. In some embodiments, inventive particles have a greatest dimension of less than 2000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 1000 nanometers (nm). In some embodiments, inventive particles have a greatest dimension of less than 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, or 100 nm. Typically, inventive particles have a greatest dimension (e.g., diameter) of 500 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g.,

diameter) of 250 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 200 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 150 nm or less. In some embodiments, inventive particles have a greatest dimension (e.g., diameter) of 100 nm or less. Smaller particles, e.g., having a greatest dimension of 50 nm or less are used in some embodiments of the invention. In some embodiments, inventive particles have a greatest dimension ranging between 25 nm and 200 nm.

[00300] Particle characterization (including e.g., characterizing morphology, dimension, etc.) is done using a variety of different techniques. Common techniques are electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry(MALDI-TOF), ultraviolet-visible spectroscopy, dual polarisation interferometry and nuclear magnetic resonance (NMR). Characterization (dimension measurements) may be made as to native particles (i.e., preloading) or after loading of the cargo (herein cargo refers to e.g., one or more components of CRISPR-Cas system e.g., CRISPR enzyme or mRNA or guide RNA, or any combination thereof, and may include additional carriers and/or excipients) to provide particles of an optimal size for delivery for any *in vitro*, *ex vivo* and/or *in vivo* application of the present invention. In certain preferred embodiments, particle dimension (e.g., diameter) characterization is based on measurements using dynamic laser scattering (DLS). Mention is made of US Patent No. 8,709,843; US Patent No. 6,007,845; US Patent No. 5,855,913; US Patent No. 5,985,309; US. Patent No. 5,543,158; and the publication by James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84, concerning particles, methods of making and using them and measurements thereof.

[00301] Particles delivery systems within the scope of the present invention may be provided in any form, including but not limited to solid, semi-solid, emulsion, or colloidal particles. As such any of the delivery systems described herein, including but not limited to, e.g., lipid-based systems, liposomes, micelles, microvesicles, exosomes, or gene gun may be provided as particle delivery systems within the scope of the present invention.

[00302] Nanoparticles

[00303] In terms of this invention, it is preferred to have one or more components of CRISPR complex, e.g., CRISPR enzyme or mRNA or guide RNA delivered using nanoparticles or lipid envelopes. Other delivery systems or vectors are may be used in conjunction with the nanoparticle aspects of the invention.

[00304] In general, a "nanoparticle" refers to any particle having a diameter of less than 1000 nm. In certain preferred embodiments, nanoparticles of the invention have a greatest dimension (e.g., diameter) of 500 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 25 nm and 200 nm. In other preferred embodiments, nanoparticles of the invention have a greatest dimension of 100 nm or less. In other preferred embodiments, nanoparticles of the invention have a greatest dimension ranging between 35 nm and 60 nm.

[00305] Nanoparticles encompassed in the present invention may be provided in different forms, e.g., as solid nanoparticles (e.g., metal such as silver, gold, iron, titanium), non-metal, lipid-based solids, polymers), suspensions of nanoparticles, or combinations thereof. Metal, dielectric, and semiconductor nanoparticles may be prepared, as well as hybrid structures (e.g., core–shell nanoparticles). Nanoparticles made of semiconducting material may also be labeled quantum dots if they are small enough (typically sub 10 nm) that quantization of electronic energy levels occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents and may be adapted for similar purposes in the present invention.

[00306] Semi-solid and soft nanoparticles have been manufactured, and are within the scope of the present invention. A prototype nanoparticle of semi-solid nature is the liposome. Various types of liposome nanoparticles are currently used clinically as delivery systems for anticancer drugs and vaccines. Nanoparticles with one half hydrophilic and the other half hydrophobic are termed Janus particles and are particularly effective for stabilizing emulsions. They can self-assemble at water/oil interfaces and act as solid surfactants.

[00307] US Patent No. 8,709,843, incorporated herein by reference, provides a drug delivery system for targeted delivery of therapeutic agent-containing particles to tissues, cells, and intracellular compartments. The invention provides targeted particles comprising comprising polymer conjugated to a surfactant, hydrophilic polymer or lipid.

[00308] US Patent No. 6,007,845, incorporated herein by reference, provides particles which have a core of a multiblock copolymer formed by covalently linking a multifunctional compound

with one or more hydrophobic polymers and one or more hydrophilic polymers, and conatin a biologically active material.

[00309] US Patent No. 5,855,913, incorporated herein by reference, provides a particulate composition having aerodynamically light particles having a tap density of less than 0.4 g/cm3 with a mean diameter of between 5 □m and 30 □m, incorporating a surfactant on the surface thereof for drug delivery to the pulmonary system.

[00310] US Patent No. 5,985,309, incorporated herein by reference, provides particles incorporating a surfactant and/or a hydrophilic or hydrophobic complex of a positively or negatively charged therapeutic or diagnostic agent and a charged molecule of opposite charge for delivery to the pulmonary system.

[00311] US. Patent No. 5,543,158, incorporated herein by reference, provides biodegradable injectable nanoparticles having a biodegradable solid core containing a biologically active material and poly(alkylene glycol) moieties on the surface.

[00312] WO2012135025 (also published as US20120251560), incorporated herein by reference, describes conjugated polyethyleneimine (PEI) polymers and conjugated aza-macrocycles (collectively referred to as "conjugated lipomer" or "lipomers"). In certain embodiments, it can envisioned that such conjugated lipomers can be used in the context of the CRISPR-Cas system to achieve in vitro, ex vivo and in vivo genomic perturbations to modify gene expression, including modulation of protein expression.

[00313] In one embodiment, the nanoparticle may be epoxide-modified lipid–polymer, advantageously 7C1 (see, e.g., James E. Dahlman and Carmen Barnes et al. Nature Nanotechnology (2014) published online 11 May 2014, doi:10.1038/nnano.2014.84). C71 was synthesized by reacting C15 epoxide-terminated lipids with PEI600 at a 14:1 molar ratio, and was formulated with C14PEG2000 to produce nanoparticles (diameter between 35 and 60 nm) that were stable in PBS solution for at least 40 days.

[00314] An epoxide-modified lipid-polymer may be utilized to deliver the CRISPR-Cas system of the present invention to pulmonary, cardiovascular or renal cells, however, one of skill in the art may adapt the system to deliver to other target organs. Dosage ranging from about 0.05 to about 0.6 mg/kg are envisioned. Dosages over several days or weeks are also envisioned, with a total dosage of about 2 mg/kg.

[00315] An advantage of the present methods is that the CRISPR system avoids off-target binding and its resulting side effects. This is achieved using systems arranged to have a high degree of sequence specificity for the target DNA.

[00316] Cas9

[00317] Cas9 optimization may be used to enhance function or to develop new functions, one can generate chimeric Cas9 proteins. Examples that the Applicants have generated are provided in Example 6. Chimeric Cas9 proteins can be made by combining fragments from different Cas9 homologs. For example, two example chimeric Cas9 proteins from the Cas9s described herein. For example, Applicants fused the N-term of St1Cas9 (fragment from this protein is in bold) with C-term of SpCas9. The benefit of making chimeric Cas9s include any or all of: reduced toxicity; improved expression in eukaryotic cells; enhanced specificity; reduced molecular weight of protein, for example, making the protein smaller by combining the smallest domains from different Cas9 homologs; and/or altering the PAM sequence requirement.

[00318] The Cas9 may be used as a generic DNA binding protein. For example, and as shown in Example 7, Applicants used Cas9 as a generic DNA binding protein by mutating the two catalytic domains (D10 and H840) responsible for cleaving both strands of the DNA target. In order to upregulate gene transcription at a target locus Applicants fused a transcriptional activation domain (VP64) to Cas9. Other transcriptional activation domains are known. As shown in Example 17, transcriptional activation is possible. As also shown in Example 17, gene repression (in this case of the beta-catenin gene) is possible using a Cas9 repressor (DNA-binding domain) that binds to the target gene sequence, thus repressing its activity.

[00319] Cas9 and one or more guide RNA can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other plasmid or viral vector types, in particular, using formulations and doses from, for example, US Patents Nos. 8,454,972 (formulations, doses for adenovirus), 8,404,658 (formulations, doses for AAV) and 5,846,946 (formulations, doses for DNA plasmids) and from clinical trials and publications regarding the clinical trials involving lentivirus, AAV and adenovirus. For examples, for AAV, the route of administration, formulation and dose can be as in US Patent No. 8,454,972 and as in clinical trials involving AAV. For Adenovirus, the route of administration, formulation and dose can be as in US Patent No. 8,404,658 and as in clinical trials involving adenovirus. For plasmid delivery, the route of administration, formulation and dose can be as in US Patent No 5,846,946 and as in clinical

studies involving plasmids. Doses may be based on or extrapolated to an average 70 kg individual, and can be adjusted for patients, subjects, mammals of different weight and species. Frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), depending on usual factors including the age, sex, general health, other conditions of the patient or subject and the particular condition or symptoms being addressed.

[00320] The viral vectors can be injected into the tissue of interest. For cell-type specific genome modification, the expression of Cas9 can be driven by a cell-type specific promoter. For example, liver-specific expression might use the Albumin promoter and neuron-specific expression might use the Synapsin I promoter.

[00321] **Transgenic animals**

[00322] Transgenic animals (models) are also provided and the following applies equally to ex vivo model tissues and collections of tissues, such as organoids, liver on a chip and so forth. Preferred examples include animals comprising Cas9, in terms of polynucleotides encoding Cas9 or the protein itself. Mice, rats and rabbits are preferred. To generate transgenic mice with the constructs, as exemplified herein one may inject pure, linear DNA into the pronucleus of a zygote from a pseudo pregnant female, e.g. a CB56 female. Founders may then be identified, genotyped, and backcrossed to CB57 mice. The constructs may then be cloned and optionally verified, for instance by Sanger sequencing. Knock outs are envisaged where for instance one or more genes are knocked out in a model. However, are knockins are also envisaged (alone or in combination). An example knockin Cas9 mouse was generated and this is exemplified, but Cas9 knockins are preferred. To generate a Cas9 knock in mice one may target the same constitutive and conditional constructs to the Rosa26 locus, as described herein (Figs. 25A-B and 26). Methods of US Patent Publication Nos. 20120017290 and 20110265198 assigned to Sangamo BioSciences, Inc. directed to targeting the Rosa locus may be modified to utilize the CRISPR Cas system of the present invention. In another embodiment, the methods of US Patent Publication No. 20130236946 assigned to Cellectis directed to targeting the Rosa locus may also be modified to utilize the CRISPR Cas system of the present invention.

[00323] Utility of the conditional Cas9 mouse: Applicants have shown in 293 cells that the Cas9 conditional expression construct can be activated by co-expression with Cre. Applicants also show that the correctly targeted R1 mESCs can have active Cas9 when Cre is expressed. Because Cas9 is followed by the P2A peptide cleavage sequence and then EGFP Applicants

identify successful expression by observing EGFP. Applicants have shown Cas9 activation in mESCs. This same concept is what makes the conditional Cas9 mouse so useful. Applicants may cross their conditional Cas9 mouse with a mouse that ubiquitously expresses Cre (ACTB-Cre line) and may arrive at a mouse that expresses Cas9 in every cell. It should only take the delivery of chimeric RNA to induce genome editing in embryonic or adult mice. Interestingly, if the conditional Cas9 mouse is crossed with a mouse expressing Cre under a tissue specific promoter, there should only be Cas9 in the tissues that also express Cre. This approach may be used to edit the genome in only precise tissues by delivering chimeric RNA to the same tissue.

[00324] As mentioned above, transgenic animals are also provided. In this regard, transgenic animals, especially mammals such as livestock (cows, sheep, goats and pigs), but also poultry and edible insects, are preferred.

[00325] **Adeno associated virus (AAV)**

[00326] In terms of *in vivo* delivery, AAV is advantageous over other viral vectors for a couple of reasons:

[00327] Low toxicity (this may be due to the purification method not requiring ultra centrifugation of cell particles that can activate the immune response)

[00328] Low probability of causing insertional mutagenesis because it doesn't integrate into the host genome.

[00329] AAV has a packaging limit of 4.5 or 4.75 Kb. This means that Cas9 as well as a promoter and transcription terminator have to be all fit into the same viral vector. Constructs larger than 4.5 or 4.75 Kb will lead to significantly reduced virus production. SpCas9 is quite large, the gene itself is over 4.1 Kb, which makes it difficult for packing into AAV. Therefore embodiments of the invention include utilizing homologs of Cas9 that are shorter. For example:

Species	Cas9 Size
Corynebacter diphtheriae	3252
Eubacterium ventriosum	3321
Streptococcus pasteurianus	3390
Lactobacillus farciminis	3378
Sphaerochaeta globus	3537
Azospirillum B510	3504
Gluconacetobacter diazotrophicus	3150
Neisseria cinerea	3246
Roseburia intestinalis	3420
Parvibaculum lavamentivorans	3111
Staphylococcus aureus	3159

Nitratifractor salsuginis DSM 16511	3396
Campylobacter lari CF89-12	3009
Streptococcus thermophilus LMD-9	3396

[00330] These species are therefore, in general, preferred Cas9 species. Applicants have shown delivery and *in vivo* mouse brain Cas9 expression data.

[00331] Two ways to package Cas9 coding nucleic acid molecules, e.g., DNA, into viral vectors to mediate genome modification *in vivo* are preferred:

[00332] To achieve NHEJ-mediated gene knockout:

[00333] Single virus vector:

Vector containing two or more expression cassettes:

Promoter-Cas9 coding nucleic acid molecule -terminator

Promoter-gRNA1-terminator

Promoter-gRNA2-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

[00334] Double virus vector:

Vector 1 containing one expression cassette for driving the expression of Cas9

Promoter-Cas9 coding nucleic acid molecule-terminator

Vector 2 containing one more expression cassettes for driving the expression of one or more guideRNAs

Promoter-gRNA1-terminator

Promoter-gRNA(N)-terminator (up to size limit of vector)

[00335] To mediate homology-directed repair. In addition to the single and double virus vector approaches described above, an additional vector is used to deliver a homology-direct repair template.

[00336] Promoter used to drive Cas9 coding nucleic acid molecule expression can include:

[00337] AAV ITR can serve as a promoter: this is advantageous for eliminating the need for an additional promoter element (which can take up space in the vector). The additional space freed up can be used to drive the expression of additional elements (gRNA, etc.). Also, ITR activity is relatively weaker, so can be used to reduce toxicity due to over expression of Cas9.

[00338] For ubiquitous expression, can use promoters: CMV, CAG, CBh, PGK, SV40, Ferritin heavy or light chains, etc.

[00339] For brain expression, can use promoters: SynapsinI for all neurons, CaMKIIalpha for excitatory neurons, GAD67 or GAD65 or VGAT for GABAergic neurons, etc.

[00340] For liver expression, can use Albumin promoter.

[00341] For lung expression, can use SP-B.

[00342] For endothelial cells, can use ICAM.

[00343] For hematopoietic cells can use IFNbeta or CD45.

[00344] For Osteoblasts can use OG-2.

[00345] Promoter used to drive guide RNA can include:

[00346] **Pol III promoters such as U6 or H1**

[00347] **Use of Pol II promoter and intronic cassettes to express gRNA**

[00348] As to AAV, the AAV can be AAV1, AAV2, AAV5 or any combination thereof. One can select the AAV of the AAV with regard to the cells to be targeted; e.g., one can select AAV serotypes 1, 2, 5 or a hybrid capsid AAV1, AAV2, AAV5 or any combination thereof for targeting brain or neuronal cells; and one can select AAV4 for targeting cardiac tissue. AAV8 is useful for delivery to the liver. The above promoters and vectors are preferred individually.

[00349] RNA delivery is also a useful method of *in vivo* delivery. Fig. 27 shows delivery and *in vivo* mouse brain Cas9 expression data. It is possible to deliver Cas9 and gRNA (and, for instance, HR repair template) into cells using liposomes or nanoparticles. Thus delivery of the CRISPR enzyme, such as a Cas9 and/or delivery of the RNAs of the invention may be in RNA form and via microvesicles, liposomes or nanoparticles. For example, Cas9 mRNA and gRNA can be packaged into liposomal particles for delivery *in vivo*. Liposomal transfection reagents such as lipofectamine from Life Technologies and other reagents on the market can effectively deliver RNA molecules into the liver.

[00350] Enhancing NHEJ or HR efficiency is also helpful for delivery. It is preferred that NHEJ efficiency is enhanced by co-expressing end-processing enzymes such as Trex2 (Dumitrache et al. Genetics. 2011 August; 188(4): 787–797). It is preferred that HR efficiency is increased by transiently inhibiting NHEJ machineries such as Ku70 and Ku86. HR efficiency can also be increased by co-expressing prokaryotic or eukaryotic homologous recombination enzymes such as RecBCD, RecA.

[00351] Various means of delivery are described herein, and further discussed in this section.

[00352] Viral delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can be delivered using adeno associated virus (AAV), lentivirus, adenovirus or other viral vector types, or combinations thereof. Cas9 and one or more guide RNAs can be packaged into one or more viral vectors. In some embodiments, the viral vector is delivered to the tissue of interest by, for example, an intramuscular injection, while other times the viral delivery is via intravenous, transdermal, intranasal, oral, mucosal, or other delivery methods. Such delivery may be either via a single dose, or multiple doses. One skilled in the art understands that the actual dosage to be delivered herein may vary greatly depending upon a variety of factors, such as the vector chose, the target cell, organism, or tissue, the general condition of the subject to be treated, the degree of transformation/modification sought, the administration route, the administration mode, the type of transformation/modification sought, etc.

[00353] Such a dosage may further contain, for example, a carrier (water, saline, ethanol, glycerol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, etc.), a diluent, a pharmaceutically-acceptable carrier (e.g., phosphate-buffered saline), a pharmaceutically-acceptable excipient, and/or other compounds known in the art. Such a dosage formulation is readily ascertainable by one skilled in the art. The dosage may further contain one or more pharmaceutically acceptable salts such as, for example, a mineral acid salt such as a hydrochloride, a hydrobromide, a phosphate, a sulfate, etc.; and the salts of organic acids such as acetates, propionates, malonates, benzoates, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, gels or gelling materials, flavorings, colorants, microspheres, polymers, suspension agents, etc. may also be present herein. In addition, one or more other conventional pharmaceutical ingredients, such as preservatives, humectants, suspending agents, surfactants, antioxidants, anticaking agents, fillers, chelating agents, coating agents, chemical stabilizers, etc. may also be present, especially if the dosage form is a reconstitutable form. Suitable exemplary ingredients include microcrystalline cellulose, carboxymethylcellulose sodium, polysorbate 80, phenylethyl alcohol, chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, parachlorophenol, gelatin, albumin and a combination thereof. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991) which is incorporated by reference herein.

[00354] In an embodiment herein the delivery is via an adenovirus, which may be at a single booster dose containing at least 1 x 10^5 particles (also referred to as particle units, pu) of adenoviral vector. In an embodiment herein, the dose preferably is at least about 1 x 10^6 particles (for example, about 1 x 10^6-1 x 10^{12} particles), more preferably at least about 1 x 10^7 particles, more preferably at least about 1 x 10^8 particles (e.g., about 1 x 10^8-1 x 10^{11} particles or about 1 x 10^8-1 x 10^{12} particles), and most preferably at least about 1 x 10^0 particles (e.g., about 1 x 10^9-1 x 10^{10} particles or about 1 x 10^9-1 x 10^{12} particles), or even at least about 1 x 10^{10} particles (e.g., about 1 x 10^{10}-1 x 10^{12} particles) of the adenoviral vector. Alternatively, the dose comprises no more than about 1 x 10^{14} particles, preferably no more than about 1 x 10^{13} particles, even more preferably no more than about 1 x 10^{12} particles, even more preferably no more than about 1 x 10^{11} particles, and most preferably no more than about 1 x 10^{10} particles (e.g., no more than about 1 x 10^9 articles). Thus, the dose may contain a single dose of adenoviral vector with, for example, about 1 x 10^6 particle units (pu), about 2 x 10^6 pu, about 4 x 10^6 pu, about 1 x 10^7 pu, about 2 x 10^7 pu, about 4 x 10^7 pu, about 1 x 10^8 pu, about 2 x 10^8 pu, about 4 x 10^8 pu, about 1 x 10^9 pu, about 2 x 10^9 pu, about 4 x 10^9 pu, about 1 x 10^{10} pu, about 2 x 10^{10} pu, about 4 x 10^{10} pu, about 1 x 10^{11} pu, about 2 x 10^{11} pu, about 4 x 10^{11} pu, about 1 x 10^{12} pu, about 2 x 10^{12} pu, or about 4 x 10^{12} pu of adenoviral vector. See, for example, the adenoviral vectors in U.S. Patent No. 8,454,972 B2 to Nabel, et. al., granted on June 4, 2013; incorporated by reference herein, and the dosages at col 29, lines 36-58 thereof. In an embodiment herein, the adenovirus is delivered via multiple doses.

[00355] In an embodiment herein, the delivery is via an AAV. A therapeutically effective dosage for in vivo delivery of the AAV to a human is believed to be in the range of from about 20 to about 50 ml of saline solution containing from about 1 x 10^{10} to about 1 x 10^{10} functional AAV/ml solution. The dosage may be adjusted to balance the therapeutic benefit against any side effects. In an embodiment herein, the AAV dose is generally in the range of concentrations of from about 1 x 10^5 to 1 x 10^{50} genomes AAV, from about 1 x 10^8 to 1 x 10^{20} genomes AAV, from about 1 x 10^{10} to about 1 x 10^{16} genomes, or about 1 x 10^{11} to about 1 x 10^{16} genomes AAV. A human dosage may be about 1 x 10^{13} genomes AAV. Such concentrations may be delivered in from about 0.001 ml to about 100 ml, about 0.05 to about 50 ml, or about 10 to about 25 ml of a carrier solution. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. See, for

example, U.S. Patent No. 8,404,658 B2 to Hajjar, et al., granted on March 26, 2013, at col. 27, lines 45-60.

[00356] In an embodiment herein the delivery is via a plasmid. In such plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response. For instance, suitable quantities of plasmid DNA in plasmid compositions can be from about 0.1 to about 2 mg, or from about 1 μg to about 10 μg.

[00357] The doses herein are based on an average 70 kg individual. The frequency of administration is within the ambit of the medical or veterinary practitioner (e.g., physician, veterinarian), or scientist skilled in the art. Mice used in experiments are about 20g. From that which is administered to a 20g mouse, one can extrapolate to a 70kg individual.

[00358] **Lentivirus**

[00359] Lentiviruses are complex retroviruses that have the ability to infect and express their genes in both mitotic and post-mitotic cells. The most commonly known lentivirus is the human immunodeficiency virus (HIV), which uses the envelope glycoproteins of other viruses to target a broad range of cell types.

[00360] Lentiviruses may be prepared as follows. After cloning pCasES10 (which contains a lentiviral transfer plasmid backbone), HEK293FT at low passage (p=5) were seeded in a T-75 flask to 50% confluence the day before transfection in DMEM with 10% fetal bovine serum and without antibiotics. After 20 hours, media was changed to OptiMEM (serum-free) media and transfection was done 4 hours later. Cells were transfected with 10 μg of lentiviral transfer plasmid (pCasES10) and the following packaging plasmids: 5 μg of pMD2.G (VSV-g pseudotype), and 7.5ug of psPAX2 (gag/pol/rev/tat). Transfection was done in 4mL OptiMEM with a cationic lipid delivery agent (50uL Lipofectamine 2000 and 100ul Plus reagent). After 6 hours, the media was changed to antibiotic-free DMEM with 10% fetal bovine serum.

[00361] Lentivirus may be purified as follows. Viral supernatants were harvested after 48 hours. Supernatants were first cleared of debris and filtered through a 0.45um low protein binding (PVDF) filter. They were then spun in a ultracentrifuge for 2 hours at 24,000 rpm. Viral pellets were resuspended in 50ul of DMEM overnight at 4C. They were then aliquotted and immediately frozen at -80C.

[00362] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see,

e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostain and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)) may be modified for the CRISPR-Cas system of the present invention.

[00363] In another embodiment, self-inactivating lentiviral vectors with an siRNA targeting a common exon shared by HIV tat/rev, a nucleolar-localizing TAR decoy, and an anti–CCR5-specific hammerhead ribozyme (see, e.g., DiGiusto et al. (2010) Sci Transl Med 2:36ra43) may be used/and or adapted to the CRISPR-Cas system of the present invention. A minimum of 2.5 × 10^6 CD34+ cells per kilogram patient weight may be collected and prestimulated for 16 to 20 hours in X-VIVO 15 medium (Lonza) containing 2 micro mol/ L-glutamine, stem cell factor (100 ng/ml), Flt-3 ligand (Flt-3L) (100 ng/ml), and thrombopoietin (10 ng/ml) (CellGenix) at a density of 2 × 10^6 cells/ml. Prestimulated cells may be transduced with lentiviral at a multiplicity of infection of 5 for 16 to 24 hours in 75-cm^2 tissue culture flasks coated with fibronectin (25 mg/cm^2) (RetroNectin,Takara Bio Inc.).

[00364] Lentiviral vectors have been disclosed as in the treatment for Parkinson's Disease, see, e.g., US Patent Publication No. 20120295960 and US Patent Nos. 7303910 and 7351585. Lentiviral vectors have also been disclosed for the treatment of ocular diseases, see e.g., US Patent Publication Nos. 20060281180, 20090007284, US20110117189; US20090017543; US20070054961, US20100317109. Lentiviral vectors have also been disclosed for delivery to the train, see, e.g., US Patent Publication Nos. US20110293571; US20110293571, US20040013648, US20070025970, US20090111106 and US Patent No. US7259015.

[00365] **RNA delivery**

[00366] RNA delivery: The CRISPR enzyme, for instance a Cas9, and/or any of the present RNAs, for instance a guide RNA, can also be delivered in the form of RNA. Cas9 mRNA can be generated using *in vitro* transcription. For example, Cas9 mRNA can be synthesized using a PCR cassette containing the following elements: T7_promoter-kozak sequence (GCCACC)-Cas9-3' UTR from beta globin-polyA tail (a string of 120 or more adenines). The cassette can be used for

transcription by T7 polymerase. Guide RNAs can also be transcribed using in vitro transcription from a cassette containing T7_promoter-GG-guide RNA sequence.

[00367] To enhance expression and reduce toxicity, the CRISPR enzyme and/or guide RNA can be modified using pseudo-U or 5-Methyl-C.

[00368] mRNA delivery methods are especially promising for liver delivery currently. In particular, for AAV8 is particularly preferred for delivery to the liver.

[00369] **Nanoparticles**

[00370] CRISPR enzyme mRNA and guide RNA may be delivered simultaneously using nanoparticles or lipid envelopes.

[00371] For example, Su X, Fricke J, Kavanagh DG, Irvine DJ ("In vitro and in vivo mRNA delivery using lipid-enveloped pH-responsive polymer nanoparticles" Mol Pharm. 2011 Jun 6;8(3):774-87. doi: 10.1021/mp100390w. Epub 2011 Apr 1) describes biodegradable core-shell structured nanoparticles with a poly(β-amino ester) (PBAE) core enveloped by a phospholipid bilayer shell. These were developed for in vivo mRNA delivery. The pH-responsive PBAE component was chosen to promote endosome disruption, while the lipid surface layer was selected to minimize toxicity of the polycation core. Such are, therefore, preferred for delivering RNA of the present invention.

[00372] In one embodiment, nanoparticles based on self assembling bioadhesive polymers are contemplated, which may be applied to oral delivery of peptides, intravenous delivery of peptides and nasal delivery of peptides, all to the brain. Other embodiments, such as oral absorption and ocular deliver of hydrophobic drugs are also contemplated. The molecular envelope technology involves an engineered polymer envelope which is protected and delivered to the site of the disease (see, e.g., Mazza, M. et al. ACSNano, 2013. 7(2): 1016-1026; Siew, A., et al. Mol Pharm, 2012. 9(1):14-28; Lalatsa, A., et al. J Contr Rel, 2012. 161(2):523-36; Lalatsa, A., et al., Mol Pharm, 2012. 9(6):1665-80; Lalatsa, A., et al. Mol Pharm, 2012. 9(6):1764-74; Garrett, N.L., et al. J Biophotonics, 2012. 5(5-6):458-68; Garrett, N.L., et al. J Raman Spect, 2012. 43(5):681-688; Ahmad, S., et al. J Royal Soc Interface 2010. 7:S423-33; Uchegbu, I.F. Expert Opin Drug Deliv, 2006. 3(5):629-40; Qu, X.,et al. Biomacromolecules, 2006. 7(12):3452-9 and Uchegbu, I.F., et al. Int J Pharm, 2001. 224:185-199). Doses of about 5 mg/kg are contemplated, with single or multiple doses, depending on the target tissue.

[00373] In one embodiment, nanoparticles that can deliver RNA to a cancer cell to stop tumor growth developed by Dan Anderson's lab at MIT may be used/and or adapted to the CRISPR Cas system of the present invention. In particular, the Anderson lab developed fully automated, combinatorial systems for the synthesis, purification, characterization, and formulation of new biomaterials and nanoformulations. See, e.g., Alabi et al., Proc Natl Acad Sci U S A. 2013 Aug 6;110(32):12881-6; Zhang et al., Adv Mater. 2013 Sep 6;25(33):4641-5; Jiang et al., Nano Lett. 2013 Mar 13;13(3):1059-64; Karagiannis et al., ACS Nano. 2012 Oct 23;6(10):8484-7; Whitehead et al., ACS Nano. 2012 Aug 28;6(8):6922-9 and Lee et al., Nat Nanotechnol. 2012 Jun 3;7(6):389-93.

[00374] US patent application 20110293703 relates to lipidoid compounds are also particularly useful in the administration of polynucleotides, which may be applied to deliver the CRISPR Cas system of the present invention. In one aspect, the aminoalcohol lipidoid compounds are combined with an agent to be delivered to a cell or a subject to form microparticles, nanoparticles, liposomes, or micelles. The agent to be delivered by the particles, liposomes, or micelles may be in the form of a gas, liquid, or solid, and the agent may be a polynucleotide, protein, peptide, or small molecule. The minoalcohol lipidoid compounds may be combined with other aminoalcohol lipidoid compounds, polymers (synthetic or natural), surfactants, cholesterol, carbohydrates, proteins, lipids, etc. to form the particles. These particles may then optionally be combined with a pharmaceutical excipient to form a pharmaceutical composition.

[00375] US Patent Publication No. 0110293703 also provides methods of preparing the aminoalcohol lipidoid compounds. One or more equivalents of an amine are allowed to react with one or more equivalents of an epoxide-terminated compound under suitable conditions to form an aminoalcohol lipidoid compound of the present invention. In certain embodiments, all the amino groups of the amine are fully reacted with the epoxide-terminated compound to form tertiary amines. In other embodiments, all the amino groups of the amine are not fully reacted with the epoxide-terminated compound to form tertiary amines thereby resulting in primary or secondary amines in the aminoalcohol lipidoid compound. These primary or secondary amines are left as is or may be reacted with another electrophile such as a different epoxide-terminated compound. As will be appreciated by one skilled in the art, reacting an amine with less than excess of epoxide-terminated compound will result in a plurality of different aminoalcohol

lipidoid compounds with various numbers of tails. Certain amines may be fully functionalized with two epoxide-derived compound tails while other molecules will not be completely functionalized with epoxide-derived compound tails. For example, a diamine or polyamine may include one, two, three, or four epoxide-derived compound tails off the various amino moieties of the molecule resulting in primary, secondary, and tertiary amines. In certain embodiments, all the amino groups are not fully functionalized. In certain embodiments, two of the same types of epoxide-terminated compounds are used. In other embodiments, two or more different epoxide-terminated compounds are used. The synthesis of the aminoalcohol lipidoid compounds is performed with or without solvent, and the synthesis may be performed at higher temperatures ranging from 30.-100 C., preferably at approximately 50.-90 C. The prepared aminoalcohol lipidoid compounds may be optionally purified. For example, the mixture of aminoalcohol lipidoid compounds may be purified to yield an aminoalcohol lipidoid compound with a particular number of epoxide-derived compound tails. Or the mixture may be purified to yield a particular stereo- or regioisomer. The aminoalcohol lipidoid compounds may also be alkylated using an alkyl halide (e.g., methyl iodide) or other alkylating agent, and/or they may be acylated.

[00376] US Patent Publication No. 0110293703 also provides libraries of aminoalcohol lipidoid compounds prepared by the inventive methods. These aminoalcohol lipidoid compounds may be prepared and/or screened using high-throughput techniques involving liquid handlers, robots, microtiter plates, computers, etc. In certain embodiments, the aminoalcohol lipidoid compounds are screened for their ability to transfect polynucleotides or other agents (e.g., proteins, peptides, small molecules) into the cell.

[00377] US Patent Publication No. 20130302401 relates to a class of poly(beta-amino alcohols) (PBAAs) has been prepared using combinatorial polymerization. The inventive PBAAs may be used in biotechnology and biomedical applications as coatings (such as coatings of films or multilayer films for medical devices or implants), additives, materials, excipients, non-biofouling agents, micropatterning agents, and cellular encapsulation agents. When used as surface coatings, these PBAAs elicited different levels of inflammation, both in vitro and in vivo, depending on their chemical structures. The large chemical diversity of this class of materials allowed us to identify polymer coatings that inhibit macrophage activation in vitro. Furthermore, these coatings reduce the recruitment of inflammatory cells, and reduce fibrosis, following the subcutaneous implantation of carboxylated polystyrene microparticles. These polymers may be

used to form polyelectrolyte complex capsules for cell encapsulation. The invention may also have many other biological applications such as antimicrobial coatings, DNA or siRNA delivery, and stem cell tissue engineering. The teachings of US Patent Publication No. 20130302401 may be applied to the CRISPR Cas system of the present invention.

[00378] In another embodiment, lipid nanoparticles (LNPs) are contemplated. In particular, an antitransthyretin small interfering RNA encapsulated in lipid nanoparticles (see, e.g., Coelho et al., N Engl J Med 2013;369:819-29) may be applied to the CRISPR Cas system of the present invention. Doses of about 0.01 to about 1 mg per kg of body weight administered intravenously are contemplated. Medications to reduce the risk of infusion-related reactions are contemplated, such as dexamethasone, acetampinophen, diphenhydramine or cetirizine, and ranitidine are contemplated. Multiple doses of about 0.3 mg per kilogram every 4 weeks for five doses are also contemplated.

[00379] LNPs have been shown to be highly effective in delivering siRNAs to the liver (see, e.g., Tabernero et al., Cancer Discovery, April 2013, Vol. 3, No. 4, pages 363-470) and are therefore contemplated for delivering CRISPR Cas to the liver. A dosage of about four doses of 6 mg/kg of the LNP (or RNA of the CRISPR-Cas system) every two weeks may be contemplated. Tabernero et al. demonstrated that tumor regression was observed after the first 2 cycles of LNPs dosed at 0.7 mg/kg, and by the end of 6 cycles the patient had achieved a partial response with complete regression of the lymph node metastasis and substantial shrinkage of the liver tumors. A complete response was obtained after 40 doses in this patient, who has remained in remission and completed treatment after receiving doses over 26 months. Two patients with RCC and extrahepatic sites of disease including kidney, lung, and lymph nodes that were progressing following prior therapy with VEGF pathway inhibitors had stable disease at all sites for approximately 8 to 12 months, and a patient with PNET and liver metastases continued on the extension study for 18 months (36 doses) with stable disease.

[00380] However, the charge of the LNP must be taken into consideration. As cationic lipids combined with negatively charged lipids to induce nonbilayer structures that facilitate intracellular delivery. Because charged LNPs are rapidly cleared from circulation following intravenous injection, ionizable cationic lipids with pKa values below 7 were developed (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). Negatively charged polymers such as siRNA oligonucleotides may be loaded into LNPs at low pH values

(e.g., pH 4) where the ionizable lipids display a positive charge. However, at physiological pH values, the LNPs exhibit a low surface charge compatible with longer circulation times. Four species of ionizable cationic lipids have been focused upon, namely 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxy-keto-N,N-dimethyl-3-aminopropane (DLinKDMA), and 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA). It has been shown that LNP siRNA systems containing these lipids exhibit remarkably different gene silencing properties in hepatocytes in vivo, with potencies varying according to the series DLinKC2-DMA>DLinKDMA>DLinDMA>>DLinDAP employing a Factor VII gene silencing model (see, e.g., Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). A dosage of 1 μg/ml levels may be contemplated, especially for a formulation containing DLinKC2-DMA.

[00381] Preparation of LNPs and CRISPR Cas encapsulation may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011). The cationic lipids 1,2-dilineoyl-3-dimethylammonium-propane (DLinDAP), 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinoleyloxyketo-N,N-dimethyl-3-aminopropane (DLinK-DMA), 1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLinKC2-DMA), (3-o-[2″-(methoxypolyethyleneglycol 2000) succinoyl]-1,2-dimyristoyl-sn-glycol (PEG-S-DMG), and R-3-[(ω-methoxy-poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-C-DOMG) may be provided by Tekmira Pharmaceuticals (Vancouver, Canada) or synthesized. Cholesterol may be purchased from Sigma (St Louis, MO). The specific CRISPR Cas RNA may be encapsulated in LNPs containing DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA (cationic lipid:DSPC:CHOL: PEGS-DMG or PEG-C-DOMG at 40:10:40:10 molar ratios). When required, 0.2% SP-DiOC18 (Invitrogen, Burlington, Canada) may be incorporated to assess cellular uptake, intracellular delivery, and biodistribution. Encapsulation may be performed by dissolving lipid mixtures comprised of cationic lipid:DSPC:cholesterol:PEG-c-DOMG (40:10:40:10 molar ratio) in ethanol to a final lipid concentration of 10 mmol/l. This ethanol solution of lipid may be added drop-wise to 50 mmol/l citrate, pH 4.0 to form multilamellar vesicles to produce a final concentration of 30% ethanol vol/vol. Large unilamellar vesicles may be formed following extrusion of multilamellar vesicles through two stacked 80 nm Nuclepore polycarbonate filters using the Extruder (Northern Lipids, Vancouver, Canada). Encapsulation may be achieved by adding RNA dissolved at 2 mg/ml in 50

mmol/l citrate, pH 4.0 containing 30% ethanol vol/vol drop-wise to extruded preformed large unilamellar vesicles and incubation at 31 °C for 30 minutes with constant mixing to a final RNA/lipid weight ratio of 0.06/1 wt/wt. Removal of ethanol and neutralization of formulation buffer were performed by dialysis against phosphate-buffered saline (PBS), pH 7.4 for 16 hours using Spectra/Por 2 regenerated cellulose dialysis membranes. Nanoparticle size distribution may be determined by dynamic light scattering using a NICOMP 370 particle sizer, the vesicle/intensity modes, and Gaussian fitting (Nicomp Particle Sizing, Santa Barbara, CA). The particle size for all three LNP systems may be ~70 nm in diameter. siRNA encapsulation efficiency may be determined by removal of free siRNA using VivaPureD MiniH columns (Sartorius Stedim Biotech) from samples collected before and after dialysis. The encapsulated RNA may be extracted from the eluted nanoparticles and quantified at 260 nm. siRNA to lipid ratio was determined by measurement of cholesterol content in vesicles using the Cholesterol E enzymatic assay from Wako Chemicals USA (Richmond, VA). PEGylated liposomes (or LNPs) can also be used for delivery.

[00382] Preparation of large LNPs may be used/and or adapted from Rosin et al, Molecular Therapy, vol. 19, no. 12, pages 1286-2200, Dec. 2011. A lipid premix solution (20.4 mg/ml total lipid concentration) may be prepared in ethanol containing DLinKC2-DMA, DSPC, and cholesterol at 50:10:38.5 molar ratios. Sodium acetate may be added to the lipid premix at a molar ratio of 0.75:1 (sodium acetate:DLinKC2-DMA). The lipids may be subsequently hydrated by combining the mixture with 1.85 volumes of citrate buffer (10 mmol/l, pH 3.0) with vigorous stirring, resulting in spontaneous liposome formation in aqueous buffer containing 35% ethanol. The liposome solution may be incubated at 37 °C to allow for time-dependent increase in particle size. Aliquots may be removed at various times during incubation to investigate changes in liposome size by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Once the desired particle size is achieved, an aqueous PEG lipid solution (stock = 10 mg/ml PEG-DMG in 35% (vol/vol) ethanol) may be added to the liposome mixture to yield a final PEG molar concentration of 3.5% of total lipid. Upon addition of PEG-lipids, the liposomes should their size, effectively quenching further growth. RNA may then be added to the empty liposomes at an siRNA to total lipid ratio of approximately 1:10 (wt:wt), followed by incubation for 30 minutes at 37 °C to form loaded LNPs. The mixture may be subsequently dialyzed overnight in PBS and filtered with a 0.45-μm syringe filter.

[00383] Spherical Nucleic Acid (SNA™) constructs and other nanoparticles (particularly gold nanoparticles) are also contemplate as a means to delivery CRISPR/Cas system to intended targets. Significant data show that AuraSense Therapeutics' Spherical Nucleic Acid (SNA™) constructs, based upon nucleic acid-functionalized gold nanoparticles, are superior to alternative platforms based on multiple key success factors, such as:

[00384] High in vivo stability. Due to their dense loading, a majority of cargo (DNA or siRNA) remains bound to the constructs inside cells, conferring nucleic acid stability and resistance to enzymatic degradation.

[00385] Deliverability. For all cell types studied (e.g., neurons, tumor cell lines, etc.) the constructs demonstrate a transfection efficiency of 99% with no need for carriers or transfection agents.

[00386] Therapeutic targeting. The unique target binding affinity and specificity of the constructs allow exquisite specificity for matched target sequences (i.e., limited off-target effects).

[00387] Superior efficacy. The constructs significantly outperform leading conventional transfection reagents (Lipofectamine 2000 and Cytofectin).

[00388] Low toxicity. The constructs can enter a variety of cultured cells, primary cells, and tissues with no apparent toxicity.

[00389] No significant immune response. The constructs elicit minimal changes in global gene expression as measured by whole-genome microarray studies and cytokine-specific protein assays.

[00390] Chemical tailorability. Any number of single or combinatorial agents (e.g., proteins, peptides, small molecules) can be used to tailor the surface of the constructs.

[00391] This platform for nucleic acid-based therapeutics may be applicable to numerous disease states, including inflammation and infectious disease, cancer, skin disorders and cardiovascular disease.

[00392] Citable literature includes: Cutler et al.,. J. Am. Chem. Soc. 2011 133:9254-9257, Hao et al., Small. 2011 7:3158-3162, Zhang et al., ACS Nano. 2011 5:6962-6970, Cutler et al., J. Am. Chem. Soc. 2012 134:1376-1391, Young et al.,. Nano Lett. 2012 12:3867-71, Zheng et al., Proc. Natl. Acad. Sci. USA. 2012 109:11975-80, Mirkin, Nanomedicine 2012 7:635-638 Zhang et al., J. Am. Chem. Soc. 2012 134:16488-1691, Weintraub, Nature 2013 495:S14-S16, Choi et

al., Proc. Natl. Acad. Sci. USA. 2013 110(19):7625-7630, Jensen et al., Sci. Transl. Med. 5, 209ra152 (2013) and Mirkin, et al., Small, doi.org/10.1002/smll.201302143.

[00393] Self-assembling nanoparticles with siRNA may be constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG), for example, as a means to target tumor neovasculature expressing integrins and used to deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby tumor angiogenesis (see, e.g., Schiffelers et al., Nucleic Acids Research, 2004, Vol. 32, No. 19). Nanoplexes may be prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. A dosage of about 100 to 200 mg of CRISPR Cas is envisioned for delivery in the self-assembling nanoparticles of Schiffelers et al.

[00394] The nanoplexes of Bartlett et al. (PNAS, September 25, 2007,vol. 104, no. 39) may also be applied to the present invention. The nanoplexes of Bartlett et al. are prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes. The DOTA-siRNA of Bartlett et al. was synthesized as follows: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA-NHSester) was ordered from Macrocyclics (Dallas, TX). The amine modified RNA sense strand with a 100-fold molar excess of DOTA-NHS-ester in carbonate buffer (pH 9) was added to a microcentrifuge tube. The contents were reacted by stirring for 4 h at room temperature. The DOTA-RNAsense conjugate was ethanol-precipitated, resuspended in water, and annealed to the unmodified antisense strand to yield DOTA-siRNA. All liquids were pretreated with Chelex-100 (Bio-Rad, Hercules, CA) to remove trace metal contaminants. Tf-targeted and nontargeted siRNA nanoparticles may be formed by using cyclodextrin-containing polycations. Typically, nanoparticles were formed in water at a charge ratio of 3 (+/-) and an siRNA concentration of 0.5 g/liter. One percent of the adamantane-PEG molecules on the surface of the targeted

nanoparticles were modified with Tf (adamantane-PEG-Tf). The nanoparticles were suspended in a 5% (wt/vol) glucose carrier solution for injection.

[00395] Davis et al. (Nature, Vol 464, 15 April 2010) conducts a siRNA clinical trial that uses a targeted nanoparticle-delivery system (clinical trial registration number NCT00689065). Patients with solid cancers refractory to standard-of-care therapies are administered doses of targeted nanoparticles on days 1, 3, 8 and 10 of a 21-day cycle by a 30-min intravenous infusion. The nanoparticles consist of a synthetic delivery system containing: (1) a linear, cyclodextrin-based polymer (CDP), (2) a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells, (3) a hydrophilic polymer (polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids), and (4) siRNA designed to reduce the expression of the RRM2 (sequence used in the clinic was previously denoted siR2B+5). The TFR has long been known to be upregulated in malignant cells, and RRM2 is an established anti-cancer target. These nanoparticles (clinical version denoted as CALAA-01) have been shown to be well tolerated in multi-dosing studies in non-human primates. Although a single patient with chronic myeloid leukaemia has been administered siRNAby liposomal delivery, Davis et al.'s clinical trial is the initial human trial to systemically deliver siRNA with a targeted delivery system and to treat patients with solid cancer. To ascertain whether the targeted delivery system can provide effective delivery of functional siRNA to human tumours, Davis et al. investigated biopsies from three patients from three different dosing cohorts; patients A, B and C, all of whom had metastatic melanoma and received CALAA-01 doses of 18, 24 and 30 mg m^{-2} siRNA, respectively. Similar doses may also be contemplated for the CRISPR Cas system of the present invention. The delivery of the invention may be achieved with nanoparticles containing a linear, cyclodextrin-based polymer (CDP), a human transferrin protein (TF) targeting ligand displayed on the exterior of the nanoparticle to engage TF receptors (TFR) on the surface of the cancer cells and/or a hydrophilic polymer (for example, polyethylene glycol (PEG) used to promote nanoparticle stability in biological fluids).

[00396] **Exosomes**

[00397] Exosomes are endogenous nano-vesicles that transport RNAs and proteins which can deliver short interfering (si)RNA to the brain in mice. To reduce immunogenicity, Alvarez-Erviti et al. (2011, Nat Biotechnol 29: 341) used self-derived dendritic cells for exosome production.

Targeting was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide. Purified exosomes were loaded with exogenous siRNA by electroporation. Intravenously injected RVG-targeted exosomes delivered GAPDH siRNA specifically to neurons, microglia, oligodendrocytes in the brain, resulting in a specific gene knockdown. Pre-exposure to RVG exosomes did not attenuate knockdown, and non-specific uptake in other tissues was not observed. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the strong mRNA (60%) and protein (62%) knockdown of BACE1, a therapeutic target in Alzheimer's disease.

[00398] To obtain a pool of immunologically inert exosomes, Alvarez-Erviti et al. harvested bone marrow from inbred C57BL/6 mice with a homogenous major histocompatibility complex (MHC) haplotype. As immature dendritic cells produce large quantities of exosomes devoid of T-cell activators such as MHC-II and CD86, Alvarez-Erviti et al. selected for dendritic cells with granulocyte/macrophage-colony stimulating factor (GM-CSF) for 7 d. Exosomes were purified from the culture supernatant the following day using well-established ultracentrifugation protocols. The exosomes produced were physically homogenous, with a size distribution peaking at 80 nm in diameter as determined by nanoparticle tracking analysis (NTA) and electron microscopy. Alvarez-Erviti et al. obtained 6-12 μg of exosomes (measured based on protein concentration) per 10^6 cells.

[00399] Next, Alvarez-Erviti et al. investigated the possibility of loading modified exosomes with exogenous cargoes using electroporation protocols adapted for nanoscale applications. As electroporation for membrane particles at the nanometer scale is not well-characterized, nonspecific Cy5-labeled siRNA was used for the empirical optimization of the electroporation protocol. The amount of encapsulated siRNA was assayed after ultracentrifugation and lysis of exosomes. Electroporation at 400 V and 125 μF resulted in the greatest retention of siRNA and was used for all subsequent experiments.

[00400] Alvarez-Erviti et al. administered 150 μg of each BACE1 siRNA encapsulated in 150 μg of RVG exosomes to normal C57BL/6 mice and compared the knockdown efficiency to four controls: untreated mice, mice injected with RVG exosomes only, mice injected with BACE1 siRNA complexed to an in vivo cationic liposome reagent and mice injected with BACE1 siRNA complexed to RVG-9R, the RVG pep tide conjugated to 9 D-arginines that electrostatically binds to the siRNA. Cortical tissue samples were analyzed 3 d after administration and a significant

protein knockdown (45%, P < 0.05, versus 62%, P < 0.01) in both siRNA-RVG-9R-treated and siRNARVG exosome-treated mice was observed, resulting from a significant decrease in BACE1 mRNA levels (66% [+ or -] 15%, P < 0.001 and 61% [+ or -] 13% respectively, P < 0.01). Moreover, Applicants demonstrated a significant decrease (55%, P < 0.05) in the total [beta]-amyloid 1-42 levels, a main component of the amyloid plaques in Alzheimer's pathology, in the RVG-exosome-treated animals. The decrease observed was greater than the β-amyloid 1-40 decrease demonstrated in normal mice after intraventricular injection of BACE1 inhibitors. Alvarez-Erviti et al. carried out 5'-rapid amplification of cDNA ends (RACE) on BACE1 cleavage product, which provided evidence of RNAi-mediated knockdown by the siRNA.

[00401] Finally, Alvarez-Erviti et al. investigated whether siRNA-RVG exosomes induced immune responses in vivo by assessing IL-6, IP-10, TNFα and IFN-α serum concentrations. Following siRNA-RVG exosome treatment, nonsignificant changes in all cytokines were registered similar to siRNA-transfection reagent treatment in contrast to siRNA-RVG-9R, which potently stimulated IL-6 secretion, confirming the immunologically inert profile of the exosome treatment. Given that exosomes encapsulate only 20% of siRNA, delivery with RVG-exosome appears to be more efficient than RVG-9R delivery as comparable mRNA knockdown and greater protein knockdown was achieved with fivefold less siRNA without the corresponding level of immune stimulation. This experiment demonstrated the therapeutic potential of RVG-exosome technology, which is potentially suited for long-term silencing of genes related to neurodegenerative diseases. The exosome delivery system of Alvarez-Erviti et al. may be applied to deliver the CRISPR-Cas system of the present invention to therapeutic targets, especially neurodegenerative diseases. A dosage of about 100 to 1000 mg of CRISPR Cas encapsulated in about 100 to 1000 mg of RVG exosomes may be contemplated for the present invention.

[00402] El-Andaloussi et al. (Nature Protocols 7,2112–2126(2012)) discloses how exosomes derived from cultured cells can be harnessed for delivery of siRNA in vitro and in vivo. This protocol first describes the generation of targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. Next, El-Andaloussi et al. explain how to purify and characterize exosomes from transfected cell supernatant. Next, El-Andaloussi et al. detail crucial steps for loading siRNA into exosomes. Finally, El-Andaloussi et al. outline how to use exosomes to efficiently deliver siRNA in vitro and in vivo in mouse brain. Examples of anticipated results in which exosome-mediated siRNA delivery is evaluated by

functional assays and imaging are also provided. The entire protocol takes ~3 weeks. Delivery or administration according to the invention may be performed using exosomes produced from self-derived dendritic cells.

[00403] In another embodiment, the plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) are contemplated. Exosomes are nano-sized vesicles (30–90nm in size) produced by many cell types, including dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumor cells. These vesicles are formed by inward budding of late endosomes and are then released to the extracellular environment upon fusion with the plasma membrane. Because exosomes naturally carry RNA between cells, this property might be useful in gene therapy.

[00404] Exosomes from plasma are prepared by centrifugation of buffy coat at 900g for 20 min to isolate the plasma followed by harvesting cell supernatants, centrifuging at 300g for 10 min to eliminate cells and at 16 500g for 30 min followed by filtration through a 0.22 mm filter. Exosomes are pelleted by ultracentrifugation at 120 000g for70 min. Chemical transfection of siRNA into exosomes is carried out according to the manufacturer's instructions in RNAi Human/Mouse Starter Kit (Quiagen, Hilden, Germany). siRNA is added to 100 ml PBS at a final concentration of 2 mmol/ml. After adding HiPerFect transfection reagent, the mixture is incubated for 10 min at RT. In order to remove the excess of micelles, the exosomes are re-isolated using aldehyde/sulfate latex beads. The chemical transfection of CRISPR Cas into exosomes may be conducted similarly to siRNA. The exosomes may be co-cultured with monocytes and lymphocytes isolated from the peripheral blood of healthy donors. Therefore, it may be contemplated that exosomes containing CRISPR Cas may be introduced to monocytes and lymphocytes of and autologously reintroduced into a human. Accordingly, delivery or administration according to the invention may beperformed using plasma exosomes.

[00405] **Liposomes**

[00406] Delivery or administration according to the invention can be performed with liposomes. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load

across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00407] Liposomes can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00408] Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure and to prevent the leakage of the liposomal inner cargo. Further, liposomes are prepared from hydrogenated egg phosphatidylcholine or egg phosphatidylcholine, cholesterol, and dicetyl phosphate, and their mean vesicle sizes were adjusted to about 50 and 100 nm. (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00409] Conventional liposome formulation is mainly comprised of natural phospholipids and lipids such as 1,2-distearoryl-sn-glycero-3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholines and monosialoganglioside. Since this formulation is made up of phospholipids only, liposomal formulations have encountered many challenges, one of the ones being the instability in plasma. Several attempts to overcome these challenges have been made, specifically in the manipulation of the lipid membrane. One of these attempts focused on the manipulation of cholesterol. Addition of cholesterol to conventional formulations reduces rapid release of the encapsulated bioactive compound into the plasma or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) increases the stability (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review).

[00410] In a particularly advantageous embodiment, Trojan Horse liposomes (also known as Molecular Trojan Horses) are desirable and protocols may be found at http://cshprotocols.cshlp.org/content/2010/4/pdb.prot5407.long. These particles allow delivery of

a transgene to the entire brain after an intravascular injection. Without being bound by limitation, it is believed that neutral lipid particles with specific antibodies conjugated to surface allow crossing of the blood brain barrier via endocytosis. Applicant postulates utilizing Trojan Horse Liposomes to deliver the CRISPR family of nucleases to the brain via an intravascular injection, which would allow whole brain transgenic animals without the need for embryonic manipulation. About 1-5 g of nucleic acid molecule, e.g., DNA, RNA may be contemplated for in vivo administration in liposomes.

[00411] In another embodiment, the CRISPR Cas system may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., Nature Biotechnology, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of a specific CRISPR Cas targeted in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks. In another embodiment, a specific CRISPR Cas encapsulated SNALP) administered by intravenous injection to at doses of abpit 1 or 2.5 mg/kg are also contemplated (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006). The SNALP formulation may contain the lipids 3-N-[(wmethoxypoly(ethylene glycol) 2000) carbamoyl] -! ,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio (see, e.g., Zimmerman et al., Nature Letters, Vol. 441, 4 May 2006).

[00412] In another embodiment, stable nucleic-acid-lipid particles (SNALPs) have proven to be effective delivery molecules to highly vascularized HepG2-derived liver tumors but not in poorly vascularized HCT-116 derived liver tumors (see, e.g., Li, Gene Therapy (2012) 19, 775–780). The SNALP liposomes may be prepared by formulating D-Lin-DMA and PEG-C-DMA with distearoylphosphatidylcholine (DSPC), Cholesterol and siRNA using a 25:1 lipid/siRNA ratio and a 48/40/10/2 molar ratio of Cholesterol/D-Lin-DMA/DSPC/PEG-C-DMA. The resulted SNALP liposomes are about 80–100 nm in size.

[00413] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich, St Louis, MO, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA), 3-N-[(w-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxypropylamine, and cationic 1,2-dilinoleyloxy-3-N,Ndimethylaminopropane (see,

e.g., Geisbert et al., Lancet 2010; 375: 1896-905). A dosage of about 2 mg/kg total CRISPR Cas per dose administered as, for example, a bolus intravenous infusion may be contemplated.

[00414] In yet another embodiment, a SNALP may comprise synthetic cholesterol (Sigma-Aldrich), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids Inc.), PEG-cDMA, and 1,2-dilinoleyloxy-3-(N;N-dimethyl)aminopropane (DLinDMA) (see, e.g., Judge, J. Clin. Invest. 119:661-673 (2009)). Formulations used for in vivo studies may comprise a final lipid/RNA mass ratio of about 9:1.

[00415] The safety profile of RNAi nanomedicines has been reviewed by Barros and Gollob of Alnylam Pharmaceuticals (see, e.g., Advanced Drug Delivery Reviews 64 (2012) 1730–1737). The stable nucleic acid lipid particle (SNALP) is comprised of four different lipids — an ionizable lipid (DLinDMA) that is cationic at low pH, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG)-lipid. The particle is approximately 80 nm in diameter and is charge-neutral at physiologic pH. During formulation, the ionizable lipid serves to condense lipid with the anionic siRNA during particle formation. When positively charged under increasingly acidic endosomal conditions, the ionizable lipid also mediates the fusion of SNALP with the endosomal membrane enabling release of siRNA into the cytoplasm. The PEG-lipid stabilizes the particle and reduces aggregation during formulation, and subsequently provides a neutral hydrophilic exterior that improves pharmacokinetic properties.

[00416] To date, two clinical programs have been initiated using SNALPsiRNA formulations. Tekmira Pharmaceuticals recently completed a phase I single-dose study of SNALP-ApoB in adult volunteers with elevated LDL cholesterol. ApoB is predominantly expressed in the liver and jejunum and is essential for the assembly and secretion of VLDL and LDL. ApoB is also succesfuly targeted by our CrISPR-Cas systems, see examples 38-39. Seventeen subjects received a single dose of SNALP-ApoB (dose escalation across 7 dose levels). There was no evidence of liver toxicity (anticipated as the potential dose-limiting toxicity based on preclinical studies). One (of two) subjects at the highest dose experienced flu-like symptoms consistent with immune system stimulation, and the decision was made to conclude the trial.

[00417] Alnylam Pharmaceuticals has similarly advanced ALN-TTR01, which employs the SNALP technology described above and targets hepatocyte production of both mutant and wild-type TTR to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) —

both caused by autosomal dominant mutations in TTR; and senile systemic amyloidosis (SSA) cause by wildtype TTR. A placebo-controlled, single dose-escalation phase I trial of ALN-TTR01 was recently completed in patients with ATTR. ALN-TTR01 was administered as a 15-minute IV infusion to 31 patients (23 with study drug and 8 with placebo) within a dose range of 0.01 to 1.0 mg/kg (based on siRNA). Treatmentwaswell tolerated with no significant increases in liver function tests. Infusion-related reactions were noted in 3 of 23 patients at≥0.4 mg/kg; all responded to slowing of the infusion rate and all continued on study. Minimal and transient elevations of serum cytokines IL-6, IP-10 and IL-1ra were noted in two patients at the highest dose of 1 mg/kg (as anticipated from preclinical and NHP studies). Lowering of serum TTR, the expected pharmacodynamics effect of ALN-TTR01, was observed at 1 mg/kg.

[00418] In yet another embodiment, a SNALP may be made by solubilizing a cationic lipid, DSPC, cholesterol and PEG-lipid were solubilized in ethanol at a molar ratio of 40:10:40:10, respectively (see, Semple et al., Nature Niotechnology, Volume 28 Number 2 February 2010, pp. 172-177). The lipid mixture was added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/ml, respectively, and allowed to equilibrate at 22 °C for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22 °C using a Lipex Extruder (Northern Lipids) until a vesicle diameter of 70–90 nm, as determined by dynamic light scattering analysis, was obtained. This generally required 1–3 passes. The siRNA (solubilized in a 50 mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35 °C) vesicles at a rate of ~5 ml/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was reached, the mixture was incubated for a further 30 min at 35 °C to allow vesicle reorganization and encapsulation of the siRNA. The ethanol was then removed and the external buffer replaced with PBS (155 mM NaCl, 3 mM Na2HPO4, 1 mM KH2PO4, pH 7.5) by either dialysis or tangential flow diafiltration. siRNA were encapsulated in SNALP using a controlled step-wise dilution method process. The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid), dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids), synthetic cholesterol (Sigma) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 μm filter before use. Mean particle sizes were 75–85 nm and 90–95% of the siRNA was encapsulated within the lipid particles. The final siRNA/lipid ratio in formulations used for in

vivo testing was ~0.15 (wt/wt). LNP-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile PBS immediately before use and the formulations were administered intravenously through the lateral tail vein in a total volume of 10 ml/kg. This method may be extrapolated to the CRISPR Cas system of the present invention.

[00419] **Other Lipids**

[00420] Other cationic lipids, such as amino lipid 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) may be utilized to encapsulate CRISPR Cas similar to SiRNA (see, e.g., Jayaraman, Angew. Chem. Int. Ed. 2012, 51, 8529 –8533). A preformed vesicle with the following lipid composition may be contemplated: amino lipid, distearoylphosphatidylcholine (DSPC), cholesterol and (R)-2,3-bis(octadecyloxy) propyl-1-(methoxy poly(ethylene glycol)2000)propylcarbamate (PEG-lipid) in the molar ratio 40/10/40/10, respectively, and a FVII siRNA/total lipid ratio of approximately 0.05 (w/w). To ensure a narrow particle size distribution in the range of 70–90 nm and a low polydispersity index of 0.11_0.04 (n=56), the particles may be extruded up to three times through 80 nm membranes prior to adding the CRISPR Cas RNA. Particles containing the highly potent amino lipid 16 may be used, in which the molar ratio of the four lipid components 16, DSPC, cholesterol and PEG-lipid (50/10/38.5/1.5) which may be further optimized to enhance in vivo activity.

[00421] Michael S D Kormann et al. ("Expression of therapeutic proteins after delivery of chemically modified mRNA in mice: Nature Biotechnology, Volume:29, Pages: 154–157 (2011) Published online 09 January 2011) describes the use of lipid envelopes to deliver RNA. Use of lipid envelopes is also preferred in the present invention.

[00422] In another embodiment, lipids may be formulated with the CRISPR Cas system of the present invention to form lipid nanoparticles (LNPs). Lipids include, but are not limited to, DLin-KC2-DMA4, C12-200 and colipids disteroylphosphatidyl choline, cholesterol, and PEG-DMG may be formulated with CRISPR Cas instead of siRNA (see, e.g., Novobrantseva, Molecular Therapy--Nucleic Acids (2012) 1, e4; doi:10.1038/mtna.2011.3) using a spontaneous vesicle formation procedure. The component molar ratio may be about 50/10/38.5/1.5 (DLin-KC2-DMA or C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG). The final lipid:siRNA weight ratio may be ~12:1 and 9:1 in the case of DLin-KC2-DMA and C12-200

lipid nanoparticles (LNPs), respectively. The formulations may have mean particle diameters of ~80 nm with >90% entrapment efficiency. A 3 mg/kg dose may be contemplated.

[00423] Tekmira has a portfolio of approximately 95 patent families, in the U.S. and abroad, that are directed to various aspects of LNPs and LNP formulations (see, e.g., U.S. Pat. Nos. 7,982,027; 7,799,565; 8,058,069; 8,283,333; 7,901,708; 7,745,651; 7,803,397; 8,101,741; 8,188,263; 7,915,399; 8,236,943 and 7,838,658 and European Pat. Nos .1766035; 1519714; 1781593 and 1664316), all of which may be used/and or adapted to the present invention.

[00424] The CRISPR Cas system may be delivered encapsulated in PLGA Microspheres such as that further described in US published applications 20130252281 and 20130245107 and 20130244279 (assigned to Moderna Therapeutics) which relate to aspects of formulation of compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid:fusogenic lipid:cholesterol:PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC. See also, Schrum et al., Delivery and Formulation of Engineered Nucleic Acids, US published application 20120251618.

[00425] Nanomerics' technology addresses bioavailability challenges for a broad range of therapeutics, including low molecular weight hydrophobic drugs, peptides, and nucleic acid based therapeutics (plasmid, siRNA, miRNA). Specific administration routes for which the technology has demonstrated clear advantages include the oral route, transport across the blood-brain-barrier, delivery to solid tumours, as well as to the eye. See, e.g., Mazza et al., 2013, ACS Nano. 2013 Feb 26;7(2):1016-26; Uchegbu and Siew, 2013, J Pharm Sci. 102(2):305-10 and Lalatsa et al., 2012, J Control Release. 2012 Jul 20;161(2):523-36.

[00426] US Patent Publication No. 20050019923 describes cationic dendrimers for delivering bioactive molecules, such as polynucleotide molecules, peptides and polypeptides and/or pharmaceutical agents, to a mammalian body. The dendrimers are suitable for targeting the delivery of the bioactive molecules to, for example, the liver, spleen, lung, kidney or heart. Dendrimers are synthetic 3-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers are synthesised from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core

(convergent approach to synthesis) and each addition of a 3-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers start from a diaminobutane core to which is added twice the number of amino groups by a double Michael addition of acrylonitrile to the primary amines followed by the hydrogenation of the nitriles. This results in a doubling of the amino groups. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups (generation 5, DAB 64). Protonable groups are usually amine groups which are able to accept protons at neutral pH. The use of dendrimers as gene delivery agents has largely focused on the use of the polyamidoamine. and phosphorous containing compounds with a mixture of amine/amide or N--P(O_2)S as the conjugating units respectively with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. Polypropylenimine dendrimers have also been studied as pH sensitive controlled release systems for drug delivery and for their encapsulation of guest molecules when chemically modified by peripheral amino acid groups. The cytotoxicity and interaction of polypropylenimine dendrimers with DNA as well as the transfection efficacy of DAB 64 has also been studied.

[00427] US Patent Publication No. 20050019923 is based upon the observation that, contrary to earlier reports, cationic dendrimers, such as polypropylenimine dendrimers, display suitable properties, such as specific targeting and low toxicity, for use in the targeted delivery of bioactive molecules, such as genetic material. In addition, derivatives of the cationic dendrimer also display suitable properties for the targeted delivery of bioactive molecules. See also, Bioactive Polymers, US published application 20080267903, which discloses "Various polymers, including cationic polyamine polymers and dendrimeric polymers, are shown to possess anti-proliferative activity, and may therefore be useful for treatment of disorders characterised by undesirable cellular proliferation such as neoplasms and tumours, inflammatory disorders (including autoimmune disorders), psoriasis and atherosclerosis. The polymers may be used alone as active agents, or as delivery vehicles for other therapeutic agents, such as drug molecules or nucleic acids for gene therapy. In such cases, the polymers' own intrinsic anti-tumour activity may complement the activity of the agent to be delivered."

[00428] **Supercharged proteins**

[00429] Supercharged proteins are a class of engineered or naturally occurring proteins with unusually high positive or negative net theoretical charge. Both supernegatively and

superpositively charged proteins exhibit a remarkable ability to withstand thermally or chemically induced aggregation. Superpositively charged proteins are also able to penetrate mammalian cells. Associating cargo with these proteins, such as plasmid DNA, siRNA, or other proteins, can enable the functional delivery of these macromolecules into mammalian cells both in vitro and in vivo. David Liu's lab reported the creation and characterization of supercharged proteins in 2007 (Lawrence et al., 2007, Journal of the American Chemical Society 129, 10110–10112).

[00430] The nonviral delivery of siRNA and plasmid DNA into mammalian cells are valuable both for research and therapeutic applications (Akinc et al., 2010, Nat. Biotech. 26, 561–569). Purified +36 GFP protein (or other superpositively charged protein) is mixed with siRNAs in the appropriate serum-free media and allowed to complex prior addition to cells. Inclusion of serum at this stage inhibits formation of the supercharged protein-siRNA complexes and reduces the effectiveness of the treatment. The following protocol has been found to be effective for a variety of cell lines (McNaughton et al., 2009, Proc. Natl. Acad. Sci. USA 106, 6111–6116). However, pilot experiments varying the dose of protein and siRNA should be performed to optimize the procedure for specific cell lines.

(1) One day before treatment, plate 1 x 10^5 cells per well in a 48-well plate.

(2) On the day of treatment, dilute purified +36 GFP protein in serumfree media to a final concentration 200nM. Add siRNA to a final concentration of 50nM. Vortex to mix and incubate at room temperature for 10min.

(3) During incubation, aspirate media from cells and wash once with PBS.

(4) Following incubation of +36 GFP and siRNA, add the protein-siRNA complexes to cells.

(5) Incubate cells with complexes at 37 C for 4h.

(6) Following incubation, aspirate the media and wash three times with 20 U/mL heparin PBS. Incubate cells with serum-containing media for a further 48h or longer depending upon the assay for knockdown.

(7) Analyze cells by immunoblot, qPCR, phenotypic assay, or other appropriate method.

[00431] It has been found +36 GFP to be an effective plasmid delivery reagent in a range of cells. As plasmid DNA is a larger cargo than siRNA, proportionately more +36 GFP protein is

required to effectively complex plasmids. For effective plasmid delivery Applicants have developed a variant of +36 GFP bearing a C-terminal HA2 peptide tag, a known endosome-disrupting peptide derived from the influenza virus hemagglutinin protein. The following protocol has been effective in a variety of cells, but as above it is advised that plasmid DNA and supercharged protein doses be optimized for specific cell lines and delivery applications.

(1) One day before treatment, plate 1 x 10^5 per well in a 48-well plate.

(2) On the day of treatment, dilute purified þ36 GFP protein in serumfree media to a final concentration 2 mM. Add 1mg of plasmid DNA. Vortex to mix and incubate at room temperature for 10min.

(3) During incubation, aspirate media from cells and wash once with PBS.

(4) Following incubation of þ36 GFP and plasmid DNA, gently add the protein-DNA complexes to cells.

(5) Incubate cells with complexes at 37 C for 4h.

(6) Following incubation, aspirate the media and wash with PBS. Incubate cells in serum-containing media and incubate for a further 24–48h.

(7) Analyze plasmid delivery (e.g., by plasmid-driven gene expression) as appropriate.

[00432] See also, e.g., McNaughton et al., Proc. Natl. Acad. Sci. USA 106, 6111-6116 (2009); Cronican et al., ACS Chemical Biology 5, 747-752 (2010); Cronican et al., Chemistry & Biology 18, 833-838 (2011); Thompson et al., Methods in Enzymology 503, 293-319 (2012); Thompson, D.B., et al., Chemistry & Biology 19 (7), 831-843 (2012). The methods of the super charged proteins may be used and/or adapted for delivery of the CRISPR Cas system of the present invention.

[00433] **Cell penetrating peptides**

[00434] In yet another embodiment, cell penetrating peptides (CPPs) are contemplated for the delivery of the CRISPR Cas system. CPPs are short peptides that facilitate cellular uptake of various molecular cargo (from nanosize particles to small chemical molecules and large fragments of DNA). The term "cargo" as used herein includes but is not limited to the group consisting of therapeutic agents, diagnostic probes, peptides, nucleic acids, antisense oligonucleotides, plasmids, proteins, nanoparticles, liposomes, chromophores, small molecules and radioactive materials. In aspects of the invention, the cargo may also comprise any

component of the CRISPR Cas system or the entire functional CRISPR Cas system. Aspects of the present invention further provide methods for delivering a desired cargo into a subject comprising: (a) preparing a complex comprising the cell penetrating peptide of the present invention and a desired cargo, and (b) orally, intraarticularly, intraperitoneally, intrathecally, intrarterially, intranasally, intraparenchymally, subcutaneously, intramuscularly, intravenously, dermally, intrarectally, or topically administering the complex to a subject. The cargo is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions.

[00435] The function of the CPPs are to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells. Cell-penetrating peptides are of different sizes, amino acid sequences, and charges but all CPPs have one distinct characteristic, which is the ability to translocate the plasma membrane and facilitate the delivery of various molecular cargoes to the cytoplasm or an organelle. CPP translocation may be classified into three main entry mechanisms: direct penetration in the membrane, endocytosis-mediated entry, and translocation through the formation of a transitory structure. CPPs have found numerous applications in medicine as drug delivery agents in the treatment of different diseases including cancer and virus inhibitors, as well as contrast agents for cell labeling. Examples of the latter include acting as a carrier for GFP, MRI contrast agents, or quantum dots. CPPs hold great potential as in vitro and in vivo delivery vectors for use in research and medicine. CPPs typically have an amino acid composition that either contains a high relative abundance of positively charged amino acids such as lysine or arginine or has sequences that contain an alternating pattern of polar/charged amino acids and non-polar, hydrophobic amino acids. These two types of structures are referred to as polycationic or amphipathic, respectively. A third class of CPPs are the hydrophobic peptides, containing only apolar residues, with low net charge or have hydrophobic amino acid groups that are crucial for cellular uptake. One of the initial CPPs discovered was the trans-activating transcriptional activator (Tat) from Human Immunodeficiency Virus 1 (HIV-1) which was found to be efficiently taken up from the surrounding media by numerous cell types in culture. Since then, the number of known CPPs has expanded considerably and small molecule synthetic analogues with more effective protein transduction properties have been generated. CPPs include but are not limited to Penetratin, Tat (48-60), Transportan, and (R-AhX-R4) (Ahx=aminohexanoyl).

[00436] U.S. patent 8,372,951provides a CPP derived from eosinophil cationic protein (ECP) which exhibits highly cell-penetrating efficiency and low toxicity. Aspects of delivering the CPP with its cargo into a vertebrate subject are also provided. Further aspects of CPPs and their delivery may be in U.S. patents 8,575,305; 8;614,194 and 8,044,019.

[00437] That CPPs can be employed to deliver the CRISPR-Cas system is also provided in the manuscript "Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA", by Suresh Ramakrishna, Abu-Bonsrah Kwaku Dad, Jagadish Beloor, et al. Genome Res. 2014 Apr 2. [Epub ahead of print], incorporated by reference in its entirety, wherein it is demonstrated that treatment with CPP-conjugated recombinant Cas9 protein and CPP-complexed guide RNAs lead to endogenous gene disruptions in human cell lines. In the paper the Cas9 protein was conjugated to CPP via a thioether bond, whereas the guide RNA was complexed with CPP, forming condensed, positively charged nanoparticles. It was shown that simultaneous and sequential treatment of human cells, including embryonic stem cells, dermal fibroblasts, HEK293T cells, HeLa cells, and embryonic carcinoma cells, with the modified Cas9 and guide RNA led to efficient gene disruptions with reduced off-target mutations relative to plasmid transfections.

[00438] **Implantable devices**

[00439] In another embodiment, implantable devices are also contemplated for delivery of the CRISPR Cas system. For example, US Patent Publication 20110195123 discloses an implantable medical device which elutes a drug locally and in prolonged period is provided, including several types of such a device, the treatment modes of implementation and methods of implantation. The device comprising of polymeric substrate, such as a matrix for example, that is used as the device body, and drugs, and in some cases additional scaffolding materials, such as metals or additional polymers, and materials to enhance visibility and imaging. The selection of drug is based on the advantageous of releasing drug locally and in prolonged period, where drug is released directly to the extracellular matrix (ECM) of the diseased area such as tumor, inflammation, degeneration or for symptomatic objectives, or to injured smooth muscle cells, or for prevention. One kind of drug is the gene silencing drugs based on RNA interference (RNAi), including but not limited to si RNA, sh RNA, or antisense RNA/DNA, ribozyme and nucleoside analogs. Therefore, this system may be used/and or adapted to the CRISPR Cas system of the present invention. The modes of implantation in some embodiments are existing implantation procedures that are

developed and used today for other treatments, including brachytherapy and needle biopsy. In such cases the dimensions of the new implant described in this invention are similar to the original implant. Typically a few devices are implanted during the same treatment procedure.

[00440] As described in US Patent Publication 20110195123, there is provided a drug delivery implantable or insertable system, including systems applicable to a cavity such as the abdominal cavity and/or any other type of administration in which the drug delivery system is not anchored or attached, comprising a biostable and/or degradable and/or bioabsorbable polymeric substrate, which may for example optionally be a matrix. It should be noted that the term "insertion" also includes implantation. The drug delivery system is preferably implemented as a "Loder" as described in US Patent Publication 20110195123.

[00441] The polymer or plurality of polymers are biocompatible, incorporating an agent and/or plurality of agents, enabling the release of agent at a controlled rate, wherein the total volume of the polymeric substrate, such as a matrix for example, in some embodiments is optionally and preferably no greater than a maximum volume that permits a therapeutic level of the agent to be reached. As a non-limiting example, such a volume is preferably within the range of 0.1 m^3 to 1000 mm^3, as required by the volume for the agent load. The Loder may optionally be larger, for example when incorporated with a device whose size is determined by functionality, for example and without limitation, a knee joint, an intra-uterine or cervical ring and the like.

[00442] The drug delivery system (for delivering the composition) is designed in some embodiments to preferably employ degradable polymers, wherein the main release mechanism is bulk erosion; or in some embodiments, non degradable, or slowly degraded polymers are used, wherein the main release mechanism is diffusion rather than bulk erosion, so that the outer part functions as membrane, and its internal part functions as a drug reservoir, which practically is not affected by the surroundings for an extended period (for example from about a week to about a few months). Combinations of different polymers with different release mechanisms may also optionally be used. The concentration gradient at the surface is preferably maintained effectively constant during a significant period of the total drug releasing period, and therefore the diffusion rate is effectively constant (termed "zero mode" diffusion). By the term "constant" it is meant a diffusion rate that is preferably maintained above the lower threshold of therapeutic effectiveness, but which may still optionally feature an initial burst and/or fluctuate, for example

increasing and decreasing to a certain degree. The diffusion rate is preferably so maintained for a prolonged period, and it can be considered constant to a certain level to optimize the therapeutically effective period, for example the effective silencing period.

[00443] The drug delivery system optionally and preferably is designed to shield the nucleotide based therapeutic agent from degradation, whether chemical in nature or due to attack from enzymes and other factors in the body of the subject.

[00444] The drug delivery system as described in US Patent Publication 20110195123 is optionally associated with sensing and/or activation appliances that are operated at and/or after implantation of the device, by non and/or minimally invasive methods of activation and/or acceleration/deceleration, for example optionally including but not limited to thermal heating and cooling, laser beams, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices.

[00445] According to some embodiments of US Patent Publication 20110195123, the site for local delivery may optionally include target sites characterized by high abnormal proliferation of cells, and suppressed apoptosis, including tumors, active and or chronic inflammation and infection including autoimmune diseases states, degenerating tissue including muscle and nervous tissue, chronic pain, degenerative sites, and location of bone fractures and other wound locations for enhancement of regeneration of tissue, and injured cardiac, smooth and striated muscle. The site for local delivery also may optionally include sites enabling performing preventive activities including pregnancy, prevention of infection and aging.

[00446] The site for implantation of the composition, or target site, preferably features a radius, area and/or volume that is sufficiently small for targeted local delivery. For example, the target site optionally has a diameter in a range of from about 0.1 mm to about 5 cm.

[00447] The location of the target site is preferably selected for maximum therapeutic efficacy. For example, the composition of the drug delivery system (optionally with a device for implantation as described above) is optionally and preferably implanted within or in the proximity of a tumor environment, or the blood supply associated thereof.

[00448] For example the composition (optionally with the device) is optionally implanted within or in the proximity to pancreas, prostate, breast, liver, via the nipple, within the vascular system and so forth.

[00449] The target location is optionally selected from the group consisting of (as non-limiting examples only, as optionally any site within the body may be suitable for implanting a Loder): 1. brain at degenerative sites like in Parkinson or Alzheimer disease at the basal ganglia, white and gray matter; 2. spine as in the case of amyotrophic lateral sclerosis (ALS); 3. uterine cervix to prevent HPV infection; 4. active and chronic inflammatory joints; 5. dermis as in the case of psoriasis; 6. sympathetic and sensoric nervous sites for analgesic effect; 7. Intra osseous implantation; 8. acute and chronic infection sites; 9. Intra vaginal; 10. Inner ear--auditory system, labyrinth of the inner ear, vestibular system; 11. Intra tracheal; 12. Intra-cardiac; coronary, epicardiac; 13. urinary bladder; 14. biliary system; 15. parenchymal tissue including and not limited to the kidney, liver, spleen; 16. lymph nodes; 17. salivary glands; 18. dental gums; 19. Intra-articular (into joints); 20. Intra-ocular; 21. Brain tissue; 22. Brain ventricles; 23. Cavities, including abdominal cavity (for example but without limitation, for ovary cancer); 24. Intra esophageal and 25. Intra rectal.

[00450] Optionally insertion of the system (for example a device containing the composition) is associated with injection of material to the ECM at the target site and the vicinity of that site to affect local pH and/or temperature and/or other biological factors affecting the diffusion of the drug and/or drug kinetics in the ECM, of the target site and the vicinity of such a site.

[00451] Optionally, according to some embodiments, the release of said agent could be associated with sensing and/or activation appliances that are operated prior and/or at and/or after insertion, by non and/or minimally invasive and/or else methods of activation and/or acceleration/deceleration, including laser beam, radiation, thermal heating and cooling, and ultrasonic, including focused ultrasound and/or RF (radiofrequency) methods or devices, and chemical activators.

[00452] According to other embodiments of US Patent Publication 20110195123, the drug preferably comprises a gene silencing biological RNAi drug, for example for localized cancer cases in breast, pancreas, brain, kidney, bladder, lung, and prostate as described below. Moreover, many drugs other than siRNA are applicable to be encapsulated in Loder, and can be used in association with this invention, as long as such drugs can be encapsulated with the Loder substrate, such as a matrix for example. Such drugs include approved drugs that are delivered today by methods other than of this invention, including Amphotericin B for fungal infection; antibiotics such as in osteomyelitis; pain killers such as narcotics; anti degenerative such as in

Alzheimer or Parkinson diseases in a Loder implanted in the vicinity of the spine in the case of back pain. Such a system may be used and/or adapted to deliver the CRISPR Cas system of the present invention.

[00453] For example, for specific applications such as prevention of growth or regrowth of smooth muscle cells (that are injured during a stenting procedure and as a result tend to proliferate), the drug may optionally be siRNA that silence smooth muscle cells, including H19 silencing, or a drug selected from the group consisting of taxol, rapamycin and rapamycin-analogs. In such cases the Loder is preferably either a Drug Eluting Stent (DES), with prolonged release at constant rate, or a dedicated device that is implanted separately, in association to the stent. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00454] As another example of a specific application, neuro and muscular degenerative diseases develop due to abnormal gene expression. Local delivery of silencing RNAs may have therapeutic properties for interfering with such abnormal gene expression. Local delivery of anti apoptotic, anti inflammatory and anti degenerative drugs including small drugs and macromolecules may also optionally be therapeutic. In such cases the Loder is applied for prolonged release at constant rate and/or through a dedicated device that is implanted separately. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00455] As yet another example of a specific application, psychiatric and cognitive disorders are treated with gene modifiers. Gene knockdown with silencing RNA is a treatment option. Loders locally delivering nucleotide based agents to central nervous system sites are therapeutic options for psychiatric and cognitive disorders including but not limited to psychosis, bi-polar diseases, neurotic disorders and behavioral maladies. The Loders could also deliver locally drugs including small drugs and macromolecules upon implantation at specific brain sites. All of this may be used and/or adapted to the CRISPR Cas system of the present invention.

[00456] As another example of a specific application, silencing of innate and/or adaptive immune mediators at local sites enables the prevention of organ transplant rejection. Local delivery of silencing RNAs and immunomodulating reagents with the Loder implanted into the transplanted organ and/or the implanted site renders local immune suppression by repelling immune cells such as CD8 activated against the transplanted organ. All of this may be used/and or adapted to the CRISPR Cas system of the present invention.

[00457] As another example of a specific application, vascular growth factors including VEGFs and angiogenin and others are essential for neovascularization. Local delivery of the factors, peptides, peptidomimetics, or suppressing their repressors is an important therapeutic modality; silencing the repressors and local delivery of the factors, peptides, macromolecules and small drugs stimulating angiogenesis with the Loder is therapeutic for peripheral, systemic and cardiac vascular disease.

[00458] The method of insertion, such as implantation, may optionally already be used for other types of tissue implantation and/or for insertions and/or for sampling tissues, optionally without modifications, or alternatively optionally only with non-major modifications in such methods. Such methods optionally include but are not limited to brachytherapy methods, biopsy, endoscopy with and/or without ultrasound, such as ERCP, stereotactic methods into the brain tissue, Laparoscopy, including implantation with a laparoscope into joints, abdominal organs, the bladder wall and body cavities.

[00459] **CRISPR enzyme mRNA and guide RNA**

[00460] CRISPR enzyme mRNA and guide RNA might also be delivered separately. CRISPR enzyme mRNA can be delivered prior to the guide RNA to give time for CRISPR enzyme to be expressed. CRISPR enzyme mRNA might be administered 1-12 hours (preferably around 2-6 hours) prior to the administration of guide RNA.

[00461] Alternatively, CRISPR enzyme mRNA and guide RNA can be administered together. Advantageously, a second booster dose of guide RNA can be administered 1-12 hours (preferably around 2-6 hours) after the initial administration of CRISPR enzyme mRNA + guide RNA.

[00462] Additional administrations of CRISPR enzyme mRNA and/or guide RNA might be useful to achieve the most efficient levels of genome modification.

[00463] For minimization of toxicity and off-target effect, it will be important to control the concentration of CRISPR enzyme mRNA and guide RNA delivered. Optimal concentrations of CRISPR enzyme mRNA and guide RNA can be determined by testing different concentrations in a cellular or animal model and using deep sequencing the analyze the extent of modification at potential off-target genomic loci. For example, for the guide sequence targeting 5'-GAGTCCGAGCAGAAGAAGAA-3' (SEQ ID NO:) in the EMX1 gene of the human genome, deep sequencing can be used to assess the level of modification at the following two off-target

loci, 1: 5'-GAGTCCTAGCAGGAGAAGAA-3' (SEQ ID NO:) and 2: 5'-GAGTCTAAGCAGAAGAAGAA-3' (SEQ ID NO:). The concentration that gives the highest level of on-target modification while minimizing the level of off-target modification should be chosen for in vivo delivery.

[00464] Alternatively, to minimize the level of toxicity and off-target effect, CRISPR enzyme nickase mRNA (for example S. pyogenes Cas9 with the D10A mutation) can be delivered with a pair of guide RNAs targeting a site of interest. The two guide RNAs need to be spaced as follows. Guide sequences in red (single underline) and blue (double underline) respectively (these examples are based on the PAM requirement for *Streptococcus pyogenes* Cas9).

Overhang length (bp)	Guide RNA design (guide sequence and PAM color coded)
14	5'- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN NNNGGNNNNNNNNNNNNNN-3' 3'- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN NNNCCNNNNNNNNNNNNNNN-5'
13	
12	5'- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN NNGGNNNNNNNNNNNNNN-3' 3'- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNN NNNCCNNNNNNNNNNNNNNN-5'
11	
10	5'- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNN NGGNNNNNNNNNNNNNNN-3' 3'-
9	

8	NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNN-5’
7	5’- NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN-3’
6	3’- NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN-5’
5	5’- NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN-3’
4	3’- NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN-5’
3	5’- NNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN-3’
2	3’- NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN-5’
1	5’- NNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN-3’
blunt	3’- NNNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNNNN-5’

2	5’- NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNGGNN NNNNNNNNNNNNNNNNNNN-3’
3	3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNCCNN NNNNNNNNNNNNNNNNNNN-5’
4	5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNGGNNN
5	NNNNNNNNNNNNNNNNNNN-3’
6	3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNCCNNN NNNNNNNNNNNNNNNNNNN-5’
7	5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNGGNNNN NNNNNNNNNNNNNNNNNNN-3’
8	3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNCCNNNN NNNNNNNNNNNNNNNNNNN-5’
12	5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNNNNGGNNNN NNNNNNNNNNNNNNNNNNN-3’
13	3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNCCNNNN NNNNNNNNNNNNNNNNNNN-5’
14	5’-

15	NNNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNGGNNNNNN NNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNCCNNNNNN
16	NNNNNNNNNNNNNNNNNNNN-5’
17	5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNGGNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNCCNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNGGNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNCCNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNGGNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNCCNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNNNNNNNNGGNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’

	3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNCCNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNCCNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNNCCNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNCCNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNNNCCNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN

	NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNNCCNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCNGGNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGNCCNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNGGCCNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNGGNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNCCGGNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNN-5’

	5’- NNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNNCCNGGNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNNCCNNGGNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNNCCNNNGGNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNNCCNNNNGGNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNN-5’ 5’- NNNNNNNNNNNNNNNNNNNNNNNNCGGNNNNNNNNNNNNNNN

	NNNNNNNNNNNNNNNNNNNN-3’ 3’- NNNNNNNNNNNNNNNNNNNNNNNCCNNNNNGGNNNNNNNNN NNNNNNNNNNNNNNNNNNN-5’

[00465] Further interrogation of the system have given Applicants evidence of the 5’ overhang (see, e.g., Ran et al., Cell. 2013 Sep 12;154(6):1380-9 and US Provisional Patent Application Serial No. 61/871,301 filed August 28, 2013). Applicants have further identified parameters that relate to efficient cleavage by the Cas9 nickase mutant when combined with two guide RNAs and these parameters include but are not limited to the length of the 5’ overhang. In embodiments of the invention the 5’ overhang is at most 200 base pairs, preferably at most 100 base pairs, or more preferably at most 50 base pairs. In embodiments of the invention the 5’ overhang is at least 26 base pairs, preferably at least 30 base pairs or more preferably 34-50 base pairs or 1-34 base pairs. In other preferred methods of the invention the first guide sequence directing cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directing cleavage of other strand near the second target sequence results in a blunt cut or a 3’ overhang. In embodiments of the invention the 3’ overhang is at most 150, 100 or 25 base pairs or at least 15, 10 or 1 base pairs. In preferred embodiments the 3’ overhang is 1-100 basepairs.

[00466] Aspects of the invention relate to the expression of the gene product being decreased or a template polynucleotide being further introduced into the DNA molecule encoding the gene product or an intervening sequence being excised precisely by allowing the two 5’ overhangs to reanneal and ligate or the activity or function of the gene product being altered or the expression of the gene product being increased. In an embodiment of the invention, the gene product is a protein.

[00467] Only sgRNA pairs creating 5' overhangs with less than 8bp overlap between the guide sequences (offset greater than -8 bp) were able to mediate detectable indel formation. Importantly, each guide used in these assays is able to efficiently induce indels when paired with wildtype Cas9, indicating that the relative positions of the guide pairs are the most important parameters in predicting double nicking activity.

[00468] Since Cas9n and Cas9H840A nick opposite strands of DNA, substitution of Cas9n with Cas9H840A with a given sgRNA pair should result in the inversion of the overhang type. For example, a pair of sgRNAs that will generate a 5' overhang with Cas9n should in principle generate the corresponding 3' overhang instead. Therefore, sgRNA pairs that lead to the generation of a 3' overhang with Cas9n might be used with Cas9H840A to generate a 5' overhang. Unexpectedly, Applicants tested Cas9H840A with a set of sgRNA pairs designed to generate both 5' and 3' overhangs (offset range from –278 to +58 bp), but were unable to observe indel formation. Further work may be needed to identify the necessary design rules for sgRNA pairing to allow double nicking by Cas9H840A.

[00469] **Liver, proprotein convertase subtilisin kexin 9 (PCSK9)**

[00470] The data shows phenotypic conversion.

[00471] Proprotein convertase subtilisin kexin 9 (PCSK9) is a member of the subtilisin serine protease family. PCSK9 is primarily expressed by the liver and is critical for the down regulation of hepatocyte LDL receptor expression. LDL-C levels in plasma are highly elevated in humans with gain of function mutations in PCSK9, classifying them as having severe hypercholesterolemia. Therefore, PCSK9 is an attractive target for CRISPR. PCS9K-targeted CRISPR may be formulated in a lipid particle and for example administered at about 15, 45, 90, 150, 250 and 400 μg/kg intraveneously (see, e.g., http://www.alnylam.com/capella/wp-content/uploads/2013/08/ALN-PCS02-001-Protocol-Lancet.pdf).

[00472] Bailey et al. (J Mol Med (Berl). 1999 Jan;77(1):244-9) discloses insulin delivery by ex-vivo somatic cell gene therapy involves the removal of non-B-cell somatic cells (e.g. fibroblasts) from a diabetic patient, and genetically altering them in vitro to produce and secrete insulin. The cells can be grown in culture and returned to the donor as a source of insulin replacement. Cells modified in this way could be evaluated before implantation, and reserve stocks could be cryopreserved. By using the patient's own cells, the procedure should obviate the need for immunosuppression and overcome the problem of tissue supply, while avoiding a

recurrence of cell destruction. Ex-vivo somatic cell gene therapy requires an accessible and robust cell type that is amenable to multiple transfections and subject to controlled proliferation. Special problems associated with the use of non-B-cell somatic cells include the processing of proinsulin to insulin, and the conferment of sensitivity to glucose-stimulated proinsulin biosynthesis and regulated insulin release. Preliminary studies using fibroblasts, pituitary cells, kidney (COS) cells and ovarian (CHO) cells suggest that these challenges could be met, and that ex-vivo somatic cell gene therapy offers a feasible approach to insulin replacement therapy. The system of Bailey et al. may be used/and or adapted to the CRISPR Cas system of the present invention for delivery to the liver.

[00473] The methods of Sato et al. (Nature Biotechnology Volume 26 Number 4 April 2008, Pages 431-442) may be applied to the CRISPR Cas system of the present invention for delivery to the liver. Sato et al. found that treatments with the siRNA-bearing vitamin A–coupled liposomes almost completely resolved liver fibrosis and prolonged survival in rats with otherwise lethal dimethylnitrosamine-induced liver cirrhosis in a dose- and duration-dependent manner. Cationic liposomes (Lipotrust) containing O,O'-ditetradecanoyl-N-(a-trimethylammonioacetyl) diethanolamine chloride (DC-6-14) as a cationic lipid, cholesterol and dioleoylphosphatidylethanolamine at a molar ratio of 4:3:3 (which has shown high transfection efficiency under serumcontaining conditions for in vitro and in vivo gene delivery) were purchased from Hokkaido System Science. The liposomes were manufactured using a freeze-dried empty liposomes method and prepared at a concentration of 1 mM (DC-16-4) by addition of double-distilled water (DDW) to the lyophilized lipid mixture under vortexing before use. To prepare VA-coupled liposomes, 200 nmol of vitamin A (retinol, Sigma) dissolved in DMSO was mixed with the liposome suspensions (100 nmol as DC-16-4) by vortexing in a 1.5 ml tube at 25 1C. To prepare VA-coupled liposomes carrying siRNAgp46 (VA-lip-siRNAgp46), a solution of siRNAgp46 (580 pmol/ml in DDW) was added to the retinol-coupled liposome solution with stirring at 25 C. The ratio of siRNA to DC-16-4 was 1:11.5 (mol/mol) and the siRNA to liposome ratio (wt/wt) was 1:1. Any free vitamin A or siRNA that was not taken up by liposomes were separated from liposomal preparations using a micropartition system (VIVASPIN 2 concentrator 30,000 MWCO PES, VIVASCIENCE). The liposomal suspension was added to the filters and centrifuged at 1,500g for 5 min 3 times at 25 1C. Fractions were collected and the material trapped in the filter was reconstituted with PBS to achieve the desired dose for in vitro

or in vivo use. Three injections of 0.75 mg/kg siRNA were given every other day to rats. The system of Sato et al. may be used/and or adapted to the CRISPR Cas system of the present invention for delivery to the liver by delivering about 0.5 to 1 mg/kg of CRISPR Cas RNA in the liposomes as described by Sato et al. to humans.

[00474] The methods of Rozema et al. (PNAS, August 7, 2007, vol. 104, no. 32) for a vehicle for the delivery of siRNA to hepatocytes both in vitro and in vivo, which Rozema et al. have named siRNA Dynamic PolyConjugates may also be applied to the present invention. Key features of the Dynamic Poly-Conjugate technology include a membrane-active polymer, the ability to reversibly mask the activity of this polymer until it reaches the acidic environment of endosomes, and the ability to target this modified polymer and its siRNA cargo specifically to hepatocytes in vivo after simple, low-pressure i.v. injection. SATA-modified siRNAs are synthesized by reaction of 5' aminemodified siRNA with 1 weight equivalents (wt eq) of Nsuccinimidyl-S-acetylthioacetate (SATA) reagent (Pierce) and 0.36 wt eq of $NaHCO_3$ in water at 4°C for 16 h. The modified siRNAs are then precipitated by the addition of 9 vol of ethanol and incubation at 80°C for 2 h. The precipitate is resuspended in 1X siRNA buffer (Dharmacon) and quantified by measuring absorbance at the 260-nm wavelength. PBAVE (30 mg/ml in 5mMTAPS, pH 9) is modified by addition of 1.5 wt % SMPT (Pierce). After a 1-h incubation, 0.8 mg of SMPT-PBAVE was added to 400 µl of isotonic glucose solution containing 5 mM TAPS (pH 9). To this solution was added 50 µg of SATA-modified siRNA. For the dose--response experiments where [PBAVE] was constant, different amounts of siRNA are added. The mixture is then incubated for 16 h. To the solution is then added 5.6 mg of Hepes free base followed by a mixture of 3.7 mg ofCDM-NAGand 1.9mg of CDM-PEG. The solution is then incubated for at least 1 h at room temperature before injection. CDM-PEG and CDM-NAG are synthesized from the acid chloride generated by using oxalyl chloride. To the acid chloride is added 1.1 molar equivalents polyethylene glycol monomethyl ether (molecular weight average of 450) to generate CDM-PEG or (aminoethoxy)ethoxy-2-(acetylamino)-2-deoxy-β-D-glucopyranoside to generate CDM-NAG. The final product is purified by using reverse-phase HPLC with a 0.1% TFA water/acetonitrile gradient. About 25 to 50 µg of siRNA was delivered to mice. The system of Rozema et al. may be applied to the CRISPR Cas system of the present invention for delivery to the liver, for example by envisioning a dosage of about 50 to about 200 mg of CRISPR Cas for delivery to a human.

[00475] **Bone**

[00476] Oakes and Lieberman (Clin Orthop Relat Res. 2000 Oct;(379 Suppl):S101-12) discusses delivery of genes to the bone. By transferring genes into cells at a specific anatomic site, the osteoinductive properties of growth factors can be used at physiologic doses for a sustained period to facilitate a more significant healing response. The specific anatomic site, the quality of the bone, and the soft-tissue envelope, influences the selection of the target cells for regional gene therapy. Gene therapy vectors delivered to a treatment site in osteoconductive carriers have yielded promising results. Several investigators have shown exciting results using ex vivo and in vivo regional gene therapy in animal models. Such a system may be used/and or adapted to the CRISPR Cas system for delivery to the bone.

[00477] **Brain**

[00478] Delivery options for the brain include encapsulation of CRISPR enzyme and guide RNA in the form of either DNA or RNA into liposomes and conjugating to molecular Trojan horses for trans-blood brain barrier (BBB) delivery. Molecular Trojan horses have been shown to be effective for delivery of B-gal expression vectors into the brain of non-human primates. The same approach can be used to delivery vectors containing CRISPR enzyme and guide RNA. For instance, Xia CF and Boado RJ, Pardridge WM ("Antibody-mediated targeting of siRNA via the human insulin receptor using avidin-biotin technology." Mol Pharm. 2009 May-Jun;6(3):747-51. doi: 10.1021/mp800194) describes how delivery of short interfering RNA (siRNA) to cells in culture, and in vivo, is possible with combined use of a receptor-specific monoclonal antibody (mAb) and avidin-biotin technology. The authors also report that because the bond between the targeting mAb and the siRNA is stable with avidin-biotin technology, and RNAi effects at distant sites such as brain are observed in vivo following an intravenous administration of the targeted siRNA.

[00479] Zhang et al. (Mol Ther. 2003 Jan;7(1):11-8.)) describe how expression plasmids encoding reporters such as luciferase were encapsulated in the interior of an "artificial virus" comprised of an 85 nm pegylated immunoliposome, which was targeted to the rhesus monkey brain in vivo with a monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb enables the liposome carrying the exogenous gene to undergo transcytosis across the blood-brain barrier and endocytosis across the neuronal plasma membrane following intravenous injection. The level of luciferase gene expression in the brain was 50-fold higher in the rhesus

monkey as compared to the rat. Widespread neuronal expression of the beta-galactosidase gene in primate brain was demonstrated by both histochemistry and confocal microscopy. The authors indicate that this approach makes feasible reversible adult transgenics in 24 hours. Accordingly, the use of immunoliposome is preferred. These may be used in conjunction with antibodies to target specific tissues or cell surface proteins.

[00480] Other means of delivery or RNA are also preferred, such as via nanoparticles (Cho, S., Goldberg, M., Son, S., Xu, Q., Yang, F., Mei, Y., Bogatyrev, S., Langer, R. and Anderson, D., Lipid-like nanoparticles for small interfering RNA delivery to endothelial cells, Advanced Functional Materials, 19: 3112-3118, 2010) or exosomes (Schroeder, A., Levins, C., Cortez, C., Langer, R., and Anderson, D., Lipid-based nanotherapeutics for siRNA delivery, Journal of Internal Medicine, 267: 9-21, 2010, PMID: 20059641).

[00481] Indeed, exosomes have been shown to be particularly useful in delivery siRNA, a system with some parallels to the CRISPR system. For instance, El-Andaloussi S, et al. ("Exosome-mediated delivery of siRNA in vitro and in vivo." Nat Protoc. 2012 Dec;7(12):2112-26. doi: 10.1038/nprot.2012.131. Epub 2012 Nov 15.) describe how exosomes are promising tools for drug delivery across different biological barriers and can be harnessed for delivery of siRNA in vitro and in vivo. Their approach is to generate targeted exosomes through transfection of an expression vector, comprising an exosomal protein fused with a peptide ligand. The exosomes are then purify and characterized from transfected cell supernatant, then siRNA is loaded into the exosomes. Delivery or administration according to the invention can be performed with exosomes, in particular but not limited to the brain.

[00482] Vitamin E (α-tocopherol) may be conjugated with CRISPR Cas and delivered to the brain along with high density lipoprotein (HDL), for example in a similar manner as was done by Uno et al. (HUMAN GENE THERAPY 22:711–719 (June 2011)) for delivering short-interfering RNA (siRNA) to the brain. Mice were infused via Osmotic minipumps (model 1007D; Alzet, Cupertino, CA) filled with phosphate-buffered saline (PBS) or free TocsiBACE or Toc-siBACE/HDL and connected with Brain Infusion Kit 3 (Alzet). A brain-infusion cannula was placed about 0.5mm posterior to the bregma at midline for infusion into the dorsal third ventricle. Uno et al. found that as little as 3 nmol of Toc-siRNA with HDL could induce a target reduction in comparable degree by the same ICV infusion method. A similar dosage of CRISPR Cas conjugated to α-tocopherol and co-administered with HDL targeted to the brain may be

contemplated for humans in the present invention, for example, about 3 nmol to about 3 μmol of CRISPR Cas targeted to the brain may becontemplated.

[00483] Zou et al. ((HUMAN GENE THERAPY 22:465-475 (April 2011)) describes a method of lentiviral-mediated delivery of short-hairpin RNAs targeting PKCγ for in vivo gene silencing in the spinal cord of rats. Zou et al. administered about 10 μl of a recombinant lentivirus having a titer of 1 x 10^9 transducing units (TU)/ml by an intrathecal catheter. A similar dosage of CRISPR Cas expressed in a lentiviral vector targeted to the brain may be contemplated for humans in the present invention, for example, about 10-50 ml of CRISPR Cas targeted to the brain in a lentivirus having a titer of 1 x 10^9 transducing units (TU)/ml may becontemplated.

[00484] **Targeted deletion, therapeutic applications**

[00485] Targeted deletion of genes is preferred. Examples are exemplified in Example 18. Preferred are, therefore, genes involved in cholesterol biosynthesis, fatty acid biosynthesis, and other metabolic disorders, genes encoding mis-folded proteins involved in amyloid and other diseases, oncogenes leading to cellular transformation, latent viral genes, and genes leading to dominant-negative disorders, amongst other disorders. As exemplified here, Applicants prefer gene delivery of a CRISPR-Cas system to the liver, brain, ocular, epithelial, hematopoetic, or another tissue of a subject or a patient in need thereof, suffering from metabolic disorders, amyloidosis and protein-aggregation related diseases, cellular transformation arising from genetic mutations and translocations, dominant negative effects of gene mutations, latent viral infections, and other related symptoms, using either viral or nanoparticle delivery system.

[00486] Therapeutic applications of the CRISPR-Cas system include Glaucoma, Amyloidosis, and Huntington's disease. These are exemplified in Example 20 and the features described therein are preferred alone or in combination.

[00487] Another example of a polyglutamine expansion disease that may be treated by the present invention includes spinocerebellar ataxia type 1 (SCA1). Upon intracerebellar injection, recombinant adenoassociated virus (AAV) vectors expressing short hairpin RNAs profoundly improve motor coordination, restored cerebellar morphology and resolved characteristic ataxin-1 inclusions in Purkinje cells of SCA1 mice (see, e.g., Xia et al., Nature Medicine, Vol. 10, No. 8, Aug. 2004). In particular, AAV1 and AAV5 vectors are preferred and AAV titers of about 1 x 10^{12} vector genomes/ml are desirable.

[00488] As an example, chronic infection by HIV-1 may be treated or prevented. In order to accomplish this, one may generate CRISPR-Cas guide RNAs that target the vast majority of the HIV-1 genome while taking into account HIV-1 strain variants for maximal coverage and effectiveness. One may accomplish delivery of the CRISPR-Cas system by conventional adenoviral or lentiviral-mediated infection of the host immune system. Depending on approach, host immune cells could be a) isolated, transduced with CRISPR-Cas, selected, and re-introduced in to the host or b) transduced in vivo by systemic delivery of the CRISPR-Cas system. The first approach allows for generation of a resistant immune population whereas the second is more likely to target latent viral reservoirs within the host. This is discussed in more detail in the Examples section.

[00489] In another example, US Patent Publication No. 20130171732 assigned to Sangamo BioSciences, Inc. relates to insertion of an anti-HIV transgene into the genome, methods of which may be applied to the CRISPR Cas system of the present invention. In another embodiment, the CXCR4 gene may be targeted and the TALE system of US Patent Publication No. 20100291048 assigned to Sangamo BioSciences, Inc. may be modified to the CRISPR Cas system of the present invention. The method of US Patent Publication Nos. 20130137104 and 20130122591 assigned to Sangamo BioSciences, Inc. and US Patent Publication No. 20100146651 assigned to Cellectis may be more generally applicable for transgene expression as it involves modifying a hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus for increasing the frequency of gene modification.

[00490] It is also envisaged that the present invention generates a gene knockout cell library. Each cell may have a single gene knocked out. This is exemplified in Example 23.

[00491] One may make a library of ES cells where each cell has a single gene knocked out, and the entire library of ES cells will have every single gene knocked out. This library is useful for the screening of gene function in cellular processes as well as diseases. To make this cell library, one may integrate Cas9 driven by an inducible promoter (e.g. doxycycline inducible promoter) into the ES cell. In addition, one may integrate a single guide RNA targeting a specific gene in the ES cell. To make the ES cell library, one may simply mix ES cells with a library of genes encoding guide RNAs targeting each gene in the human genome. One may first introduce a single BxB1 attB site into the AAVS1 locus of the human ES cell. Then one may use the BxB1 integrase to facilitate the integration of individual guide RNA genes into the BxB1 attB site in

AAVS1 locus. To facilitate integration, each guide RNA gene may be contained on a plasmid that carries of a single attP site. This way BxB1 will recombine the attB site in the genome with the attP site on the guide RNA containing plasmid. To generate the cell library, one may take the library of cells that have single guide RNAs integrated and induce Cas9 expression. After induction, Cas9 mediates double strand break at sites specified by the guide RNA.

[00492] Chronic administration of protein therapeutics may elicit unacceptable immune responses to the specific protein. The immunogenicity of protein drugs can be ascribed to a few immunodominant helper T lymphocyte (HTL) epitopes. Reducing the MHC binding affinity of these HTL epitopes contained within these proteins can generate drugs with lower immunogenicity (Tangri S, et al. ("Rationally engineered therapeutic proteins with reduced immunogenicity" J Immunol. 2005 Mar 15;174(6):3187-96.) In the present invention, the immunogenicity of the CRISPR enzyme in particular may be reduced following the approach first set out in Tangri et al with respect to erythropoietin and subsequently developed. Accordingly, directed evolution or rational design may be used to reduce the immunogenicity of the CRISPR enzyme (for instance a Cas9) in the host species (human or other species).

[00493] In Example 28, Applicants used 3 guideRNAs of interest and able to visualize efficient DNA cleavage *in vivo* occurring only in a small subset of cells. Essentially, what Applicants have shown here is targeted *in vivo* cleavage. In particular, this provides proof of concept that specific targeting in higher organisms such as mammals can also be achieved. It also highlights multiplex aspect in that multiple guide sequences (i.e. separate targets) can be used simultaneously (in the sense of co-delivery). In other words, Applicants used a multiple approach, with several different sequences targeted at the same time, but independently.

[00494] A suitable example of a protocol for producing AAV, a preferred vector of the invention is provided in Example 34.

[00495] Trinucleotide repeat disorders are preferred conditions to be treated. These are also exemplified herein.

[00496] For example, US Patent Publication No. 20110016540, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with trinucleotide repeat expansion disorders. Trinucleotide repeat expansion disorders are complex, progressive disorders that involve developmental neurobiology and often affect cognition as well as sensori-motor functions.

[00497] Trinucleotide repeat expansion proteins are a diverse set of proteins associated with susceptibility for developing a trinucleotide repeat expansion disorder, the presence of a trinucleotide repeat expansion disorder, the severity of a trinucleotide repeat expansion disorder or any combination thereof. Trinucleotide repeat expansion disorders are divided into two categories determined by the type of repeat. The most common repeat is the triplet CAG, which, when present in the coding region of a gene, codes for the amino acid glutamine (Q). Therefore, these disorders are referred to as the polyglutamine (polyQ) disorders and comprise the following diseases: Huntington Disease (HD); Spinobulbar Muscular Atrophy (SBMA); Spinocerebellar Ataxias (SCA types 1, 2, 3, 6, 7, and 17); and Dentatorubro-Pallidoluysian Atrophy (DRPLA). The remaining trinucleotide repeat expansion disorders either do not involve the CAG triplet or the CAG triplet is not in the coding region of the gene and are, therefore, referred to as the non-polyglutamine disorders. The non-polyglutamine disorders comprise Fragile X Syndrome (FRAXA); Fragile XE Mental Retardation (FRAXE); Friedreich Ataxia (FRDA); Myotonic Dystrophy (DM); and Spinocerebellar Ataxias (SCA types 8, and 12).

[00498] The proteins associated with trinucleotide repeat expansion disorders are typically selected based on an experimental association of the protein associated with a trinucleotide repeat expansion disorder to a trinucleotide repeat expansion disorder. For example, the production rate or circulating concentration of a protein associated with a trinucleotide repeat expansion disorder may be elevated or depressed in a population having a trinucleotide repeat expansion disorder relative to a population lacking the trinucleotide repeat expansion disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with trinucleotide repeat expansion disorders may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00499] Non-limiting examples of proteins associated with trinucleotide repeat expansion disorders include AR (androgen receptor), FMR1 (fragile X mental retardation 1), HTT (huntingtin), DMPK (dystrophia myotonica-protein kinase), FXN (frataxin), ATXN2 (ataxin 2), ATN1 (atrophin 1), FEN1 (flap structure-specific endonuclease 1), TNRC6A (trinucleotide

repeat containing 6A), PABPN1 (poly(A) binding protein, nuclear 1), JPH3 (junctophilin 3), MED15 (mediator complex subunit 15), ATXN1 (ataxin 1), ATXN3 (ataxin 3), TBP (TATA box binding protein), CACNA1A (calcium channel, voltage-dependent, P/Q type, alpha 1A subunit), ATXN80S (ATXN8 opposite strand (non-protein coding)), PPP2R2B (protein phosphatase 2, regulatory subunit B, beta), ATXN7 (ataxin 7), TNRC6B (trinucleotide repeat containing 6B), TNRC6C (trinucleotide repeat containing 6C), CELF3 (CUGBP, Elav-like family member 3), MAB21L1 (mab-21-like 1 (C. elegans)), MSH2 (mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)), TMEM185A (transmembrane protein 185A), SIX5 (SIX homeobox 5), CNPY3 (canopy 3 homolog (zebrafish)), FRAXE (fragile site, folic acid type, rare, fra(X)(q28) E), GNB2 (guanine nucleotide binding protein (G protein), beta polypeptide 2), RPL14 (ribosomal protein L14), ATXN8 (ataxin 8), INSR (insulin receptor), TTR (transthyretin), EP400 (E1A binding protein p400), GIGYF2 (GRB10 interacting GYF protein 2), OGG1 (8-oxoguanine DNA glycosylase), STC1 (stanniocalcin 1), CNDP1 (carnosine dipeptidase 1 (metallopeptidase M20 family)), C10orf2 (chromosome 10 open reading frame 2), MAML3 mastermind-like 3 (Drosophila), DKC1 (dyskeratosis congenita 1, dyskerin), PAXIP1 (PAX interacting (with transcription-activation domain) protein 1), CASK (calcium/calmodulin-dependent serine protein kinase (MAGUK family)), MAPT (microtubule-associated protein tau), SP1 (Sp1 transcription factor), POLG (polymerase (DNA directed), gamma), AFF2 (AF4/FMR2 family, member 2), THBS1 (thrombospondin 1), TP53 (tumor protein p53), ESR1 (estrogen receptor 1), CGGBP1 (CGG triplet repeat binding protein 1), ABT1 (activator of basal transcription 1), KLK3 (kallikrein-related peptidase 3), PRNP (prion protein), JUN (jun oncogene), KCNN3 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3), BAX (BCL2-associated X protein), FRAXA (fragile site, folic acid type, rare, fra(X)(q27.3) A (macroorchidism, mental retardation)), KBTBD10 (kelch repeat and BTB (POZ) domain containing 10), MBNL1 (muscleblind-like (Drosophila)), RAD51 (RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)), NCOA3 (nuclear receptor coactivator 3), ERDA1 (expanded repeat domain, CAG/CTG 1), TSC1 (tuberous sclerosis 1), COMP (cartilage oligomeric matrix protein), GCLC (glutamate-cysteine ligase, catalytic subunit), RRAD (Ras-related associated with diabetes), MSH3 (mutS homolog 3 (E. coli)), DRD2 (dopamine receptor D2), CD44 (CD44 molecule (Indian blood group)), CTCF (CCCTC-binding factor (zinc finger protein)), CCND1 (cyclin D1), CLSPN (claspin homolog (Xenopus laevis)), MEF2A (myocyte enhancer factor

2A), PTPRU (protein tyrosine phosphatase, receptor type, U), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), TRIM22 (tripartite motif-containing 22), WT1 (Wilms tumor 1), AHR (aryl hydrocarbon receptor), GPX1 (glutathione peroxidase 1), TPMT (thiopurine S-methyltransferase), NDP (Norrie disease (pseudoglioma)), ARX (aristaless related homeobox), MUS81 (MUS81 endonuclease homolog (S. cerevisiae)), TYR (tyrosinase (oculocutaneous albinism IA)), EGR1 (early growth response 1), UNG (uracil-DNA glycosylase), NUMBL (numb homolog (Drosophila)-like), FABP2 (fatty acid binding protein 2, intestinal), EN2 (engrailed homeobox 2), CRYGC (crystallin, gamma C), SRP14 (signal recognition particle 14 kDa (homologous Alu RNA binding protein)), CRYGB (crystallin, gamma B), PDCD1 (programmed cell death 1), HOXA1 (homeobox A1), ATXN2L (ataxin 2-like), PMS2 (PMS2 postmeiotic segregation increased 2 (S. cerevisiae)), GLA (galactosidase, alpha), CBL (Cas-Br-M (murine) ecotropic retroviral transforming sequence), FTH1 (ferritin, heavy polypeptide 1), IL12RB2 (interleukin 12 receptor, beta 2), OTX2 (orthodenticle homeobox 2), HOXA5 (homeobox A5), POLG2 (polymerase (DNA directed), gamma 2, accessory subunit), DLX2 (distal-less homeobox 2), SIRPA (signal-regulatory protein alpha), OTX1 (orthodenticle homeobox 1), AHRR (aryl-hydrocarbon receptor repressor), MANF (mesencephalic astrocyte-derived neurotrophic factor), TMEM158 (transmembrane protein 158 (gene/pseudogene)), and ENSG00000078687.

[00500] Preferred proteins associated with trinucleotide repeat expansion disorders include HTT (Huntingtin), AR (androgen receptor), FXN (frataxin), Atxn3 (ataxin), Atxn1 (ataxin), Atxn2 (ataxin), Atxn7 (ataxin), Atxn10 (ataxin), DMPK (dystrophia myotonica-protein kinase), Atn1 (atrophin 1), CBP (creb binding protein), VLDLR (very low density lipoprotein receptor), and any combination thereof.

[00501] According to another aspect, a method of gene therapy for the treatment of a subject having a mutation in the CFTR gene is provided and comprises administering a therapeutically effective amount of a CRISPR-Cas gene therapy particle, optionally via a biocompatible pharmaceutical carrier, to the cells of a subject. Preferably, the target DNA comprises the mutation deltaF508. In general, it is of preferred that the mutation is repaired to the wildtype. In this case, the mutation is a deletion of the three nucleotides that comprise the codon for phenylalanine (F) at position 508. Accordingly, repair in this instance requires reintroduction of the missing codon into the mutant.

[00502] To implement this Gene Repair Strategy, it is preferred that an adenovirus/AAV vector system is introduced into the host cell, cells or patient. Preferably, the system comprises a Cas9 (or Cas9 nickase) and the guide RNA along with a adenovirus/AAV vector system comprising the homology repair template containing the F508 residue. This may be introduced into the subject via one of the methods of delivery discussed earlier. The CRISPR-Cas system may be guided by the CFTRdelta 508 chimeric guide RNA. It targets a specific site of the CFTR genomic locus to be nicked or cleaved. After cleavage, the repair template is inserted into the cleavage site via homologous recombination correcting the deletion that results in cystic fibrosis or causes cystic fibrosis related symptoms. This strategy to direct delivery and provide systemic introduction of CRISPR systems with appropriate guide RNAs can be employed to target genetic mutations to edit or otherwise manipulate genes that cause metabolic, liver, kidney and protein diseases and disorders such as those in Table B.

[00503] Genome editing

[00504] The CRISPR/Cas9 systems of the present invention can be used to correct genetic mutations that were previously attempted with limited success using TALEN and ZFN. For example, WO2013163628 A2, Genetic Correction of Mutated Genes, published application of Duke University describes efforts to correct, for example, a frameshift mutation which causes a premature stop codon and a truncated gene product that can be corrected via nuclease mediated non-homologous end joining such as those responsible for Duchenne Muscular Dystrophy, ("DMD") a recessive, fatal, X-linked disorder that results in muscle degeneration due to mutations in the dystrophin gene. The majority of dystrophin mutations that cause DMD are deletions of exons that disrupt the reading frame and cause premature translation termination in the dystrophin gene. Dystrophin is a cytoplasmic protein that provides structural stability to the dystroglycan complex of the cell membrane that is responsible for regulating muscle cell integrity and function. The dystrophin gene or "DMD gene" as used interchangeably herein is 2.2 megabases at locus Xp21. The primary transcription measures about 2,400 kb with the mature mRNA being about 14 kb. 79 exons code for the protein which is over 3500 amino acids. Exon 51 is frequently adjacent to frame-disrupting deletions in DMD patients and has been targeted in clinical trials for oligonucleotide-based exon skipping. A clinical trial for the exon 51 skipping compound eteplirsen recently reported a significant functional benefit across 48 weeks, with an

average of 47% dystrophin positive fibers compared to baseline. Mutations in exon 51 are ideally suited for permanent correction by NHEJ-based genome editing.

[00505] The methods of US Patent Publication No. 20130145487 assigned to Cellectis, which relates to meganuclease variants to cleave a target sequence from the human dystrophin gene (DMD), may also be modified to for the CRISPR Cas system of the present invention.

[00506] **Blood**

[00507] The present invention also contemplates delivering the CRISPR-Cas system to the blood.

[00508] The plasma exosomes of Wahlgren et al. (Nucleic Acids Research, 2012, Vol. 40, No. 17 e130) were previously described and may be utilized to deliver the CRISPR Cas system to the blood.

[00509] The CRISPR Cas system of the present invention is also contemplated to treat hemoglobinopathies, such as thalassemias and sickle cell disease. See, e.g., International Patent Publication No. WO 2013/126794 for potential targets that may be targeted by the CRISPR Cas system of the present invention.

[00510] US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 assigned to Cellectis, relates to CREI variants , wherein at least one of the two I-CreI monomers has at least two substitutions, one in each of the two functional subdomains of the LAGLIDADG core domain situated respectively from positions 26 to 40 and 44 to 77 of I-CreI, said variant being able to cleave a DNA target sequence from the human interleukin-2 receptor gamma chain (IL2RG) gene also named common cytokine receptor gamma chain gene or gamma C gene. The target sequences identified in US Patent Publication Nos. 20110225664, 20110091441, 20100229252, 20090271881 and 20090222937 may be utilized for the CRISPR Cas system of the present invention.

[00511] Severe Combined Immune Deficiency (SCID) results from a defect in lymphocytes T maturation, always associated with a functional defect in lymphocytes B (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109). Overall incidence is estimated to 1 in 75 000 births. Patients with untreated SCID are subject to multiple opportunist micro-organism infections, and do generally not live beyond one year. SCID can be treated by allogenic hematopoietic stem cell transfer, from a familial donor. Histocompatibility with the donor can vary widely. In the case of Adenosine Deaminase (ADA)

deficiency, one of the SCID forms, patients can be treated by injection of recombinant Adenosine Deaminase enzyme.

[00512] Since the ADA gene has been shown to be mutated in SCID patients (Giblett et al., Lancet, 1972, 2, 1067-1069), several other genes involved in SCID have been identified (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109). There are four major causes for SCID: (i) the most frequent form of SCID, SCID-X1 (X-linked SCID or X-SCID), is caused by mutation in the IL2RG gene, resulting in the absence of mature T lymphocytes and NK cells. IL2RG encodes the gamma C protein (Noguchi, et al., Cell, 1993, 73, 147-157), a common component of at least five interleukin receptor complexes. These receptors activate several targets through the JAK3 kinase (Macchi et al., Nature, 1995, 377, 65-68), which inactivation results in the same syndrome as gamma C inactivation; (ii) mutation in the ADA gene results in a defect in purine metabolism that is lethal for lymphocyte precursors, which in turn results in the quasi absence of B, T and NK cells; (iii) V(D)J recombination is an essential step in the maturation of immunoglobulins and T lymphocytes receptors (TCRs). Mutations in Recombination Activating Gene 1 and 2 (RAG1 and RAG2) and Artemis, three genes involved in this process, result in the absence of mature T and B lymphocytes; and (iv) Mutations in other genes such as CD45, involved in T cell specific signaling have also been reported, although they represent a minority of cases (Cavazzana-Calvo et al., Annu. Rev. Med., 2005, 56, 585-602; Fischer et al., Immunol. Rev., 2005, 203, 98-109).

[00513] Since when their genetic bases have been identified, the different SCID forms have become a paradigm for gene therapy approaches (Fischer et al., Immunol. Rev., 2005, 203, 98-109) for two major reasons. First, as in all blood diseases, an ex vivo treatment can be envisioned. Hematopoietic Stem Cells (HSCs) can be recovered from bone marrow, and keep their pluripotent properties for a few cell divisions. Therefore, they can be treated in vitro, and then reinjected into the patient, where they repopulate the bone marrow. Second, since the maturation of lymphocytes is impaired in SCID patients, corrected cells have a selective advantage. Therefore, a small number of corrected cells can restore a functional immune system. This hypothesis was validated several times by (i) the partial restoration of immune functions associated with the reversion of mutations in SCID patients (Hirschhorn et al., Nat. Genet., 1996, 13, 290-295; Stephan et al., N. Engl. J. Med., 1996, 335, 1563-1567; Bousso et al., Proc. Natl., Acad. Sci. USA, 2000, 97, 274-278; Wada et al., Proc. Natl. Acad. Sci. USA, 2001, 98, 8697-

8702; Nishikomori et al., Blood, 2004, 103, 4565-4572), (ii) the correction of SCID-X1 deficiencies in vitro in hematopoietic cells (Candotti et al., Blood, 1996, 87, 3097-3102; Cavazzana-Calvo et al., Blood, 1996, Blood, 88, 3901-3909; Taylor et al., Blood, 1996, 87, 3103-3107; Hacein-Bey et al., Blood, 1998, 92, 4090-4097), (iii) the correction of SCID-X1 (Soudais et al., Blood, 2000, 95, 3071-3077; Tsai et al., Blood, 2002, 100, 72-79), JAK-3 (Bunting et al., Nat. Med., 1998, 4, 58-64; Bunting et al., Hum. Gene Ther., 2000, 11, 2353-2364) and RAG2 (Yates et al., Blood, 2002, 100, 3942-3949) deficiencies in vivo in animal models and (iv) by the result of gene therapy clinical trials (Cavazzana-Calvo et al., Science, 2000, 288, 669-672; Aiuti et al., Nat. Med., 2002; 8, 423-425; Gaspar et al., Lancet, 2004, 364, 2181-2187).

[00514] US Patent Publication No. 20110182867 assigned to the Children's Medical Center Corporation and the President and Fellows of Harvard College relates to methods and uses of modulating fetal hemoglobin expression (HbF) in a hematopoietic progenitor cells via inhibitors of BCL11A expression or activity, such as RNAi and antibodies. The targets disclosed in US Patent Publication No. 20110182867, such as BCL11A, may be targeted by the CRISPR Cas system of the present invention for modulating fetal hemoglobin expression. See also Bauer et al. (Science 11 October 2013: Vol. 342 no. 6155 pp. 253-257) and Xu et al. (Science 18 November 2011: Vol. 334 no. 6058 pp. 993-996) for additional BCL11A targets.

[00515] **Ears**

[00516] The present invention also contemplates delivering the CRISPR-Cas system to one or both ears.

[00517] Researchers are looking into whether gene therapy could be used to aid current deafness treatments—namely, cochlear implants. Deafness is often caused by lost or damaged hair cells that cannot relay signals to auditory neurons. In such cases, cochlear implants may be used to respond to sound and transmit electrical signals to the nerve cells. But these neurons often degenerate and retract from the cochlea as fewer growth factors are released by impaired hair cells.

[00518] US patent application 20120328580 describes injection of a pharmaceutical composition into the ear (e.g., auricular administration), such as into the luminae of the cochlea (e.g., the Scala media, Sc vestibulae, and Sc tympani), e.g., using a syringe, e.g., a single-dose syringe. For example, one or more of the compounds described herein can be administered by

intratympanic injection (e.g., into the middle ear), and/or injections into the outer, middle, and/or inner ear. Such methods are routinely used in the art, for example, for the administration of steroids and antibiotics into human ears. Injection can be, for example, through the round window of the ear or through the cochlear capsule. Other inner ear administration methods are known in the art (see, e.g., Salt and Plontke, Drug Discovery Today, 10:1299-1306, 2005).

[00519] In another mode of administration, the pharmaceutical composition can be administered in situ, via a catheter or pump. A catheter or pump can, for example, direct a pharmaceutical composition into the cochlear luminae or the round window of the ear and/or the lumen of the colon. Exemplary drug delivery apparatus and methods suitable for administering one or more of the compounds described herein into an ear, e.g., a human ear, are described by McKenna et al., (U.S. Publication No. 2006/0030837) and Jacobsen et al., (U.S. Pat. No. 7,206,639). In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient during a surgical procedure. In some embodiments, a catheter or pump can be positioned, e.g., in the ear (e.g., the outer, middle, and/or inner ear) of a patient without the need for a surgical procedure.

[00520] Alternatively or in addition, one or more of the compounds described herein can be administered in combination with a mechanical device such as a cochlear implant or a hearing aid, which is worn in the outer ear. An exemplary cochlear implant that is suitable for use with the present invention is described by Edge et al., (U.S. Publication No. 2007/0093878).

[00521] In some embodiments, the modes of administration described above may be combined in any order and can be simultaneous or interspersed.

[00522] Alternatively or in addition, the present invention may be administered according to any of the Food and Drug Administration approved methods, for example, as described in CDER Data Standards Manual, version number 004 (which is available at fda.give/cder/dsm/DRG/drg00301.htm).

[00523] In general, the cell therapy methods described in US patent application 20120328580 can be used to promote complete or partial differentiation of a cell to or towards a mature cell type of the inner ear (e.g., a hair cell) in vitro. Cells resulting from such methods can then be transplanted or implanted into a patient in need of such treatment. The cell culture methods required to practice these methods, including methods for identifying and selecting suitable cell types, methods for promoting complete or partial differentiation of selected cells, methods for

identifying complete or partially differentiated cell types, and methods for implanting complete or partially differentiated cells are described below.

[00524] Cells suitable for use in the present invention include, but are not limited to, cells that are capable of differentiating completely or partially into a mature cell of the inner ear, e.g., a hair cell (e.g., an inner and/or outer hair cell), when contacted, e.g., in vitro, with one or more of the compounds described herein. Exemplary cells that are capable of differentiating into a hair cell include, but are not limited to stem cells (e.g., inner ear stem cells, adult stem cells, bone marrow derived stem cells, embryonic stem cells, mesenchymal stem cells, skin stem cells, iPS cells, and fat derived stem cells), progenitor cells (e.g., inner ear progenitor cells), support cells (e.g., Deiters' cells, pillar cells, inner phalangeal cells, tectal cells and Hensen's cells), and/or germ cells. The use of stem cells for the replacement of inner ear sensory cells is described in Li et al., (U.S. Publication No. 2005/0287127) and Li et al., (U.S. patent Ser. No. 11/953,797). The use of bone marrow derived stem cells for the replacement of inner ear sensory cells is described in Edge et al., PCT/US2007/084654. iPS cells are described, e.g., at Takahashi et al., Cell, Volume 131, Issue 5, Pages 861-872 (2007); Takahashi and Yamanaka, Cell 126, 663-76 (2006); Okita et al., Nature 448, 260-262 (2007); Yu, J. et al., Science 318(5858):1917-1920 (2007); Nakagawa et al., Nat. Biotechnol. 26:101-106 (2008); and Zaehres and Scholer, Cell 131(5):834-835 (2007).

[00525] Such suitable cells can be identified by analyzing (e.g., qualitatively or quantitatively) the presence of one or more tissue specific genes. For example, gene expression can be detected by detecting the protein product of one or more tissue-specific genes. Protein detection techniques involve staining proteins (e.g., using cell extracts or whole cells) using antibodies against the appropriate antigen. In this case, the appropriate antigen is the protein product of the tissue-specific gene expression. Although, in principle, a first antibody (i.e., the antibody that binds the antigen) can be labeled, it is more common (and improves the visualization) to use a second antibody directed against the first (e.g., an anti-IgG). This second antibody is conjugated either with fluorochromes, or appropriate enzymes for colorimetric reactions, or gold beads (for electron microscopy), or with the biotin-avidin system, so that the location of the primary antibody, and thus the antigen, can be recognized.

[00526] The CRISPR Cas molecules of the present invention may be delivered to the ear by direct application of pharmaceutical composition to the outer ear, with compositions modified

from US Published application, 20110142917. In some embodiments the pharmaceutical composition is applied to the ear canal. Delivery to the ear may also be refered to as aural or otic delivery.

[00527] In some embodiments the RNA molecules of the invention are delivered in liposome or lipofectin formulations and the like and can be prepared by methods well known to those skilled in the art. Such methods are described, for example, in U.S. Pat. Nos. 5,593,972, 5,589,466, and 5,580,859, which are herein incorporated by reference.

[00528] Delivery systems aimed specifically at the enhanced and improved delivery of siRNA into mammalian cells have been developed, (see, for example, Shen et al FEBS Let. 2003, 539:111-114; Xia et al., Nat. Biotech. 2002, 20:1006-1010; Reich et al., Mol. Vision. 2003, 9: 210-216; Sorensen et al., J. Mol. Biol. 2003, 327: 761-766; Lewis et al., Nat. Gen. 2002, 32: 107-108 and Simeoni et al., NAR 2003, 31, 11: 2717-2724) and may be applied to the present invention. siRNA has recently been successfully used for inhibition of gene expression in primates (see for example. Tolentino et al., Retina 24(4):660 which may also be applied to the present invention.

[00529] Qi et al. discloses methods for efficient siRNA transfection to the inner ear through the intact round window by a novel proteidic delivery technology which may be applied to the CRISPR Cas system of the present invention (see, e.g., Qi et al., Gene Therapy (2013), 1-9). In particular, a TAT double stranded RNA-binding domains (TAT-DRBDs), which can transfect Cy3-labeled siRNA into cells of the inner ear, including the inner and outer hair cells, crista ampullaris, macula utriculi and macula sacculi, through intact round-window permeation was successful for delivering double stranded siRNAs in vivo for treating various inner ear ailments and preservation of hearing function. About 40 µl of 10mM RNA may be contemplated as the dosage for administration to the ear.

[00530] According to Rejali et al. (Hear Res. 2007 Jun;228(1-2):180-7), cochlear implant function can be improved by good preservation of the spiral ganglion neurons, which are the target of electrical stimulation by the implant and brain derived neurotrophic factor (BDNF) has previously been shown to enhance spiral ganglion survival in experimentally deafened ears. Rejali et al. tested a modified design of the cochlear implant electrode that includes a coating of fibroblast cells transduced by a viral vector with a BDNF gene insert. To accomplish this type of ex vivo gene transfer, Rejali et al. transduced guinea pig fibroblasts with an adenovirus with a

BDNF gene cassette insert, and determined that these cells secreted BDNF and then attached BDNF-secreting cells to the cochlear implant electrode via an agarose gel, and implanted the electrode in the scala tympani. Rejali et al. determined that the BDNF expressing electrodes were able to preserve significantly more spiral ganglion neurons in the basal turns of the cochlea after 48 days of implantation when compared to control electrodes and demonstrated the feasibility of combining cochlear implant therapy with ex vivo gene transfer for enhancing spiral ganglion neuron survival. Such a system may be applied to the CRISPR Cas system of the present invention for delivery to the ear.

[00531] Mukherjea et al. (Antioxidants & Redox Signaling, Volume 13, Number 5, 2010) document that knockdown of NOX3 using short interfering (si) RNA abrogated cisplatin ototoxicity, as evidenced by protection of OHCs from damage and reduced threshold shifts in auditory brainstem responses (ABRs). Different doses of siNOX3 (0.3, 0.6, and 0.9 μg) were administered to rats and NOX3 expression was evaluated by real time RT-PCR. The lowest dose of NOX3 siRNA used (0.3 μg) did not show any inhibition of NOX3 mRNA when compared to transtympanic administration of scrambled siRNA or untreated cochleae. However, administration of the higher doses of NOX3 siRNA (0.6 and 0.9 μg) reduced NOX3 expression compared to control scrambled siRNA. Such a system may be applied to the CRISPR Cas system of the present invention for transtympanic administration with a dosage of about 2 mg to about 4 mg of CRISPR Cas for administration to a human.

[00532] Jung et al. (Molecular Therapy, vol. 21 no. 4, 834–841 apr. 2013) demonstrate that Hes5 levels in the utricle decreased after the application of siRNA and that the number of hair cells in these utricles was significantly larger than following control treatment. The data suggest that siRNA technology may be useful for inducing repair and regeneration in the inner ear and that the Notch signaling pathway is a potentially useful target for specific gene expression inhibition. Jung et al. injected 8 μg of Hes5 siRNA in 2 μl volume, prepared by adding sterile normal saline to the lyophilized siRNA to a vestibular epithelium of the ear. Such a system may be applied to the CRISPR Cas system of the present invention for administration to the vestibular epithelium of the ear with a dosage of about 1 to about 30 mg of CRISPR Cas for administration to a human.

[00533] **Eyes**

[00534] The present invention also contemplates delivering the CRISPR-Cas system to one or both eyes.

[00535] In yet another aspect of the invention, the CRISPR-Cas system may be used to correct ocular defects that arise from several genetic mutations further described in Genetic Diseases of the Eye, Second Edition, edited by Elias I. Traboulsi, Oxford University Press, 2012.

[00536] For administration to the eye, lentiviral vectors, in particular equine infectious anemia viruses (EIAV) are particularly preferred.

[00537] In another embodiment, minimal non-primate lentiviral vectors based on the equine infectious anemia virus (EIAV) are also contemplated, especially for ocular gene therapy (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). The vectors are contemplated to have cytomegalovirus (CMV) promoter driving expression of the target gene. Intracameral, subretinal, intraocular and intravitreal injections are all contemplated (see, e.g., Balagaan, J Gene Med 2006; 8: 275 – 285, Published online 21 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jgm.845). Intraocular injections may be performed with the aid of an operating microscope. For subretinal and intravitreal injections, eyes may be prolapsed by gentle digital pressure and fundi visualised using a contact lens system consisting of a drop of a coupling medium solution on the cornea covered with a glass microscope slide coverslip. For subretinal injections, the tip of a 10-mm 34-gauge needle, mounted on a 5-µl Hamilton syringe may be advanced under direct visualisation through the superior equatorial sclera tangentially towards the posterior pole until the aperture of the needle was visible in the subretinal space. Then, 2 µl of vector suspension may be injected to produce a superior bullous retinal detachment, thus confirming subretinal vector administration. This approach creates a self-sealing sclerotomy allowing the vector suspension to be retained in the subretinal space until it is absorbed by the RPE, usually within 48 h of the procedure. This procedure may be repeated in the inferior hemisphere to produce an inferior retinal detachment. This technique results in the exposure of approximately 70% of neurosensory retina and RPE to the vector suspension. For intravitreal injections, the needle tip may be advanced through the sclera 1 mm posterior to the corneoscleral limbus and 2 µl of vector suspension injected into the vitreous cavity. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 µl of vector

suspension may be injected. For intracameral injections, the needle tip may be advanced through a corneoscleral limbal paracentesis, directed towards the central cornea, and 2 µl of vector suspension may be injected. These vectors may be injected at titres of either 1.0–1.4 × 10^{10} or 1.0–1.4 × 10^{9} transducing units (TU)/ml.

[00538] In another embodiment, RetinoStat®, an equine infectious anemia virus-based lentiviral gene therapy vector that expresses angiostatic proteins endostain and angiostatin that is delivered via a subretinal injection for the treatment of the web form of age-related macular degeneration is also contemplated (see, e.g., Binley et al., HUMAN GENE THERAPY 23:980–991 (September 2012)). Such a vector may be modified for the CRISPR-Cas system of the present invention. Each eye may be treated with either RetinoStat® at a dose of 1.1 x 10^{5} transducing units per eye (TU/eye) in a total volume of 100 µl.

[00539] In another embodiment, an E1-, partial E3-, E4-deleted adenoviral vector may be contemplated for delivery to the eye. Twenty-eight patients with advanced neovascular age-related macular degeneration (AMD) were given a single intravitreous injection of an E1-, partial E3-, E4-deleted adenoviral vector expressing human pigment ep- ithelium-derived factor (AdPEDF.11) (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Doses ranging from 10^{6} to $10^{9.5}$ particle units (PU) were investigated and there were no serious adverse events related to AdPEDF.11 and no dose-limiting toxicities (see, e.g., Campochiaro et al., Human Gene Therapy 17:167-176 (February 2006)). Adenoviral vector-mediated ocular gene transfer appears to be a viable approach for the treatment of ocular disorders and could be applied to the CRISPR Cas system.

[00540] In another embodiment, the sd-rxRNA® system of RXi Pharmaceuticals may be used/and or adapted for delivering CRISPR Cas to the eye. In this system, a single intravitreal administration of 3 µg of sd-rxRNA results in sequence-specific reduction of PPIB mRNA levels for 14 days. The the sd-rxRNA® system may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 3 to 20 mg of CRISPR administered to a human.

[00541] Millington-Ward et al. (Molecular Therapy, vol. 19 no. 4, 642–649 apr. 2011) describes adeno-associated virus (AAV) vectors to deliver an RNA interference (RNAi)-based rhodopsin suppressor and a codon-modified rhodopsin replacement gene resistant to suppression due to nucleotide alterations at degenerate positions over the RNAi target site. An injection of

either 6.0 x 10^8 vp or 1.8 x 10^{10} vp AAV were subretinally injected into the eyes by Millington-Ward et al. The AAV vectors of Millington-Ward et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 2 x 10^{11} to about 6 x 10^{13} vp administered to a human.

[00542] Dalkara et al. (Sci Transl Med 5, 189ra76 (2013)) also relates to in vivo directed evolution to fashion an AAV vector that delivers wild-type versions of defective genes throughout the retina after noninjurious injection into the eyes' vitreous humor. Dalkara describes a a 7mer peptide display library and an AAV library constructed by DNA shuffling of cap genes from AAV1, 2, 4, 5, 6, 8, and 9. The rcAAV libraries and rAAV vectors expressing GFP under a CAG or Rho promoter were packaged and and deoxyribonuclease-resistant genomic titers were obtained through quantitative PCR. The libraries were pooled, and two rounds of evolution were performed, each consisting of initial library diversification followed by three in vivo selection steps. In each such step, P30 rho-GFP mice were intravitreally injected with 2 ml of iodixanol-purified, phosphate-buffered saline (PBS)–dialyzed library with a genomic titer of about 1 × 10^{12} vg/ml. The AAV vectors of Dalkara et al. may be applied to the CRISPR Cas system of the present invention, contemplating a dose of about 1 x 10^{15} to about 1 x 10^{16} vg/ml administered to a human.

[00543] In another embodiment, the rhodopsin gene may be targeted for the treatment of retinitis pigmentosa (RP), wherein the system of US Patent Publication No. 20120204282 assigned to Sangamo BioSciences, Inc. may be modified in accordance of the CRISPR Cas system of the present invention.

[00544] In another embodiment, the methods of US Patent Publication No. 20130183282 assigned to Cellectis, which is directed to methods of cleaving a target sequence from the human rhodopsin gene, may also be modified to the CRISPR Cas system of the present invention.

[00545] US Patent Publication No. 20130202678 assigned to Academia Sinica relates to methods for treating retinopathies and sight-threatening ophthalmologic disorders relating to delivering of the Puf-A gene (which is expressed in retinal ganglion and pigmented cells of eye tissues and displays a unique anti-apoptotic activity) to the sub-retinal or intravitreal space in the eye. In particular, desirable targets are zgc:193933, prdm1a, spata2, tex10, rbb4, ddx3, zp2.2, Blimp-1 and HtrA2, all of which may be targeted by the CRISPR Cas system of the present invention.

[00546] Wu (Cell Stem Cell,13:659–62, 2013) designed a guide RNA that led Cas9 to a single base pair mutation that causes cataracts in mice, where it induced DNA cleavage. Then using either the other wild-type allele or oligos given to the zygotes repair mechanisms corrected the sequence of the broken allele and corrected the cataract-causing genetic defect in mutant mouse.

[00547] US Patent Publication No. 20120159653, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with macular degeration (MD). Macular degeneration (MD) is the primary cause of visual impairment in the elderly, but is also a hallmark symptom of childhood diseases such as Stargardt disease, Sorsby fundus, and fatal childhood neurodegenerative diseases, with an age of onset as young as infancy. Macular degeneration results in a loss of vision in the center of the visual field (the macula) because of damage to the retina. Currently existing animal models do not recapitulate major hallmarks of the disease as it is observed in humans. The available animal models comprising mutant genes encoding proteins associated with MD also produce highly variable phenotypes, making translations to human disease and therapy development problematic.

[00548] One aspect of US Patent Publication No. 20120159653 relates to editing of any chromosomal sequences that encode proteins associated with MD which may be applied to the CRISPR Cas system of the present invention. The proteins associated with MD are typically selected based on an experimental association of the protein associated with MD to an MD disorder. For example, the production rate or circulating concentration of a protein associated with MD may be elevated or depressed in a population having an MD disorder relative to a population lacking the MD disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the proteins associated with MD may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00549] By way of non-limiting example, proteins associated with MD include but are not limited to the following proteins: (ABCA4) ATP-binding cassette, sub-family A (ABC1), member 4 ACHM1 achromatopsia (rod monochromacy) 1 ApoE Apolipoprotein E (ApoE) C1QTNF5 (CTRP5) C1q and tumor necrosis factor related protein 5 (C1QTNF5) C2

Complement component 2 (C2) C3 Complement components (C3) CCL2 Chemokine (C-C motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C motif) receptor 2 (CCR2) CD36 Cluster of Differentiation 36 CFB Complement factor B CFH Complement factor CFH H CFHR1 complement factor H-related 1 CFHR3 complement factor H-related 3 CNGB3 cyclic nucleotide gated channel beta 3 CP ceruloplasmin (CP) CRP C reactive protein (CRP) CST3 cystatin C or cystatin 3 (CST3) CTSD Cathepsin D (CTSD) CX3CR1 chemokine (C-X3-C motif) receptor 1 ELOVL4 Elongation of very long chain fatty acids 4 ERCC6 excision repair cross-complementing rodent repair deficiency, complementation group 6 FBLN5 Fibulin-5 FBLN5 Fibulin 5 FBLN6 Fibulin 6 FSCN2 fascin (FSCN2) HMCN1 Hemicentrin 1 HMCN1 hemicentin 1 HTRA1 HtrA serine peptidase 1 (HTRA1) HTRA1 HtrA serine peptidase 1 IL-6 Interleukin 6 IL-8 Interleukin 8 LOC387715 Hypothetical protein PLEKHA1 Pleckstrin homology domain-containing family A member 1 (PLEKHA1) PROM1 Prominin 1(PROM1 or CD133) PRPH2 Peripherin-2 RPGR retinitis pigmentosa GTPase regulator SERPING1 serpin peptidase inhibitor, clade G, member 1 (C1- inhibitor) TCOF1 Treacle TIMP3 Metalloproteinase inhibitor 3 (TIMP3) TLR3 Toll-like receptor 3

[00550] The identity of the protein associated with MD whose chromosomal sequence is edited can and will vary. In preferred embodiments, the proteins associated with MD whose chromosomal sequence is edited may be the ATP-binding cassette, sub-family A (ABC1) member 4 protein (ABCA4) encoded by the ABCR gene, the apolipoprotein E protein (APOE) encoded by the APOE gene, the chemokine (C-C motif) Ligand 2 protein (CCL2) encoded by the CCL2 gene, the chemokine (C-C motif) receptor 2 protein (CCR2) encoded by the CCR2 gene, the ceruloplasmin protein (CP) encoded by the CP gene, the cathepsin D protein (CTSD) encoded by the CTSD gene, or the metalloproteinase inhibitor 3 protein (TIMP3) encoded by the TIMP3 gene. In an exemplary embodiment, the genetically modified animal is a rat, and the edited chromosomal sequence encoding the protein associated with MD may be: (ABCA4) ATP-binding cassette, NM_000350 sub-family A (ABC1), member 4 APOE Apolipoprotein E NM_138828 (APOE) CCL2 Chemokine (C-C NM_031530 motif) Ligand 2 (CCL2) CCR2 Chemokine (C-C NM_021866 motif) receptor 2 (CCR2) CP ceruloplasmin (CP) NM_012532 CTSD Cathepsin D (CTSD) NM_134334 TIMP3 Metalloproteinase NM_012886 inhibitor 3 (TIMP3) The animal or cell may comprise 1, 2, 3, 4, 5, 6, 7 or more disrupted chromosomal

sequences encoding a protein associated with MD and zero, 1, 2, 3, 4, 5, 6, 7 or more chromosomally integrated sequences encoding the disrupted protein associated with MD.

[00551] The edited or integrated chromosomal sequence may be modified to encode an altered protein associated with MD. Several mutations in MD-related chromosomal sequences have been associated with MD. Non-limiting examples of mutations in chromosomal sequences associated with MD include those that may cause MD including in the ABCR protein, E471K (i.e. glutamate at position 471 is changed to lysine), R1129L (i.e. arginine at position 1129 is changed to leucine), T1428M (i.e. threonine at position 1428 is changed to methionine), R1517S (i.e. arginine at position 1517 is changed to serine), I1562T (i.e. isoleucine at position 1562 is changed to threonine), and G1578R (i.e. glycine at position 1578 is changed to arginine); in the CCR2 protein, V64I (i.e. valine at position 192 is changed to isoleucine); in CP protein, G969B (i.e. glycine at position 969 is changed to asparagine or aspartate); in TIMP3 protein, S156C (i.e. serine at position 156 is changed to cysteine), G166C (i.e. glycine at position 166 is changed to cysteine), G167C (i.e. glycine at position 167 is changed to cysteine), Y168C (i.e. tyrosine at position 168 is changed to cysteine), S170C (i.e. serine at position 170 is changed to cysteine), Y172C (i.e. tyrosine at position 172 is changed to cysteine) and S181C (i.e. serine at position 181 is changed to cysteine). Other associations of genetic variants in MD-associated genes and disease are known in the art.

[00552] **Heart**

[00553] The present invention also contemplates delivering the CRISPR-Cas system to the heart. For the heart, a myocardium tropic adena-associated virus (AAVM) is preferred, in particular AAVM41 which showed preferential gene transfer in the heart (see, e.g., Lin-Yanga et al., PNAS, March 10, 2009, vol. 106, no. 10). Administration may be systemic or local. A dosage of about 1-10 x 10^{14} vector genomes are contemplated for systemic administration. See also, e.g., Eulalio et al. (2012) Nature 492: 376 and Somasuntharam et al. (2013) Biomaterials 34: 7790.

[00554] For example, US Patent Publication No. 20110023139, describes use of zinc finger nucleases to genetically modify cells, animals and proteins associated with cardiovascular disease. Cardiovascular diseases generally include high blood pressure, heart attacks, heart failure, and stroke and TIA. Any chromosomal sequence involved in cardiovascular disease or the protein encoded by any chromosomal sequence involved in cardiovascular disease may be utilized in the methods described in this disclosure. The cardiovascular-related proteins are

typically selected based on an experimental association of the cardiovascular-related protein to the development of cardiovascular disease. For example, the production rate or circulating concentration of a cardiovascular-related protein may be elevated or depressed in a population having a cardiovascular disorder relative to a population lacking the cardiovascular disorder. Differences in protein levels may be assessed using proteomic techniques including but not limited to Western blot, immunohistochemical staining, enzyme linked immunosorbent assay (ELISA), and mass spectrometry. Alternatively, the cardiovascular-related proteins may be identified by obtaining gene expression profiles of the genes encoding the proteins using genomic techniques including but not limited to DNA microarray analysis, serial analysis of gene expression (SAGE), and quantitative real-time polymerase chain reaction (Q-PCR).

[00555] By way of example, the chromosomal sequence may comprise, but is not limited to, IL1B (interleukin 1, beta), XDH (xanthine dehydrogenase), TP53 (tumor protein p53), PTGIS (prostaglandin 12 (prostacyclin) synthase), MB (myoglobin), IL4 (interleukin 4), ANGPT1 (angiopoietin 1), ABCG8 (ATP-binding cassette, sub-family G (WHITE), member 8), CTSK (cathepsin K), PTGIR (prostaglandin 12 (prostacyclin) receptor (IP)), KCNJ11 (potassium inwardly-rectifying channel, subfamily J, member 11), INS (insulin), CRP (C-reactive protein, pentraxin-related), PDGFRB (platelet-derived growth factor receptor, beta polypeptide), CCNA2 (cyclin A2), PDGFB (platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)), KCNJ5 (potassium inwardly-rectifying channel, subfamily J, member 5), KCNN3 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3), CAPN10 (calpain 10), PTGES (prostaglandin E synthase), ADRA2B (adrenergic, alpha-2B-, receptor), ABCG5 (ATP-binding cassette, sub-family G (WHITE), member 5), PRDX2 (peroxiredoxin 2), CAPN5 (calpain 5), PARP14 (poly (ADP-ribose) polymerase family, member 14), MEX3C (mex-3 homolog C (C. elegans)), ACE angiotensin I converting enzyme (peptidyl-dipeptidase A) 1), TNF (tumor necrosis factor (TNF superfamily, member 2)), IL6 (interleukin 6 (interferon, beta 2)), STN (statin), SERPINE1 (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1), ALB (albumin), ADIPOQ (adiponectin, C1Q and collagen domain containing), APOB (apolipoprotein B (including Ag(x) antigen)), APOE (apolipoprotein E), LEP (leptin), MTHFR (5,10-methylenetetrahydrofolate reductase (NADPH)), APOA1 (apolipoprotein A-I), EDN1 (endothelin 1), NPPB (natriuretic peptide precursor B), NOS3 (nitric oxide synthase 3 (endothelial cell)), PPARG (peroxisome

proliferator-activated receptor gamma), PLAT (plasminogen activator, tissue), PTGS2 (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)), CETP (cholesteryl ester transfer protein, plasma), AGTR1 (angiotensin II receptor, type 1), HMGCR (3-hydroxy-3-methylglutaryl-Coenzyme A reductase), IGF1 (insulin-like growth factor 1 (somatomedin C)), SELE (selectin E), REN (renin), PPARA (peroxisome proliferator-activated receptor alpha), PON1 (paraoxonase 1), KNG1 (kininogen 1), CCL2 (chemokine (C-C motif) ligand 2), LPL (lipoprotein lipase), VWF (von Willebrand factor), F2 (coagulation factor II (thrombin)), ICAM1 (intercellular adhesion molecule 1), TGFB1 (transforming growth factor, beta 1), NPPA (natriuretic peptide precursor A), IL10 (interleukin 10), EPO (erythropoietin), SOD1 (superoxide dismutase 1, soluble), VCAM1 (vascular cell adhesion molecule 1), IFNG (interferon, gamma), LPA (lipoprotein, Lp(a)), MPO (myeloperoxidase), ESR1 (estrogen receptor 1), MAPK1 (mitogen-activated protein kinase 1), HP (haptoglobin), F3 (coagulation factor III (thromboplastin, tissue factor)), CST3 (cystatin C), COG2 (component of oligomeric golgi complex 2), MMP9 (matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)), SERPINC1 (serpin peptidase inhibitor, clade C (antithrombin), member 1), F8 (coagulation factor VIII, procoagulant component), HMOX1 (heme oxygenase (decycling) 1), APOC3 (apolipoprotein C-III), IL8 (interleukin 8), PROK1 (prokineticin 1), CBS (cystathionine-beta-synthase), NOS2 (nitric oxide synthase 2, inducible), TLR4 (toll-like receptor 4), SELP (selectin P (granule membrane protein 140 kDa, antigen CD62)), ABCA1 (ATP-binding cassette, sub-family A (ABC1), member 1), AGT (angiotensinogen (serpin peptidase inhibitor, clade A, member 8)), LDLR (low density lipoprotein receptor), GPT (glutamic-pyruvate transaminase (alanine aminotransferase)), VEGFA (vascular endothelial growth factor A), NR3C2 (nuclear receptor subfamily 3, group C, member 2), IL18 (interleukin 18 (interferon-gamma-inducing factor)), NOS1 (nitric oxide synthase 1 (neuronal)), NR3C1 (nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)), FGB (fibrinogen beta chain), HGF (hepatocyte growth factor (hepapoietin A; scatter factor)), IL1A (interleukin 1, alpha), RETN (resistin), AKT1 (v-akt murine thymoma viral oncogene homolog 1), LIPC (lipase, hepatic), HSPD1 (heat shock 60 kDa protein 1 (chaperonin)), MAPK14 (mitogen-activated protein kinase 14), SPP1 (secreted phosphoprotein 1), ITGB3 (integrin, beta 3 (platelet glycoprotein 111a, antigen CD61)), CAT (catalase), UTS2 (urotensin 2), THBD (thrombomodulin), F10 (coagulation factor X), CP (ceruloplasmin (ferroxidase)), TNFRSF11B

(tumor necrosis factor receptor superfamily, member 11b), EDNRA (endothelin receptor type A), EGFR (epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)), MMP2 (matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)), PLG (plasminogen), NPY (neuropeptide Y), RHOD (ras homolog gene family, member D), MAPK8 (mitogen-activated protein kinase 8), MYC (v-myc myelocytomatosis viral oncogene homolog (avian)), FN1 (fibronectin 1), CMA1 (chymase 1, mast cell), PLAU (plasminogen activator, urokinase), GNB3 (guanine nucleotide binding protein (G protein), beta polypeptide 3), ADRB2 (adrenergic, beta-2-, receptor, surface), APOA5 (apolipoprotein A-V), SOD2 (superoxide dismutase 2, mitochondrial), F5 (coagulation factor V (proaccelerin, labile factor)), VDR (vitamin D (1,25-dihydroxyvitamin D3) receptor), ALOX5 (arachidonate 5-lipoxygenase), HLA-DRB1 (major histocompatibility complex, class II, DR beta 1), PARP1 (poly (ADP-ribose) polymerase 1), CD40LG (CD40 ligand), PON2 (paraoxonase 2), AGER (advanced glycosylation end product-specific receptor), IRS1 (insulin receptor substrate 1), PTGS1 (prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)), ECE1 (endothelin converting enzyme 1), F7 (coagulation factor VII (serum prothrombin conversion accelerator)), URN (interleukin 1 receptor antagonist), EPHX2 (epoxide hydrolase 2, cytoplasmic), IGFBP1 (insulin-like growth factor binding protein 1), MAPK10 (mitogen-activated protein kinase 10), FAS (Fas (TNF receptor superfamily, member 6)), ABCB1 (ATP-binding cassette, sub-family B (MDR/TAP), member 1), JUN (jun oncogene), IGFBP3 (insulin-like growth factor binding protein 3), CD14 (CD14 molecule), PDE5A (phosphodiesterase 5A, cGMP-specific), AGTR2 (angiotensin II receptor, type 2), CD40 (CD40 molecule, TNF receptor superfamily member 5), LCAT (lecithin-cholesterol acyltransferase), CCR5 (chemokine (C-C motif) receptor 5), MMP1 (matrix metallopeptidase 1 (interstitial collagenase)), TIMP1 (TIMP metallopeptidase inhibitor 1), ADM (adrenomedullin), DYT10 (dystonia 10), STAT3 (signal transducer and activator of transcription 3 (acute-phase response factor)), MMP3 (matrix metallopeptidase 3 (stromelysin 1, progelatinase)), ELN (elastin), USF1 (upstream transcription factor 1), CFH (complement factor H), HSPA4 (heat shock 70 kDa protein 4), MMP12 (matrix metallopeptidase 12 (macrophage elastase)), MME (membrane metallo-endopeptidase), F2R (coagulation factor II (thrombin) receptor), SELL (selectin L), CTSB (cathepsin B), ANXA5 (annexin A5), ADRB1 (adrenergic, beta-1-, receptor), CYBA (cytochrome b-245, alpha polypeptide), FGA (fibrinogen alpha chain), GGT1 (gamma-

glutamyltransferase 1), LIPG (lipase, endothelial), HIF1A (hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)), CXCR4 (chemokine (C-X-C motif) receptor 4), PROC (protein C (inactivator of coagulation factors Va and VIIIa)), SCARB1 (scavenger receptor class B, member 1), CD79A (CD79a molecule, immunoglobulin-associated alpha), PLTP (phospholipid transfer protein), ADD1 (adducin 1 (alpha)), FGG (fibrinogen gamma chain), SAA1 (serum amyloid A1), KCNH2 (potassium voltage-gated channel, subfamily H (eag-related), member 2), DPP4 (dipeptidyl-peptidase 4), G6PD (glucose-6-phosphate dehydrogenase), NPR1 (natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)), VTN (vitronectin), KIAA0101 (KIAA0101), FOS (FBJ murine osteosarcoma viral oncogene homolog), TLR2 (toll-like receptor 2), PPIG (peptidylprolyl isomerase G (cyclophilin G)), IL1R1 (interleukin 1 receptor, type I), AR (androgen receptor), CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), SERPINA1 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1), MTR (5-methyltetrahydrofolate-homocysteine methyltransferase), RBP4 (retinol binding protein 4, plasma), APOA4 (apolipoprotein A-IV), CDKN2A (cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)), FGF2 (fibroblast growth factor 2 (basic)), EDNRB (endothelin receptor type B), ITGA2 (integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)), CABIN1 (calcineurin binding protein 1), SHBG (sex hormone-binding globulin), HMGB1 (high-mobility group box 1), HSP90B2P (heat shock protein 90 kDa beta (Grp94), member 2 (pseudogene)), CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4), GJA1 (gap junction protein, alpha 1, 43 kDa), CAV1 (caveolin 1, caveolae protein, 22 kDa), ESR2 (estrogen receptor 2 (ER beta)), LTA (lymphotoxin alpha (TNF superfamily, member 1)), GDF15 (growth differentiation factor 15), BDNF (brain-derived neurotrophic factor), CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), NGF (nerve growth factor (beta polypeptide)), SP1 (Sp1 transcription factor), TGIF1 (TGFB-induced factor homeobox 1), SRC (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)), EGF (epidermal growth factor (beta-urogastrone)), PIK3CG (phosphoinositide-3-kinase, catalytic, gamma polypeptide), HLA-A (major histocompatibility complex, class I, A), KCNQ1 (potassium voltage-gated channel, KQT-like subfamily, member 1), CNR1 (cannabinoid receptor 1 (brain)), FBN1 (fibrillin 1), CHKA (choline kinase alpha), BEST1 (bestrophin 1), APP (amyloid beta (A4) precursor protein), CTNNB1 (catenin (cadherin-associated protein), beta 1, 88 kDa), IL2

(interleukin 2), CD36 (CD36 molecule (thrombospondin receptor)), PRKAB1 (protein kinase, AMP-activated, beta 1 non-catalytic subunit), TPO (thyroid peroxidase), ALDH7A1 (aldehyde dehydrogenase 7 family, member A1), CX3CR1 (chemokine (C-X3-C motif) receptor 1), TH (tyrosine hydroxylase), F9 (coagulation factor IX), GH1 (growth hormone 1), TF (transferrin), HFE (hemochromatosis), IL17A (interleukin 17A), PTEN (phosphatase and tensin homolog), GSTM1 (glutathione S-transferase mu 1), DMD (dystrophin), GATA4 (GATA binding protein 4), F13A1 (coagulation factor XIII, A1 polypeptide), TTR (transthyretin), FABP4 (fatty acid binding protein 4, adipocyte), PON3 (paraoxonase 3), APOC1 (apolipoprotein C-I), INSR (insulin receptor), TNFRSF1B (tumor necrosis factor receptor superfamily, member 1B), HTR2A (5-hydroxytryptamine (serotonin) receptor 2A), CSF3 (colony stimulating factor 3 (granulocyte)), CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9), TXN (thioredoxin), CYP11B2 (cytochrome P450, family 11, subfamily B, polypeptide 2), PTH (parathyroid hormone), CSF2 (colony stimulating factor 2 (granulocyte-macrophage)), KDR (kinase insert domain receptor (a type III receptor tyrosine kinase)), PLA2G2A (phospholipase A2, group IIA (platelets, synovial fluid)), B2M (beta-2-microglobulin), THBS1 (thrombospondin 1), GCG (glucagon), RHOA (ras homolog gene family, member A), ALDH2 (aldehyde dehydrogenase 2 family (mitochondrial)), TCF7L2 (transcription factor 7-like 2 (T-cell specific, HMG-box)), BDKRB2 (bradykinin receptor B2), NFE2L2 (nuclear factor (erythroid-derived 2)-like 2), NOTCH1 (Notch homolog 1, translocation-associated (Drosophila)), UGT1A1 (UDP glucuronosyltransferase 1 family, polypeptide A1), IFNA1 (interferon, alpha 1), PPARD (peroxisome proliferator-activated receptor delta), SIRT1 (sirtuin (silent mating type information regulation 2 homolog) 1 (S. cerevisiae)), GNRH1 (gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)), PAPPA (pregnancy-associated plasma protein A, pappalysin 1), ARR3 (arrestin 3, retinal (X-arrestin)), NPPC (natriuretic peptide precursor C), AHSP (alpha hemoglobin stabilizing protein), PTK2 (PTK2 protein tyrosine kinase 2), IL13 (interleukin 13), MTOR (mechanistic target of rapamycin (serine/threonine kinase)), ITGB2 (integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)), GSTT1 (glutathione S-transferase theta 1), IL6ST (interleukin 6 signal transducer (gp130, oncostatin M receptor)), CPB2 (carboxypeptidase B2 (plasma)), CYP1A2 (cytochrome P450, family 1, subfamily A, polypeptide 2), HNF4A (hepatocyte nuclear factor 4, alpha), SLC6A4 (solute carrier family 6 (neurotransmitter transporter, serotonin), member 4), PLA2G6 (phospholipase A2, group VI (cytosolic, calcium-

independent)), TNFSF11 (tumor necrosis factor (ligand) superfamily, member 11), SLC8A1 (solute carrier family 8 (sodium/calcium exchanger), member 1), F2RL1 (coagulation factor II (thrombin) receptor-like 1), AKR1A1 (aldo-keto reductase family 1, member A1 (aldehyde reductase)), ALDH9A1 (aldehyde dehydrogenase 9 family, member A1), BGLAP (bone gamma-carboxyglutamate (gla) protein), MTTP (microsomal triglyceride transfer protein), MTRR (5-methyltetrahydrofolate-homocysteine methyltransferase reductase), SULT1A3 (sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3), RAGE (renal tumor antigen), C4B (complement component 4B (Chido blood group), P2RY12 (purinergic receptor P2Y, G-protein coupled, 12), RNLS (renalase, FAD-dependent amine oxidase), CREB1 (cAMP responsive element binding protein 1), POMC (proopiomelanocortin), RAC1 (ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)), LMNA (lamin NC), CD59 (CD59 molecule, complement regulatory protein), SCN5A (sodium channel, voltage-gated, type V, alpha subunit), CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), MIF (macrophage migration inhibitory factor (glycosylation-inhibiting factor)), MMP13 (matrix metallopeptidase 13 (collagenase 3)), TIMP2 (TIMP metallopeptidase inhibitor 2), CYP19A1 (cytochrome P450, family 19, subfamily A, polypeptide 1), CYP21A2 (cytochrome P450, family 21, subfamily A, polypeptide 2), PTPN22 (protein tyrosine phosphatase, non-receptor type 22 (lymphoid)), MYH14 (myosin, heavy chain 14, non-muscle), MBL2 (mannose-binding lectin (protein C) 2, soluble (opsonic defect)), SELPLG (selectin P ligand), AOC3 (amine oxidase, copper containing 3 (vascular adhesion protein 1)), CTSL1 (cathepsin L1), PCNA (proliferating cell nuclear antigen), IGF2 (insulin-like growth factor 2 (somatomedin A)), ITGB1 (integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)), CAST (calpastatin), CXCL12 (chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)), IGHE (immunoglobulin heavy constant epsilon), KCNE1 (potassium voltage-gated channel, Isk-related family, member 1), TFRC (transferrin receptor (p90, CD71)), COL1A1 (collagen, type I, alpha 1), COL1A2 (collagen, type I, alpha 2), IL2RB (interleukin 2 receptor, beta), PLA2G10 (phospholipase A2, group X), ANGPT2 (angiopoietin 2), PROCR (protein C receptor, endothelial (EPCR)), NOX4 (NADPH oxidase 4), HAMP (hepcidin antimicrobial peptide), PTPN11 (protein tyrosine phosphatase, non-receptor type 11), SLC2A1 (solute carrier family 2 (facilitated glucose transporter), member 1), IL2RA (interleukin 2 receptor, alpha), CCL5 (chemokine (C-C motif) ligand 5), IRF1 (interferon regulatory factor 1), CFLAR (CASP8 and

FADD-like apoptosis regulator), CALCA (calcitonin-related polypeptide alpha), EIF4E (eukaryotic translation initiation factor 4E), GSTP1 (glutathione S-transferase pi 1), JAK2 (Janus kinase 2), CYP3A5 (cytochrome P450, family 3, subfamily A, polypeptide 5), HSPG2 (heparan sulfate proteoglycan 2), CCL3 (chemokine (C-C motif) ligand 3), MYD88 (myeloid differentiation primary response gene (88)), VIP (vasoactive intestinal peptide), SOAT1 (sterol O-acyltransferase 1), ADRBK1 (adrenergic, beta, receptor kinase 1), NR4A2 (nuclear receptor subfamily 4, group A, member 2), MMP8 (matrix metallopeptidase 8 (neutrophil collagenase)), NPR2 (natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)), GCH1 (GTP cyclohydrolase 1), EPRS (glutamyl-prolyl-tRNA synthetase), PPARGC1A (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha), F12 (coagulation factor XII (Hageman factor)), PECAM1 (platelet/endothelial cell adhesion molecule), CCL4 (chemokine (C-C motif) ligand 4), SERPINA3 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3), CASR (calcium-sensing receptor), GJA5 (gap junction protein, alpha 5, 40 kDa), FABP2 (fatty acid binding protein 2, intestinal), TTF2 (transcription termination factor, RNA polymerase II), PROS1 (protein S (alpha)), CTF1 (cardiotrophin 1), SGCB (sarcoglycan, beta (43 kDa dystrophin-associated glycoprotein)), YME1L1 (YME1-like 1 (S. cerevisiae)), CAMP (cathelicidin antimicrobial peptide), ZC3H12A (zinc finger CCCH-type containing 12A), AKR1B1 (aldo-keto reductase family 1, member B1 (aldose reductase)), DES (desmin), MMP7 (matrix metallopeptidase 7 (matrilysin, uterine)), AHR (aryl hydrocarbon receptor), CSF1 (colony stimulating factor 1 (macrophage)), HDAC9 (histone deacetylase 9), CTGF (connective tissue growth factor), KCNMA1 (potassium large conductance calcium-activated channel, subfamily M, alpha member 1), UGT1A (UDP glucuronosyltransferase 1 family, polypeptide A complex locus), PRKCA (protein kinase C, alpha), COMT (catechol-.beta.-methyltransferase), S100B (S100 calcium binding protein B), EGR1 (early growth response 1), PRL (prolactin), IL15 (interleukin 15), DRD4 (dopamine receptor D4), CAMK2G (calcium/calmodulin-dependent protein kinase II gamma), SLC22A2 (solute carrier family 22 (organic cation transporter), member 2), CCL11 (chemokine (C-C motif) ligand 11), PGF (B321 placental growth factor), THPO (thrombopoietin), GP6 (glycoprotein VI (platelet)), TACR1 (tachykinin receptor 1), NTS (neurotensin), HNF1A (HNF1 homeobox A), SST (somatostatin), KCND1 (potassium voltage-gated channel, Shal-related subfamily, member 1), LOC646627 (phospholipase inhibitor), TBXAS1 (thromboxane A synthase 1 (platelet)), CYP2J2 (cytochrome

P450, family 2, subfamily J, polypeptide 2), TBXA2R (thromboxane A2 receptor), ADH1C (alcohol dehydrogenase 1C (class I), gamma polypeptide), ALOX12 (arachidonate 12-lipoxygenase), AHSG (alpha-2-HS-glycoprotein), BHMT (betaine-homocysteine methyltransferase), GJA4 (gap junction protein, alpha 4, 37 kDa), SLC25A4 (solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4), ACLY (ATP citrate lyase), ALOX5AP (arachidonate 5-lipoxygenase-activating protein), NUMA1 (nuclear mitotic apparatus protein 1), CYP27B1 (cytochrome P450, family 27, subfamily B, polypeptide 1), CYSLTR2 (cysteinyl leukotriene receptor 2), SOD3 (superoxide dismutase 3, extracellular), LTC4S (leukotriene C4 synthase), UCN (urocortin), GHRL (ghrelin/obestatin prepropeptide), APOC2 (apolipoprotein C-II), CLEC4A (C-type lectin domain family 4, member A), KBTBD10 (kelch repeat and BTB (POZ) domain containing 10), TNC (tenascin C), TYMS (thymidylate synthetase), SHC1 (SHC (Src homology 2 domain containing) transforming protein 1), LRP1 (low density lipoprotein receptor-related protein 1), SOCS3 (suppressor of cytokine signaling 3), ADH1B (alcohol dehydrogenase 1B (class I), beta polypeptide), KLK3 (kallikrein-related peptidase 3), HSD11B1 (hydroxysteroid (11-beta) dehydrogenase 1), VKORC1 (vitamin K epoxide reductase complex, subunit 1), SERPINB2 (serpin peptidase inhibitor, clade B (ovalbumin), member 2), TNS1 (tensin 1), RNF19A (ring finger protein 19A), EPOR (erythropoietin receptor), ITGAM (integrin, alpha M (complement component 3 receptor 3 subunit)), PITX2 (paired-like homeodomain 2), MAPK7 (mitogen-activated protein kinase 7), FCGR3A (Fc fragment of IgG, low affinity 111a, receptor (CD16a)), LEPR (leptin receptor), ENG (endoglin), GPX1 (glutathione peroxidase 1), GOT2 (glutamic-oxaloacetic transaminase 2, mitochondrial (aspartate aminotransferase 2)), HRH1 (histamine receptor H1), NR112 (nuclear receptor subfamily 1, group I, member 2), CRH (corticotropin releasing hormone), HTR1A (5-hydroxytryptamine (serotonin) receptor 1A), VDAC1 (voltage-dependent anion channel 1), HPSE (heparanase), SFTPD (surfactant protein D), TAP2 (transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)), RNF123 (ring finger protein 123), PTK2B (PTK2B protein tyrosine kinase 2 beta), NTRK2 (neurotrophic tyrosine kinase, receptor, type 2), IL6R (interleukin 6 receptor), ACHE (acetylcholinesterase (Yt blood group)), GLP1R (glucagon-like peptide 1 receptor), GHR (growth hormone receptor), GSR (glutathione reductase), NQO1 (NAD(P)H dehydrogenase, quinone 1), NR5A1 (nuclear receptor subfamily 5, group A, member 1), GJB2 (gap junction protein, beta 2, 26 kDa), SLC9A1 (solute carrier family 9 (sodium/hydrogen

exchanger), member 1), MAOA (monoamine oxidase A), PCSK9 (proprotein convertase subtilisin/kexin type 9), FCGR2A (Fc fragment of IgG, low affinity IIa, receptor (CD32)), SERPINF1 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1), EDN3 (endothelin 3), DHFR (dihydrofolate reductase), GAS6 (growth arrest-specific 6), SMPD1 (sphingomyelin phosphodiesterase 1, acid lysosomal), UCP2 (uncoupling protein 2 (mitochondrial, proton carrier)), TFAP2A (transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)), C4BPA (complement component 4 binding protein, alpha), SERPINF2 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2), TYMP (thymidine phosphorylase), ALPP (alkaline phosphatase, placental (Regan isozyme)), CXCR2 (chemokine (C-X-C motif) receptor 2), SLC39A3 (solute carrier family 39 (zinc transporter), member 3), ABCG2 (ATP-binding cassette, sub-family G (WHITE), member 2), ADA (adenosine deaminase), JAK3 (Janus kinase 3), HSPA1A (heat shock 70 kDa protein 1A), FASN (fatty acid synthase), FGF1 (fibroblast growth factor 1 (acidic)), F11 (coagulation factor XI), ATP7A (ATPase, Cu++ transporting, alpha polypeptide), CR1 (complement component (3b/4b) receptor 1 (Knops blood group)), GFAP (glial fibrillary acidic protein), ROCK1 (Rho-associated, coiled-coil containing protein kinase 1), MECP2 (methyl CpG binding protein 2 (Rett syndrome)), MYLK (myosin light chain kinase), BCHE (butyrylcholinesterase), LIPE (lipase, hormone-sensitive), PRDX5 (peroxiredoxin 5), ADORA1 (adenosine A1 receptor), WRN (Werner syndrome, RecQ helicase-like), CXCR3 (chemokine (C-X-C motif) receptor 3), CD81 (CD81 molecule), SMAD7 (SMAD family member 7), LAMC2 (laminin, gamma 2), MAP3K5 (mitogen-activated protein kinase kinase kinase 5), CHGA (chromogranin A (parathyroid secretory protein 1)), IAPP (islet amyloid polypeptide), RHO (rhodopsin), ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1), PTHLH (parathyroid hormone-like hormone), NRG1 (neuregulin 1), VEGFC (vascular endothelial growth factor C), ENPEP (glutamyl aminopeptidase (aminopeptidase A)), CEBPB (CCAAT/enhancer binding protein (C/EBP), beta), NAGLU (N-acetylglucosaminidase, alpha-), F2RL3 (coagulation factor II (thrombin) receptor-like 3), CX3CL1 (chemokine (C-X3-C motif) ligand 1), BDKRB1 (bradykinin receptor B1), ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif, 13), ELANE (elastase, neutrophil expressed), ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2), CISH (cytokine inducible SH2-containing protein), GAST (gastrin), MYOC (myocilin, trabecular meshwork inducible

glucocorticoid response), ATP1A2 (ATPase, Na+/K+ transporting, alpha 2 polypeptide), NF1 (neurofibromin 1), GJB1 (gap junction protein, beta 1, 32 kDa), MEF2A (myocyte enhancer factor 2A), VCL (vinculin), BMPR2 (bone morphogenetic protein receptor, type II (serine/threonine kinase)), TUBB (tubulin, beta), CDC42 (cell division cycle 42 (GTP binding protein, 25 kDa)), KRT18 (keratin 18), HSF1 (heat shock transcription factor 1), MYB (v-myb myeloblastosis viral oncogene homolog (avian)), PRKAA2 (protein kinase, AMP-activated, alpha 2 catalytic subunit), ROCK2 (Rho-associated, coiled-coil containing protein kinase 2), TFPI (tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)), PRKG1 (protein kinase, cGMP-dependent, type I), BMP2 (bone morphogenetic protein 2), CTNND1 (catenin (cadherin-associated protein), delta 1), CTH (cystathionase (cystathionine gamma-lyase)), CTSS (cathepsin S), VAV2 (vav 2 guanine nucleotide exchange factor), NPY2R (neuropeptide Y receptor Y2), IGFBP2 (insulin-like growth factor binding protein 2, 36 kDa), CD28 (CD28 molecule), GSTA1 (glutathione S-transferase alpha 1), PPIA (peptidylprolyl isomerase A (cyclophilin A)), APOH (apolipoprotein H (beta-2-glycoprotein I)), S100A8 (S100 calcium binding protein A8), IL11 (interleukin 11), ALOX15 (arachidonate 15-lipoxygenase), FBLN1 (fibulin 1), NR1H3 (nuclear receptor subfamily 1, group H, member 3), SCD (stearoyl-CoA desaturase (delta-9-desaturase)), GIP (gastric inhibitory polypeptide), CHGB (chromogranin B (secretogranin 1)), PRKCB (protein kinase C, beta), SRD5A1 (steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)), HSD11B2 (hydroxysteroid (11-beta) dehydrogenase 2), CALCRL (calcitonin receptor-like), GALNT2 (UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)), ANGPTL4 (angiopoietin-like 4), KCNN4 (potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4), PIK3C2A (phosphoinositide-3-kinase, class 2, alpha polypeptide), HBEGF (heparin-binding EGF-like growth factor), CYP7A1 (cytochrome P450, family 7, subfamily A, polypeptide 1), HLA-DRB5 (major histocompatibility complex, class II, DR beta 5), BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3), GCKR (glucokinase (hexokinase 4) regulator), S100A12 (S100 calcium binding protein A12), PADI4 (peptidyl arginine deiminase, type IV), HSPA14 (heat shock 70 kDa protein 14), CXCR1 (chemokine (C-X-C motif) receptor 1), H19 (H19, imprinted maternally expressed transcript (non-protein coding)), KRTAP19-3 (keratin associated protein 19-3), IDDM2 (insulin-dependent diabetes mellitus 2), RAC2 (ras-related C3 botulinum toxin

substrate 2 (rho family, small GTP binding protein Rac2)), RYR1 (ryanodine receptor 1 (skeletal)), CLOCK (clock homolog (mouse)), NGFR (nerve growth factor receptor (TNFR superfamily, member 16)), DBH (dopamine beta-hydroxylase (dopamine beta-monooxygenase)), CHRNA4 (cholinergic receptor, nicotinic, alpha 4), CACNA1C (calcium channel, voltage-dependent, L type, alpha 1C subunit), PRKAG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit), CHAT (choline acetyltransferase), PTGDS (prostaglandin D2 synthase 21 kDa (brain)), NR1H2 (nuclear receptor subfamily 1, group H, member 2), TEK (TEK tyrosine kinase, endothelial), VEGFB (vascular endothelial growth factor B), MEF2C (myocyte enhancer factor 2C), MAPKAPK2 (mitogen-activated protein kinase-activated protein kinase 2), TNFRSF11A (tumor necrosis factor receptor superfamily, member 11a, NFKB activator), HSPA9 (heat shock 70 kDa protein 9 (mortalin)), CYSLTR1 (cysteinyl leukotriene receptor 1), MAT1A (methionine adenosyltransferase I, alpha), OPRL1 (opiate receptor-like 1), IMPA1 (inositol(myo)-1(or 4)-monophosphatase 1), CLCN2 (chloride channel 2), DLD (dihydrolipoamide dehydrogenase), PSMA6 (proteasome (prosome, macropain) subunit, alpha type, 6), PSMB8 (proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)), CHI3L1 (chitinase 3-like 1 (cartilage glycoprotein-39)), ALDH1B1 (aldehyde dehydrogenase 1 family, member B1), PARP2 (poly (ADP-ribose) polymerase 2), STAR (steroidogenic acute regulatory protein), LBP (lipopolysaccharide binding protein), ABCC6 (ATP-binding cassette, sub-family C(CFTR/MRP), member 6), RGS2 (regulator of G-protein signaling 2, 24 kDa), EFNB2 (ephrin-B2), GJB6 (gap junction protein, beta 6, 30 kDa), APOA2 (apolipoprotein A-II), AMPD1 (adenosine monophosphate deaminase 1), DYSF (dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)), FDFT1 (farnesyl-diphosphate farnesyltransferase 1), EDN2 (endothelin 2), CCR6 (chemokine (C-C motif) receptor 6), GJB3 (gap junction protein, beta 3, 31 kDa), IL1RL1 (interleukin 1 receptor-like 1), ENTPD1 (ectonucleoside triphosphate diphosphohydrolase 1), BBS4 (Bardet-Biedl syndrome 4), CELSR2 (cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)), F11R (F11 receptor), RAPGEF3 (Rap guanine nucleotide exchange factor (GEF) 3), HYAL1 (hyaluronoglucosaminidase 1), ZNF259 (zinc finger protein 259), ATOX1 (ATX1 antioxidant protein 1 homolog (yeast)), ATF6 (activating transcription factor 6), KHK (ketohexokinase (fructokinase)), SAT1 (spermidine/spermine N1-acetyltransferase 1), GGH (gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)), TIMP4 (TIMP metallopeptidase inhibitor 4), SLC4A4

(solute carrier family 4, sodium bicarbonate cotransporter, member 4), PDE2A (phosphodiesterase 2A, cGMP-stimulated), PDE3B (phosphodiesterase 3B, cGMP-inhibited), FADS1 (fatty acid desaturase 1), FADS2 (fatty acid desaturase 2), TMSB4X (thymosin beta 4, X-linked), TXNIP (thioredoxin interacting protein), LIMS1 (LIM and senescent cell antigen-like domains 1), RHOB (ras homolog gene family, member B), LY96 (lymphocyte antigen 96), FOXO1 (forkhead box O1), PNPLA2 (patatin-like phospholipase domain containing 2), TRH (thyrotropin-releasing hormone), GJC1 (gap junction protein, gamma 1, 45 kDa), SLC17A5 (solute carrier family 17 (anion/sugar transporter), member 5), FTO (fat mass and obesity associated), GJD2 (gap junction protein, delta 2, 36 kDa), PSRC1 (proline/serine-rich coiled-coil 1), CASP12 (caspase 12 (gene/pseudogene)), GPBAR1 (G protein-coupled bile acid receptor 1), PXK (PX domain containing serine/threonine kinase), IL33 (interleukin 33), TRIB1 (tribbles homolog 1 (Drosophila)), PBX4 (pre-B-cell leukemia homeobox 4), NUPR1 (nuclear protein, transcriptional regulator, 1), 15-Sep(15 kDa selenoprotein), CILP2 (cartilage intermediate layer protein 2), TERC (telomerase RNA component), GGT2 (gamma-glutamyltransferase 2), MT-CO1 (mitochondrially encoded cytochrome c oxidase I), and UOX (urate oxidase, pseudogene).

[00556] In an additional embodiment, the chromosomal sequence may further be selected from Pon1 (paraoxonase 1), LDLR (LDL receptor), ApoE (Apolipoprotein E), Apo B-100 (Apolipoprotein B-100), ApoA (Apolipoprotein(a)), ApoA1 (Apolipoprotein A1), CBS (Cystathione B-synthase), Glycoprotein IIb/IIb, MTHRF (5,10-methylenetetrahydrofolate reductase (NADPH), and combinations thereof. In one iteration, the chromosomal sequences and proteins encoded by chromosomal sequences involved in cardiovascular disease may be chosen from Cacna1C, Sod1, Pten, Ppar(alpha), Apo E, Leptin, and combinations thereof.

[00557] **Kidneys**

[00558] The present invention also contemplates delivering the CRISPR-Cas system to the kidney. Delivery strategies to induce cellular uptake of the therapeutic nucleic acid include physical force or vector systems such as viral-, lipid- or complex- based delivery, or nanocarriers. From the initial applications with less possible clinical relevance, when nucleic acids were addressed to renal cells with hydrodynamic high pressure injection systemically, a wide range of gene therapeutic viral and non-viral carriers have been applied already to target posttranscriptional events in different animal kidney disease models in vivo (Csaba Révész and Péter Hamar (2011). Delivery Methods to Target RNAs in the Kidney, Gene Therapy

Applications, Prof. Chunsheng Kang (Ed.), ISBN: 978-953-307-541-9, InTech, Available from: http://www.intechopen.com/books/gene-therapy-applications/delivery-methods-to-target-rnas-in-the-kidney). Delivery methods to the kidney are summarized as follows:

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Hydrodynamic / Lipid	TransIT In Vivo Gene Delivery System, DOTAP	p85α	Acute renal injury	Ischemia-reperfusion	Uptake, biodistribution	Larson et al., Surgery, (Aug 2007), Vol. 142, No. 2, pp. (262-269)
Hydrodynamic / Lipid	Lipofectamine 2000	Fas	Acute renal injury	Ischemia-reperfusion	Blood urea nitrogen, Fas Immunohistochemistry, apoptosis, histological scoring	Hamar et al., Proc Natl Acad Sci, (Oct 2004), Vol. 101, No. 41, pp. (14883-14888)
Hydrodynamic	n.a.	Apoptosis cascade elements	Acute renal injury	Ischemia-reperfusion	n.a.	Zheng et al., Am J Pathol, (Oct 2008), Vol. 173, No. 4, pp. (973–980)
Hydrodynamic	n.a.	Nuclear factor kappa-b (NFkB)	Acute renal injury	Ischemia-reperfusion	n.a.	Feng et al., Transplantation, (May 2009), Vol. 87, No. 9, pp. (1283–1289)
Hydrodynamic /Viral	Lipofectamine 2000	Apoptosis antagonizing transcription factor (AATF)	Acute renal injury	Ischemia-reperfusion	Apoptosis, oxidative stress, caspase activation, membrane lipid peroxidation	Xie & Guo, Am Soc Nephrol, (Dec 2006), Vol. 17, No. 12, pp. (3336–3346)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Hydrodynamic	pBAsi mU6 Neo/ TransIT-EE Hydrodynamic Delivery System	Gremlin	Diabetic nephropathy	Streptozotozin - induced diabetes	Proteinuria, serum creatinine, glomerular and tubular diameter, collagen type IV/BMP7 expression	Q. Zhang et al., PloS ONE, (Jul 2010), Vol. 5, No. 7, e11709, pp. (1-13)
Viral/Lipid	pSUPER vector/Lipofectamine	TGF-β type II receptor	Interstitial renal fibrosis	Unilateral urethral obstruction	α-SMA expression, collagen content,	Kushibikia et al., J Controlled Release, (Jul 2005), Vol. 105, No. 3, pp. (318-331)
Viral	Adeno-associated virus-2	Mineral corticoid receptor	Hyper-tension caused renal damage	Cold-induced hypertension	blood pressure, serum albumin, serum urea nitrogen, serum creatinine, kidney weight, urinary sodium	Wang et al., Gene Therapy, (Jul 2006), Vol. 13, No. 14, pp. (1097-1103)
Hydrodynamic /Viral	pU6 vector	Luciferase	n.a.	n.a.	uptake	Kobayashi et al., Journal of Pharmacology and Experimental Therapeutics, (Feb 2004), Vol. 308, No. 2, pp. (688-693)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Lipid	Lipoproteins, albumin	apoB1, apoM	n.a.	n.a.	Uptake, binding affinity to lipoproteins and albumin	Wolfrum et al., Nature Biotechnology, (Sep 2007), Vol. 25, No. 10, pp. (1149-1157)
Lipid	Lipofectamine 2000	p53	Acute renal injury	Ischemic and cisplatin-induced acute injury	Histological scoring, apoptosis	Molitoris et al., J Am Soc Nephrol, (Aug 2009), Vol. 20, No. 8, pp. (1754-1764)
Lipid	DOTAP/DOPE, DOTAP/DOPE/DOPE-PEG2000	COX-2	Breast adeno-carcinoma	MDA-MB-231 breast cancer xenograft-bearing mouse	Cell viability, uptake	Mikhaylova et al., Cancer Gene Therapy, (Mar 2011), Vol. 16, No. 3, pp. (217-226)
Lipid	Cholesterol	12/15-lipoxygenase	Diabetic nephro-pathy	Streptozotocin - induced diabetes	Albuminuria, urinary creatinine, histology, type I and IV collagen, TGF-β, fibronectin, plasminogen activator inhibitor 1	Yuan et al., Am J Physiol Renal Physiol, (Jun 2008), Vol. 295, pp. (F605-F617)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Lipid	Lipofectamine 2000	Mitochondrial membrane 44 (TIM44)	Diabetic nephro-pathy	Streptozotocin - induced diabetes	Cell proliferation and apoptosis, histology, ROS, mitochondrial import of Mn-SOD and glutathione peroxidase, cellular membrane polarization	Y. Zhang et al., J Am Soc Nephrol, (Apr 2006), Vol. 17, No. 4, pp. (1090–1101)
Hydrodynamic / Lipid	Proteolipo-some	RLIP76	Renal carcinoma	Caki-2 kidney cancer xenograft-bearing mouse	uptake	Singhal et al., Cancer Res, (May 2009), Vol. 69, No. 10, pp. (4244-4251)
Polymer	PEGylated PEI	Luciferase pGL3	n.a.	n.a.	Uptake, biodistribution, erythrocyte aggregation	Malek et al., Toxicology and Applied Pharmacology, (Apr 2009), Vol. 236, No. 1, pp. (97-108)
Polymer	PEGylated poly-L-lysine	MAPK1	Lupus glomerulo - nephritis	Glomerulo-nephritis	Proteinuria, glomerulosclerosis, TGF- β, fibronectin, plasminogen activator inhibitor 1	Shimizu et al., J Am Soc Nephrology, (Apr 2010), Vol. 21, No. 4, pp. (622-633)
Polymer/Nano particle	Hyaluronic acid/ Quantum dot/ PEI	VEGF	Kidney cancer/ melanoma	B16F1 melanoma tumor-bearing mouse	Biodistribution, citotoxicity, tumor volume, endocytosis	Jiang et al., Molecular Pharmaceutics, (May-Jun 2009), Vol. 6, No. 3, pp. (727-737)

Delivery method	Carrier	Target RNA	Disease	Model	Functional assays	Author
Polymer/Nano particle	PEGylated polycapro-lactone nanofiber	GAPDH	n.a.	n.a.	cell viability, uptake	Cao et al, J Controlled Release, (Jun 2010), Vol. 144, No. 2, pp. (203-212)
Aptamer	Spiegelmer mNOX-E36	CC chemokine ligand 2	Glomerulo sclerosis	Uninephrecto- mized mouse	urinary albumin, urinary creatinine, histopathology, glomerular filtration rate, macrophage count, serum Ccl2, Mac-2+, Ki-67+	Ninichuk et al., Am J Pathol, (Mar 2008), Vol. 172, No. 3, pp. (628-637)
Aptamer	Aptamer NOX-F37	vasopressin (AVP)	Congestive heart failure	n.a.	Binding affinity to D-AVP, Inhibition of AVP Signaling, Urine osmolality and sodium concentration,	Purschke et al., Proc Natl Acad Sci, (Mar 2006), Vol. 103, No. 13, pp. (5173-5178)

[00559] Yuan et al. (Am J Physiol Renal Physiol 295: F605–F617, 2008) investigated whether in vivo delivery of small interfering RNAs (siRNAs) targeting the 12/15-lipoxygenase (12/15-LO) pathway of arachidonate acid metabolism can ameliorate renal injury and diabetic nephropathy (DN) in a streptozotocininjected mouse model of type 1 diabetes. To achieve greater in vivo access and siRNA expression in the kidney, Yuan et al. used double-stranded 12/15-LO siRNA oligonucleotides conjugated with cholesterol. About 400 μg of siRNA was injected subcutaneously into mice. The method of Yuang et al. may be applied to the CRISPR Cas system of the present invention contemplating a 1-2 g subcutaneous injection of CRISPR Cas conjugated with cholesterol to a human for delivery to the kidneys.

[00560] Molitoris et al. (J Am Soc Nephrol 20: 1754–1764, 2009) exploited proximal tubule cells (PTCs), as the site of oligonucleotide reabsorption within the kidney to test the efficacy of siRNA targeted to p53, a pivotal protein in the apoptotic pathway, to prevent kidney injury. Naked synthetic siRNA to p53 injected intravenously 4 h after ischemic injury maximally protected both PTCs and kidney function. Molitoris et al.'s data indicates that rapid delivery of siRNA to proximal tubule cells follows intravenous administration. For dose-response analysis, rats were injected with doses of siP53, 0.33; 1, 3, or 5mg/kg, given at the same four time points, resulting in cumulative doses of 1.32; 4, 12, and 20 mg/kg, respectively. All siRNA doses tested produced a SCr reducing effect on day one with higher doses being effective over approximately five days compared with PBS-treated ischemic control rats. The 12 and 20 mg/kg cumulative doses provided the best protective effect. The method of Molitoris et al. may be applied to the CRISPR Cas system of the present invention contemplating 12 and 20 mg/kg cumulative doses to a human for delivery to the kidneys.

[00561] Thompson et al. (Nucleic Acid Therapeutics, Volume 22, Number 4, 2012) reports the toxicological and pharmacokinetic properties of the synthetic, small interfering RNA I5NP following intravenous administration in rodents and nonhuman primates. I5NP is designed to act via the RNA interference (RNAi) pathway to temporarily inhibit expression of the pro-apoptotic protein p53 and is being developed to protect cells from acute ischemia/reperfusion injuries such as acute kidney injury that can occur during major cardiac surgery and delayed graft function that can occur following renal transplantation. Doses of 800mg/kg I5NP in rodents, and 1,000 mg/kg I5NP in nonhuman primates, were required to elicit adverse effects, which in the monkey were isolated to direct effects on the blood that included a sub-clinical activation of complement and slightly increased clotting times. In the rat, no additional adverse effects were observed with a rat analogue of I5NP, indicating that the effects likely represent class effects of synthetic RNA duplexes rather than toxicity related to the intended pharmacologic activity of I5NP. Taken together, these data support clinical testing of intravenous administration of I5NP for the preservation of renal function following acute ischemia/reperfusion injury. The no observed adverse effect level (NOAEL) in the monkey was 500 mg/kg. No effects on cardiovascular, respiratory, and neurologic parameters were observed in monkeys following i.v. administration at dose levels up to 25 mg/kg. Therefore, a similar dosage may be contemplated for intravenous administration of CRISPR Cas to the kidneys of a human.

[00562] Shimizu et al. (J Am Soc Nephrol 21: 622–633, 2010) developed a system to target delivery of siRNAs to glomeruli via poly(ethylene glycol)-poly(L-lysine)-based vehicles. The siRNA/nanocarrier complex was approximately 10 to 20 nm in diameter, a size that would allow it to move across the fenestrated endothelium to access to the mesangium. After intraperitoneal injection of fluorescence-labeled siRNA/nanocarrier complexes, Shimizu et al. detected siRNAs in the blood circulation for a prolonged time. Repeated intraperitoneal administration of a mitogen-activated protein kinase 1 (MAPK1) siRNA/nanocarrier complex suppressed glomerular MAPK1 mRNA and protein expression in a mouse model of glomerulonephritis. For the investigation of siRNA accumulation, Cy5-labeled siRNAs complexed with PIC nanocarriers (0.5 ml, 5 nmol of siRNA content), naked Cy5-labeled siRNAs (0.5 ml, 5 nmol), or Cy5-labeled siRNAs encapsulated in HVJ-E (0.5 ml, 5 nmol of siRNA content) were administrated to BALB-c mice. The method of Shimizu et al. may be applied to the CRISPR Cas system of the present invention contemplating a dose of about of 10-20 μmol CRISPR Cas complexed with nanocarriers in about 1-2 liters to a human for intraperitoneal administration and delivery to the kidneys.

[00563] Lungs

[00564] The present invention also contemplates delivering the CRISPR-Cas system to oneor both lungs.

[00565] Although AAV-2-based vectors were originally proposed for CFTR delivery to CF airways, other serotypes such as AAV-1, AAV-5, AAV-6, and AAV-9 exhibit improved gene transfer efficiency in a variety of models of the lung epithelium (see, e.g., Li et al., Molecular Therapy, vol. 17 no. 12, 2067-2077 Dec 2009). AAV-1 was demonstrated to be ~100-fold more efficient than AAV-2 and AAV-5 at transducing human airway epithelial cells in vitro,5 although AAV-1 transduced murine tracheal airway epithelia in vivo with an efficiency equal to that of AAV-5. Other studies have shown that AAV-5 is 50-fold more efficient than AAV-2 at gene delivery to human airway epithelium (HAE) in vitro and significantly more efficient in the mouse lung airway epithelium in vivo. AAV-6 has also been shown to be more efficient than AAV-2 in human airway epithelial cells in vitro and murine airways in vivo.8 The more recent isolate, AAV-9, was shown to display greater gene transfer efficiency than AAV-5 in murine nasal and alveolar epithelia in vivo with gene expression detected for over 9 months suggesting AAV may enable long-term gene expression in vivo, a desirable property for a CFTR gene

delivery vector. Furthermore, it was demonstrated that AAV-9 could be readministered to the murine lung with no loss of CFTR expression and minimal immune consequences. CF and non-CF HAE cultures may be inoculated on the apical surface with 100 µl of AAV vectors for hours (see, e.g., Li et al., Molecular Therapy, vol. 17 no. 12, 2067-2077 Dec 2009). The MOI may vary from 1×10^3 to 4×10^5 vector genomes/cell, depending on virus concentration and purposes of the experiments. The above cited vectors are contemplated for the delivery and/or administration of the invention.

[00566] Zamora et al. (Am J Respir Crit Care Med Vol 183. pp 531–538, 2011) reported an example of the application of an RNA interference therapeutic to the treatment of human infectious disease and also a randomized trial of an antiviral drug in respiratory syncytial virus (RSV)-infected lung transplant recipients. Zamora et al. performed a randomized, double-blind, placebocontrolled trial in LTX recipients with RSV respiratory tract infection. Patients were permitted to receive standard of care for RSV. Aerosolized ALN-RSV01 (0.6 mg/kg) or placebo was administered daily for 3 days. This study demonstrates that an RNAi therapeutic targeting RSV can be safely administered to LTX recipients with RSV infection. Three daily doses of ALN-RSV01 did not result in any exacerbation of respiratory tract symptoms or impairment of lung function and did not exhibit any systemic proinflammatory effects, such as induction of cytokines or CRP. Pharmacokinetics showed only low, transient systemic exposure after inhalation, consistent with preclinical animal data showing that ALN-RSV01, administered intravenously or by inhalation, is rapidly cleared from the circulation through exonucleasemediated digestion and renal excretion. The method of Zamora et al. may be applied to the CRISPR Cas system of the present invention and an aerosolized CRISPR Cas, for example with a dosage of 0.6 mg/kg, may be contemplated for the present invention.

[00567] For an example of CFTRdelta508 chimeric guide RNA, see Example 22 which demonstrates gene transfer or gene delivery of a CRISPR-Cas system in airways of subject or a patient in need thereof, suffering from cystic fibrosis or from cystic fibrosis (CF) related symptoms, using adeno-associated virus (AAV) particles. In particular, they exemplify a repair strategy for Cystic Fibrosis delta F508 mutation. This type of strategy should apply across all organisms. With particular reference to CF, suitable patients may include: Human, non-primate human, canine, feline, bovine, equine and other domestic animals. In this instance, Applicants

utilized a CRISPR-Cas system comprising a Cas9 enzyme to target deltaF508 or other CFTR-inducing mutations.

[00568] The treated subjects in this instance receive pharmaceutically effective amount of aerosolized AAV vector system per lung endobronchially delivered while spontaneously breathing. As such, aerosolized delivery is preferred for AAV delivery in general. An adenovirus or an AAV particle may be used for delivery. Suitable gene constructs, each operably linked to one or more regulatory sequences, may be cloned into the delivery vector. In this instance, the following constructs are provided as examples: Cbh or EF1a promoter for Cas9, U6 or H1 promoter for chimeric guide RNA),: A preferred arrangement is to use a CFTRdelta508 targeting chimeric guide, a repair template for deltaF508 mutation and a codon optimized Cas9 enzyme (preferred Cas9s are those with nuclease or nickase activity) with optionally one or more nuclear localization signal or sequence(s) (NLS(s)), e.g., two (2) NLSs. Constructs without NLS are also envisaged.

[00569] In order to identify the Cas9 target site, Applicants analyzed the human CFTR genomic locus and identified the Cas9 target site. Preferably, in general and in this CF case, the PAM may contain a NGG or a NNAGAAW motif.

[00570] Accordingly, in the case of CF, the present method comprises manipulation of a target sequence in a genomic locus of interest comprising

delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises

(a) a guide sequence capable of hybridizing to the CF target sequence in a suitable mammalian cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,
wherein components I and II are located on the same or different vectors of the system,
wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and
wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence. In respect of CF, preferred target DNA sequences comprise the CFTRdelta508 mutation. A preferred PAM is described above. A preferred CRISPR enzyme is any Cas (described herein, but particularly that described in Example 22).

[00571] Alternatives to CF include any genetic disorder and examples of these are well known. Another preferred method or use of the invention is for correcting defects in the EMP2A and EMP2B genes that have been identified to be associated with Lafora disease.

[00572] In some embodiments, a "guide sequence" may be distinct from "guide RNA". A guide sequence may refer to an approx. 20bp sequence, within the guide RNA, that specifies the target site.

[00573] In some embodiments, the Cas9 is (or is derived from) SpCas9. In such embodiments, preferred mutations are at any or all or positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 or corresponding positions in other Cas9s (which may be ascertained for instance by standard sequence comparison tools. In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. The same (or conservative substitutions of these mutations) at corresponding positions in other Cas9s are also preferred. Particularly preferred are D10 and H840 in SpCas9. However, in other Cas9s, residues corresponding to SpCas9 D10 and H840 are also preferred. These are advantageous as they provide nickase activity. Such mutations may be applied to all aspects of the present invention, not only treatment of CF.

[00574] Schwank et al. (Cell Stem Cell, 13:653–58, 2013) used CRISPR/Cas9 to correct a defect associated with cystic fibrosis in human stem cells. The team's target was the gene for an ion channel, cystic fibrosis transmembrane conductor receptor (CFTR). A deletion in CFTR causes the protein to misfold in cystic fibrosis patients. Using cultured intestinal stem cells developed from cell samples from two children with cystic fibrosis, Schwank et al. were able to

correct the defect using CRISPR along with a donor plasmid containing the reparative sequence to be inserted. The researchers then grew the cells into intestinal "organoids," or miniature guts, and showed that they functioned normally. In this case, about half of clonal organoids underwent the proper genetic correction.

[00575] **Muscles**

[00576] The present invention also contemplates delivering the CRISPR-Cas system to muscle(s).

[00577] Bortolanza et al. (Molecular Therapy vol. 19 no. 11, 2055–2064 Nov. 2011) shows that systemic delivery of RNA interference expression cassettes in the FRG1 mouse, after the onset of facioscapulohumeral muscular dystrophy (FSHD), led to a dose-dependent long-term FRG1 knockdown without signs of toxicity. Bortolanza et al. found that a single intravenous injection of 5×10^{12} vg of rAAV6-sh1FRG1 rescues muscle histopathology and muscle function of FRG1 mice. In detail, 200 µl containing 2×10^{12} or 5×10^{12} vg of vector in physiological solution were injected into the tail vein using a 25-gauge Terumo syringe. The method of Bortolanza et al. may be applied to an AAV expressing CRISPR Cas and injected into humans at a dosage of about 2×10^{15} or 2×10^{16} vg of vector.

[00578] Dumonceaux et al. (Molecular Therapy vol. 18 no. 5, 881–887 May 2010) inhibit the myostatin pathway using the technique of RNA interference directed against the myostatin receptor AcvRIIb mRNA (sh-AcvRIIb). The restoration of a quasi-dystrophin was mediated by the vectorized U7 exon-skipping technique (U7-DYS). Adeno-associated vectors carrying either the sh-AcvrIIb construct alone, the U7-DYS construct alone, or a combination of both constructs were injected in the tibialis anterior (TA) muscle of dystrophic mdx mice. The injections were performed with 10^{11} AAV viral genomes. The method of Dumonceaux et al. may be applied to an AAV expressing CRISPR Cas and injected into humans, for example, at a dosage of about 10^{14} to about 10^{15} vg of vector.

[00579] Kinouchi et al. (Gene Therapy (2008) 15, 1126–1130) report the effectiveness of in vivo siRNA delivery into skeletal muscles of normal or diseased mice through nanoparticle formation of chemically unmodified siRNAs with atelocollagen (ATCOL). ATCOL-mediated local application of siRNA targeting myostatin, a negative regulator of skeletal muscle growth, in mouse skeletal muscles or intravenously, caused a marked increase in the muscle mass within a few weeks after application. These results imply that ATCOL-mediated application of siRNAs

is a powerful tool for future therapeutic use for diseases including muscular atrophy. Mst-siRNAs (final concentration, 10 mM) were mixed with ATCOL (final concentration for local administration, 0.5%) (AteloGene, Kohken, Tokyo, Japan) according to the manufacturer's instructions. After anesthesia of mice (20-week-old male C57BL/6) by Nembutal (25 mg/kg, i.p.), the Mst-siRNA/ATCOL complex was injected into the masseter and biceps femoris muscles. The method of Kinouchi et al. may be applied to CRISPR Cas and injected into a human, for example, at a dosage of about 500 to 1000 ml of a 40 μM solution into the muscle.

[00580] Hagstrom et al. (Molecular Therapy Vol. 10, No. 2, August 2004) describe an intravascular, nonviral methodology that enables efficient and repeatable delivery of nucleic acids to muscle cells (myofibers) throughout the limb muscles of mammals. The procedure involves the injection of naked plasmid DNA or siRNA into a distal vein of a limb that is transiently isolated by a tourniquet or blood pressure cuff. Nucleic acid delivery to myofibers is facilitated by its rapid injection in sufficient volume to enable extravasation of the nucleic acid solution into muscle tissue. High levels of transgene expression in skeletal muscle were achieved in both small and large animals with minimal toxicity. Evidence of siRNA delivery to limb muscle was also obtained. For plasmid DNA intravenous injection into a rhesus monkey, a threeway stopcock was connected to two syringe pumps (Model PHD 2000; Harvard Instruments), each loaded with a single syringe. Five minutes after a papaverine injection, pDNA (15.5 to 25.7 mg in 40 –100 ml saline) was injected at a rate of 1.7 or 2.0 ml/s. This could be scaled up for plasmid DNA expressing CRISPR Cas of the present invention with an injection of about 300 to 500 mg in 800 to 2000 ml saline for a human. For adenoviral vector injections into a rat, 2×10^9 infectious particles were injected in 3 ml of normal saline solution (NSS). This could be scaled up for an adenoviral vector expressing CRISPR Cas of the present invention with an injection of about 1×10^{13} infectious particles were injected in 10 liters of NSS for a human. For siRNA, a rat was injected into the great saphenous vein with 12.5 μg of a siRNA and a primate was injected injected into the great saphenous vein with 750 μg of a siRNA. This could be scaled up for a CRISPR Cas of the present invention, for example, with an injection of about 15 to about 50 mg into the great saphenous vein of a human.

[00581] **Skin**

[00582] The present invention also contemplates delivering the CRISPR-Cas system to the skin.

[00583] Hickerson et al. (Molecular Therapy—Nucleic Acids (2013) 2, e129) relates to a motorized microneedle array skin delivery device for delivering self-delivery (sd)-siRNA to human and murine skin. The primary challenge to translating siRNA-based skin therapeutics to the clinic is the development of effective delivery systems. Substantial effort has been invested in a variety of skin delivery technologies with limited success. In a clinical study in which skin was treated with siRNA, the exquisite pain associated with the hypodermic needle injection precluded enrollment of additional patients in the trial, highlighting the need for improved, more "patient-friendly" (i.e., little or no pain) delivery approaches. Microneedles represent an efficient way to deliver large charged cargos including siRNAs across the primary barrier, the stratum corneum, and are generally regarded as less painful than conventional hypodermic needles. Motorized "stamp type" microneedle devices, including the motorized microneedle array (MMNA) device used by Hickerson et al., have been shown to be safe in hairless mice studies and cause little or no pain as evidenced by (i) widespread use in the cosmetic industry and (ii) limited testing in which nearly all volunteers found use of the device to be much less painful than a flushot, suggesting siRNA delivery using this device will result in much less pain than was experienced in the previous clinical trial using hypodermic needle injections. The MMNA device (marketed as Triple-M or Tri-M by Bomtech Electronic Co, Seoul, South Korea) was adapted for delivery of siRNA to mouse and human skin. sd-siRNA solution (up to 300 µl of 0.1 mg/ml RNA) was introduced into the chamber of the disposable Tri-M needle cartridge (Bomtech), which was set to a depth of 0.1 mm. For treating human skin, deidentified skin (obtained immediately following surgical procedures) was manually stretched and pinned to a cork platform before treatment. All intradermal injections were performed using an insulin syringe with a 28-gauge 0.5-inch needle. The MMNA device and method of Hickerson et al. could be used and/or adapted to deliver the CRISPR Cas of the present invention, for example, at a dosage of up to 300 µl of 0.1 mg/ml CRISPR Cas to the skin.

[00584] Leachman et al. (Molecular Therapy, vol. 18 no. 2, 442–446 Feb. 2010) relates to a phase Ib clinical trial for treatment of a rare skin disorder pachyonychia congenita (PC), an autosomal dominant syndrome that includes a disabling plantar keratoderma, utilizing the first short-interfering RNA (siRNA)-based therapeutic for skin. This siRNA, called TD101, specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA without affecting wild-type K6a mRNA. The dose-escalation schedule is presented below:

Week	Dose no.	Days	Volume (ml)	Concentration of TD101 (mg/ml)	Total dose TD101 (mg)
1	1--2	1--7	0.1	1.0	0.10
2	3--4	8--14	0.25	1.0	0.25
3	5--8	15--21	0.50	1.0	0.50
4	7--8	22--28	1.0	1.0	1.0
5	9--10	29--35	1.5	1.0	1.5
6	11--12	36--42	2.0	1.0	2.0
7	13--14	43--49	2.0	1.5	3.0
8	15--16	50--56	2.0	2.0	4.0
9	17--18	57--63	2.0	2.5	5.0
10	19--20	64--70	2.0	3.0	6.0
11	21--22	71--77	2.0	3.5	7.0
12	23--24	78--84	2.0	4.0	8.0
13	25--26	85--91	2.0	4.5	9.0
14	27--28	92--98	2.0	5.0	10.0
15	29--30	99--105	2.0	6.0	12.0
16	31--32	106--112	2.0	7.0	14.0
17	33	113--119	2.0	8.5	17.0

[00585] Initially, 0.1 ml of a 1.0 mg/ml solution of TD101 or vehicle alone (Dulbecco's phosphate-buffered saline without calcium or magnesium) was administered to symmetric calluses. Six rising dose-volumes were completed without an adverse reaction to the increases: 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 ml of a 1.0 mg/ml solution of TD101 solution per injection. As the highest planned volume (2.0 ml) was well tolerated, the concentration of TD101 was then increased each week from 1 mg/ml up to a final concentration of 8.5 mg/ml. Similar dosages are contemplated for the administration of a CRISPR Cas that specifically and potently targets the keratin 6a (K6a) N171K mutant mRNA.

[00586] Zheng et al. (PNAS, July 24, 2012, vol. 109, no. 30, 11975–11980) show that spherical nucleic acid nanoparticle conjugates (SNA-NCs), gold cores surrounded by a dense shell of highly oriented, covalently immobilized siRNA, freely penetrate almost 100% of keratinocytes in vitro, mouse skin, and human epidermis within hours after application. Zheng et al. demonstrated that a single application of 25 nM epidermal growth factor receptor (EGFR) SNA-NCs for 60 h demonstrate effective gene knockdown in human skin. A similar dosage may be contemplated for CRISPR Cas immobilized in SNA-NCs for administration to the skin.

[00587] **Hepatitis viruses**

[00588] The present invention may also be applied to treat hepatitis B virus (HBV). However, the CRISPR Cas system must be adapted to avoid the shortcomings of RNAi, such as the risk of oversatring endogenous small RNA pathways, by for example, optimizing dose and sequence

(see, e.g., Grimm et al., Nature vol. 441, 26 May 2006). For example, low doses, such as about 1-10 x 10^{14} particles per humane are contemplated.

[00589] In another embodiment, the CRISPR Cas system directed against HBV may be administered in liposomes, such as a stable nucleic-acid-lipid particle (SNALP) (see, e.g., Morrissey et al., Nature Biotechnology, Vol. 23, No. 8, August 2005). Daily intravenous injections of about 1, 3 or 5 mg/kg/day of CRISPR Cas targeted to HBV RNA in a SNALP are contemplated. The daily treatment may be over about three days and then weekly for about five weeks.

[00590] In another embodiment, the system of Chen et al. (Gene Therapy (2007) 14, 11–19) may be used/and or adapted for the CRISPR Cas system of the present invention. Chen et al. use a double-stranded adenoassociated virus 8-pseudotyped vector (dsAAV2/8) to deliver shRNA. A single administration of dsAAV2/8 vector (1 x 10^{12} vector genomes per mouse), carrying HBV-specific shRNA, effectively suppressed the steady level of HBV protein, mRNA and replicative DNA in liver of HBV transgenic mice, leading to up to 2–3 $\log_{10}$ decrease in HBV load in the circulation. Significant HBV suppression sustained for at least 120 days after vector administration. The therapeutic effect of shRNA was target sequence dependent and did not involve activation of interferon. For the present invention, a CRISPR Cas system directed to HBV may be cloned into an AAV vector, such as a dsAAV2/8 vector and administered to a human, for example, at a dosage of about 1 x 10^{15} vector genomes to about 1 x 10^{16} vector genomes per human.

[00591] In another embodiment, the method of Wooddell et al. (Molecular Therapy vol. 21 no. 5, 973–985 May 2013) may be used/and or adapted to the CRISPR Cas system of the present invention. Woodell et al. show that simple coinjection of a hepatocyte-targeted, N-acetylgalactosamine-conjugated melittin-like peptide (NAG-MLP) with a liver-tropic cholesterol-conjugated siRNA (chol-siRNA) targeting coagulation factor VII (F7) results in efficient F7 knockdown in mice and nonhuman primates without changes in clinical chemistry or induction of cytokines. Using transient and transgenic mouse models of HBV infection, Wooddell et al. show that a single coinjection of NAG-MLP with potent chol-siRNAs targeting conserved HBV sequences resulted in multilog repression of viral RNA, proteins, and viral DNA with long duration of effect. Intraveinous coinjections, for example, of about 6 mg/kg of NAG-MLP and 6 mg/kg of HBV specific CRISPR Cas may be envisioned for the present invention. In

the alternative, about 3 mg/kg of NAG-MLP and 3 mg/kg of HBV specific CRISPR Cas may be delivered on day one, followed by administration of about about 2-3 mg/kg of NAG-MLP and 2-3 mg/kg of HBV specific CRISPR Cas two weeks later.

[00592] The present invention may also be applied to treat hepatitis C virus (HCV). The methods of Roelvinki et al. (Molecular Therapy vol. 20 no. 9, 1737-1749 Sep 2012) may be applied to the CRISPR Cas system. For example, an AAV vector such as AAV8 may be a contemplated vector and for example a dosage of about 1.25×10^{11} to 1.25×10^{13} vector genomes per kilogram body weight (vg/kg) may be contemplated.

[00593] In yet another embodiment, CRISPR-Cas9-mediated genome editing can be used to correct a disease mutation and/or phenotype. That CRISPR-Cas9-mediated genome editing can be used to correct a disease mutation and/or phenotype in the liver and or hepatocytes is illustrated in the manuscript entitled "Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype" by Hao Yin *et al.* published at Nature Biotechnology published online 30 March 2014; corrected online 31 March 2014, available at the website nature.com/doifinder/10.1038/nbt.2884, incorporated herein by reference in its entirety. The paper relates to CRISPR-Cas9–mediated correction of a Fah mutation in hepatocytes in a mouse model of the human disease hereditary tyrosinemia. It was shown that delivery of components of the CRISPR-Cas9 system by hydrodynamic injection resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. It was further shown that expansion of Fah-positive hepatocytes rescued the body weight loss phenotype.

[00594] It will be readily apparent that a host of other diseases can be treated in a similar fashion. Some examples of genetic diseases caused by mutations are provided herein, but many more are known. The above strategy can be applied to these diseases.

[00595] **Huntington's Disease (HD)**

[00596] RNA interference (RNAi) offers therapeutic potential for this disorder by reducing the expression of *HTT*, the disease-causing gene of Huntington's disease (see, e.g., McBride et al., Molecular Therapy vol. 19 no. 12 Dec. 2011, pp. 2152-2162), therefore Applicant postulates that it may be used/and or adapted to the CRISPR-Cas system. The CRISPR-Cas system may be generated using an algorithm to reduce the off-targeting potential of antisense sequences. The CRISPR-Cas sequences may target either a sequence in exon 52 of mouse, rhesus or human huntingtin and expressed in a viral vector, such as AAV. Animals, including humans, may be

injected with about three microinjections per hemisphere (six injections total): the first 1 mm rostral to the anterior commissure (12 µl) and the two remaining injections (12 µl and 10 µl, respectively) spaced 3 and 6 mm caudal to the first injection with 1e12 vg/ml of AAV at a rate of about 1 µl/minute, and the needle was left in place for an additional 5 minutes to allow the injectate to diffuse from the needle tip.

[00597] DiFiglia et al. (PNAS, October 23, 2007, vol. 104, no. 43, 17204–17209) observed that single administration into the adult striatum of an siRNA targeting Htt can silence mutant Htt, attenuate neuronal pathology, and delay the abnormal behavioral phenotype observed in a rapid-onset, viral transgenic mouse model of HD. DiFiglia injected mice intrastriatally with 2 µl of Cy3-labeled cc-siRNA-Htt or unconjugated siRNA-Htt at 10 µM. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 5-10 ml of 10 µM CRISPR Cas targeted to Htt may be injected intrastriatally.

[00598] In another example, Boudreau et al. (Molecular Therapy vol. 17 no. 6 june 2009) injects 5 µl of recombinant AAV serotype 2/1 vectors expressing htt-specific RNAi virus (at 4 x 10^{12} viral genomes/ml) into the straiatum. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 10-20 ml of 4 x 10^{12} viral genomes/ml) CRISPR Cas targeted to Htt may be injected intrastriatally.

[00599] In another example, a CRISPR Cas targetd to HTT may be administered continuously (see, e.g., Yu et al., Cell 150, 895–908, August 31, 2012). Yu et al. utilizes osmotic pumps delivering 0.25 ml/hr (Model 2004) to deliver 300 mg/day of ss-siRNA or phosphate-buffered saline (PBS) (Sigma Aldrich) for 28 days, and pumps designed to deliver 0.5 µl/hr (Model 2002) were used to deliver 75 mg/day of the positive control MOE ASO for 14 days. Pumps (Durect Corporation) were filled with ss-siRNA or MOE diluted in sterile PBS and then incubated at 37 C for 24 or 48 (Model 2004) hours prior to implantation. Mice were anesthetized with 2.5% isofluorane, and a midline incision was made at the base of the skull. Using stereotaxic guides, a cannula was implanted into the right lateral ventricle and secured with Loctite adhesive. A catheter attached to an Alzet osmotic mini pump was attached to the cannula, and the pump was placed subcutaneously in the midscapular area. The incision was closed with 5.0 nylon sutures. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 500 to 1000 g/day CRISPR Cas targeted to Htt may be administered.

[00600] In another example of continuous infusion, Stiles et al. (Experimental Neurology 233 (2012) 463–471) implanted an intraparenchymal catheter with a titanium needle tip into the right putamen. The catheter was connected to a SynchroMed® II Pump (Medtronic Neurological, Minneapolis, MN) subcutaneously implanted in the abdomen. After a 7 day infusion of phosphate buffered saline at 6 μL/day, pumps were re-filled with test article and programmed for continuous delivery for 7 days. About 2.3 to 11.52 mg/d of siRNA were infused at varying infusion rates of about 0.1 to 0.5 μL/min. A similar dosage of CRISPR Cas targeted to Htt may be contemplated for humans in the present invention, for example, about 20 to 200 mg/day CRISPR Cas targeted to Htt may be administered.

[00601] In another example, the methods of US Patent Publication No. 20130253040 assigned to Sangamo may also be also be adapted from TALES to the CRISPR Cas system of the present invention for treating Huntington's Disease.

[00602] **Nucleic acids, amino acids and proteins**

[00603] The invention uses nucleic acids to bind target DNA sequences. This is advantageous as nucleic acids are much easier and cheaper to produce than proteins, and the specificity can be varied according to the length of the stretch where homology is sought. Complex 3-D positioning of multiple fingers, for example is not required.

[00604] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. The term also encompasses nucleic-acid-like structures with synthetic backbones, see, e.g., WO 97/03211; WO 96/39154. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide

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WHAT IS CLAIMED IS:

1. A method of modifying an organism or a non-human organism by manipulation of a post-mitotic cell target sequence in a genomic locus of interest, to thereby invoke a phenotypic change in the cell, comprising

delivering a non-naturally occurring or engineered composition comprising :

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a guide sequence capable of hybridizing to a post-mitotic cell target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding a CRISPR enzyme, optionally comprising at least one or more nuclear localization sequences,

wherein (a), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the post-mitotic cell target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the post-mitotic cell target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA,

wherein the polynucleotide sequence encoding the CRISPR enzyme is operably linked to regulatory sequence(s) expression of the CRISPR enzyme, whereby with expression of the CRISPR enzyme there is assembly of the CRISPR complex in the post-mitotic cell, and manipulation thereof.

2. The method of claim 1, wherein any or all of the polynucleotide sequence encoding a CRISPR enzyme, guide sequence, tracr mate sequence or tracr sequence, is/are RNA.

3. The method of claim 1 or 2, wherein the polynucleotides encoding the sequence encoding a CRISPR enzyme, the guide sequence, tracr mate sequence or tracr sequence is/are RNA and are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

4. The method of any of claims 1 to 3, wherein the polynucleotides are comprised within a vector system comprising one or more vectors.

5. A method of modifying an organism or a non-human organism by manipulation of a post-mitotic cell target sequence in a genomic locus of interest comprising

delivering a non-naturally occurring or engineered composition comprising a viral vector system comprising one or more viral vectors operably encoding a composition for expression thereof, wherein the composition comprises:

a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(A) a guide sequence capable of hybridizing to a kidney target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, optionally comprising at least one or more nuclear localization sequences,

wherein (A), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the kidney target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the kidney target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

6. The method of claim 5, wherein one or more of the viral vectors are delivered via liposomes, nanoparticles, exosomes, microvesicles, or a gene-gun.

7. A method of treating or inhibiting a condition caused by a defect in a kidney target sequence in a genomic locus of interest in a subject or a non-human subject in need thereof comprising modifying the subject or a non-human subject by manipulation of the kidney target

sequence and wherein the condition is susceptible to treatment or amelioration by manipulation of the kidney target sequence comprising providing treatment comprising:

delivering a non-naturally occurring or engineered composition comprising an AAV or lentivirus vector system, comprising one or more AAV or lentivirus vectors operably encoding a composition for expression thereof, wherein the kidney target sequence is manipulated by the composition when expressed, wherein the composition comprises:

(A) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises

(A) a guide sequence capable of hybridizing to a kidney target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences,

wherein (A), (b) and (c) are arranged in a 5' to 3' orientation,

wherein components I and II are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the kidney target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the kidney target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence,

or

(B) a non-naturally occurring or engineered composition comprising a vector system comprising one or more vectors comprising

I. a first regulatory element operably linked to

(A) a guide sequence capable of hybridizing to a kidney target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and

III. a third regulatory element operably linked to a tracr sequence,

wherein components I, II and III are located on the same or different vectors of the system,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the kidney target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the kidney target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence.

8. The method of any preceding claim, wherein the method is carried out in vitro, and/or ex vivo.

9. The method of any preceding claim including inducing expression.

10. The method of any preceding claim wherein the organism or subject is a eukaryote.

11. The method of claim 10 wherein the organism or subject is a non-human eukaryote.

12. The method of any of claims 1 to 11 wherein the organism or subject is a mammal or a non-human mammal.

13. The method of any of claims 4 to 8 wherein the viral vector is an AAV or lentiviral vector.

14. The method according to any preceding claim wherein the CRISPR enzyme is a Cas9.

15. The method according to any preceding claim wherein expression of the guide sequence is under the control of the T7 promoter and is driven by the expression of T7 polymerase.

16. A method of delivering a CRISPR enzyme of any preceding claim, comprising delivering to a cell mRNA encoding the CRISPR enzyme.

17. The method of any one of claims 1 to 16, wherein the polynucleotide or enzyme coding sequence encoding the CRISPR enzyme is delivered to the cell by delivering mRNA encoding the CRISPR enzyme to the cell.

18. A method of preparing the AAV or lentivirus vector of claim 7 comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding

for the AAV or lentivirus into AAV-infected or lentivirus-infected cells, and supplying AAV AAV or lentivirus rep and/or cap and/or helper nucleic acid molecules obligatory for replication and packaging of the AAV or lentivirus.

19. A method of preparing an AAV or lentivirus vector for use in the method of claim 7, comprising transfecting plasmid(s) containing or consisting essentially of nucleic acid molecule(s) coding for the AAV or lentivirus into AAV-infected or lentivirus-infected cells, and supplying AAV AAV or lentivirus rep and/or cap and/or helper nucleic acid molecules obligatory for replication and packaging of the AAV or lentivirus.

20. The method of claim 18 or 19 wherein the AAV or lentivirus rep and/or cap obligatory for replication and packaging of the AAV or lentivirus are supplied by transfecting the cells with helper plasmid(s) or helper virus(es).

21. The method of claim 20 wherein the helper virus is a poxvirus, adenovirus, lentivirus, herpesvirus or baculovirus.

22. The method of claim 21 wherein the poxvirus is a vaccinia virus.

23. The method of any of claims 18 to 22 wherein the cells are mammalian cells.

24. The method of any of claims 18 to 22 wherein the cells are insect cells and the helper virus (where present) is baculovirus.

25. The method of any of claims 1 to 15 wherein the kidney target sequence is flanked at its 3' end or followed by 5'-NRG (where N is any Nucleotide), or where the CRISPR enzyme is (or is derived from) a genus belonging to the group consisting of *Corynebacter, Sutterella, Legionella, Treponema, Filifactor, Eubacterium, Streptococcus, Lactobacillus, Mycoplasma, Bacteroides, Flaviivola, Flavobacterium, Sphaerochaeta, Azospirillum, Gluconacetobacter, Neisseria, Roseburia, Parvibaculum, Staphylococcus, Nitratifractor, Mycoplasma* and *Campylobacter*.

26. A composition as defined in any of claims 1-25 for use in medicine or in therapy.

27. A composition as defined in any of claims 1-25 for use in a method of modifying an organism or a non-human organism by manipulation of a kidney target sequence in a genomic locus of interest or in a method of treating or inhibiting a condition caused by a defect in a kidney target sequence in a genomic locus of interest.

28. Use of a composition as defined in any of claims 1-25 in *ex vivo* gene or genome editing.

29. Use of a composition as defined in any of claims 1-25 in the manufacture of a medicament for *ex vivo* gene or genome editing or for use in a method of modifying an organism or a non-human organism by manipulation of a kidney target sequence in a genomic locus of interest or in a method of treating or inhibiting a condition caused by a defect in a kidney target sequence in a genomic locus of interest.

30. A composition comprising:

(A) - I. a CRISPR-Cas system RNA polynucleotide sequence, wherein the polynucleotide sequence comprises:

(A) a guide sequence capable of hybridizing to a kidney target sequence in a eukaryotic cell,

(b) a tracr mate sequence, and

(c) a tracr sequence, and

II. a polynucleotide sequence encoding a CRISPR enzyme, optionally comprising at least one or more nuclear localization sequences,

wherein (A), (b) and (c) are arranged in a 5' to 3' orientation,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the kidney target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the kidney target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA,

or

(B) I. polynucleotides comprising:

(A) a guide sequence capable of hybridizing to a kidney target sequence in a eukaryotic cell, and

(b) at least one or more tracr mate sequences,

II. a polynucleotide sequence encoding a CRISPR enzyme, and

III. a polynucleotide sequence comprising a tracr sequence,

wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the kidney target sequence, and

wherein the CRISPR complex comprises the CRISPR enzyme complexed with (1) the guide sequence that is hybridized to the kidney target sequence, and (2) the tracr mate sequence that is hybridized to the tracr sequence, and the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA;

for use in medicine or therapy; or for use in a method of modifying an organism or a non-human organism by manipulation of a kidney target sequence in a genomic locus of interest; or for use in a method of treating or inhibiting a condition caused by a defect in a kidney target sequence in a genomic locus of interest; or for use in *ex vivo* gene or genome editing.

31. The composition of claim 30, wherein the polynucleotides are comprised within a vector system comprising one or more vectors.

32. The method, use or composition of any of the preceding claims, wherein the CRISPR-Cas system RNA is a chimeric RNA (chiRNA).

33. The method, use or composition of any of the preceding claims, wherein the CRISPR-Cas system is a multiplexed CRISPR enzyme system further comprising multiple chimeras and/or multiple multiguide sequences and a single tracr sequence.

34. The method, use or composition according any of the preceding claims, wherein the CRISPR enzyme is a nuclease directing cleavage of both strands at the location of the target sequence.

35. The method, use or composition according to any of the preceding claims, wherein the CRISPR enzyme comprises one or more mutations.

36The method, use or composition according to claim35, wherein the CRISPR enzyme comprises one or more mutations D10A, E762A, H840A, N854A, N863A or D986A.

37. The method, use or composition according to claim 35 wherein the one or more mutations is in a RuvC1 domain of the CRISPR enzyme.

38. The method, use or composition according to claim34, wherein the CRISPR enzyme is a nickase directing cleavage at the location of the target sequence.

39. The method, use or composition according to claim38, wherein the nickase is a double nickase.

40. The method, use or composition according to any preceding claim further comprising at least two or more NLS.

41. The method, use or composition according to any preceding claim, wherein the CRISPR enzyme has one or more mutations in a catalytic domain, wherein when transcribed, the tracr mate sequence hybridizes to the tracr sequence and the guide sequence directs sequence-specific binding of a CRISPR complex to the target sequence, and wherein the enzyme further comprises a functional domain.

42. The method, use or composition according to claim41, wherein the functional domain is a transcriptional activation domain.

43. The method, use or composition according to claim42, wherein the transcriptional activation domain is VP64.

44. The method of any one of claims 1-25 or 32-43 further comprising minimizing off-target modifications by manipulation of a first and a second target sequence on opposite strands of a DNA duplex in a genomic locus of interest in a cell comprising

delivering a non-naturally occurring or engineered composition comprising :

I. a CRISPR-Cas system chimeric RNA (chiRNA) polynucleotide sequence, wherein the polynucleotide sequence comprises:

(a) a first guide sequence capable of hybridizing to the first target sequence,

(b) a first tracr mate sequence,

(c) a first tracr sequence,

(d) a second guide sequence capable of hybridizing to the second target sequence,

(e) a second tracr mate sequence, and

(f) a second tracr sequence, and

optionally, wherein a linker sequence is present between the first tracr sequence and the second guide sequence, whereby the first guide sequence and the second guide sequence are in tandem; and

II. a polynucleotide sequence encoding a CRISPR enzyme comprising at least one or more nuclear localization sequences, wherein (a), (b), (c), (d), (e) and (f) are arranged in a 5' to 3' orientation, wherein the polynucleotide sequence comprises a linker sequence between the first tracr sequence and the second guide sequence, whereby the first guide sequence and the second guide sequence are in tandem, and wherein when transcribed, the first and the second

tracr mate sequence hybridize to the first and second tracr sequence respectively and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively,

or

II. a second regulatory element operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, and wherein components I and II are located on the same or different vectors of the system, and when transcribed, a first tracr mate sequence hybridizes to a first tracr sequence and the first and the second guide sequence directs sequence-specific binding of a first and a second CRISPR complex to the first and second target sequences respectively;

wherein the first CRISPR complex comprises the CRISPR enzyme complexed with (1) the first guide sequence that is hybridized to the first target sequence, and (2) the first tracr mate sequence that is hybridized to the first tracr sequence,

wherein the second CRISPR complex comprises the CRISPR enzyme complexed with (1) the second guide sequence that is hybridized to the second target sequence, and (2) the second tracr mate sequence that is hybridized to the second tracr sequence,

wherein the polynucleotide sequence encoding a CRISPR enzyme is DNA or RNA, and

wherein the first guide sequence directs cleavage of one strand of the DNA duplex near the first target sequence and the second guide sequence directs cleavage of other strand near the second target sequence inducing a double strand break, thereby modifying the organism or the non-human organism by minimizing off-target modifications.

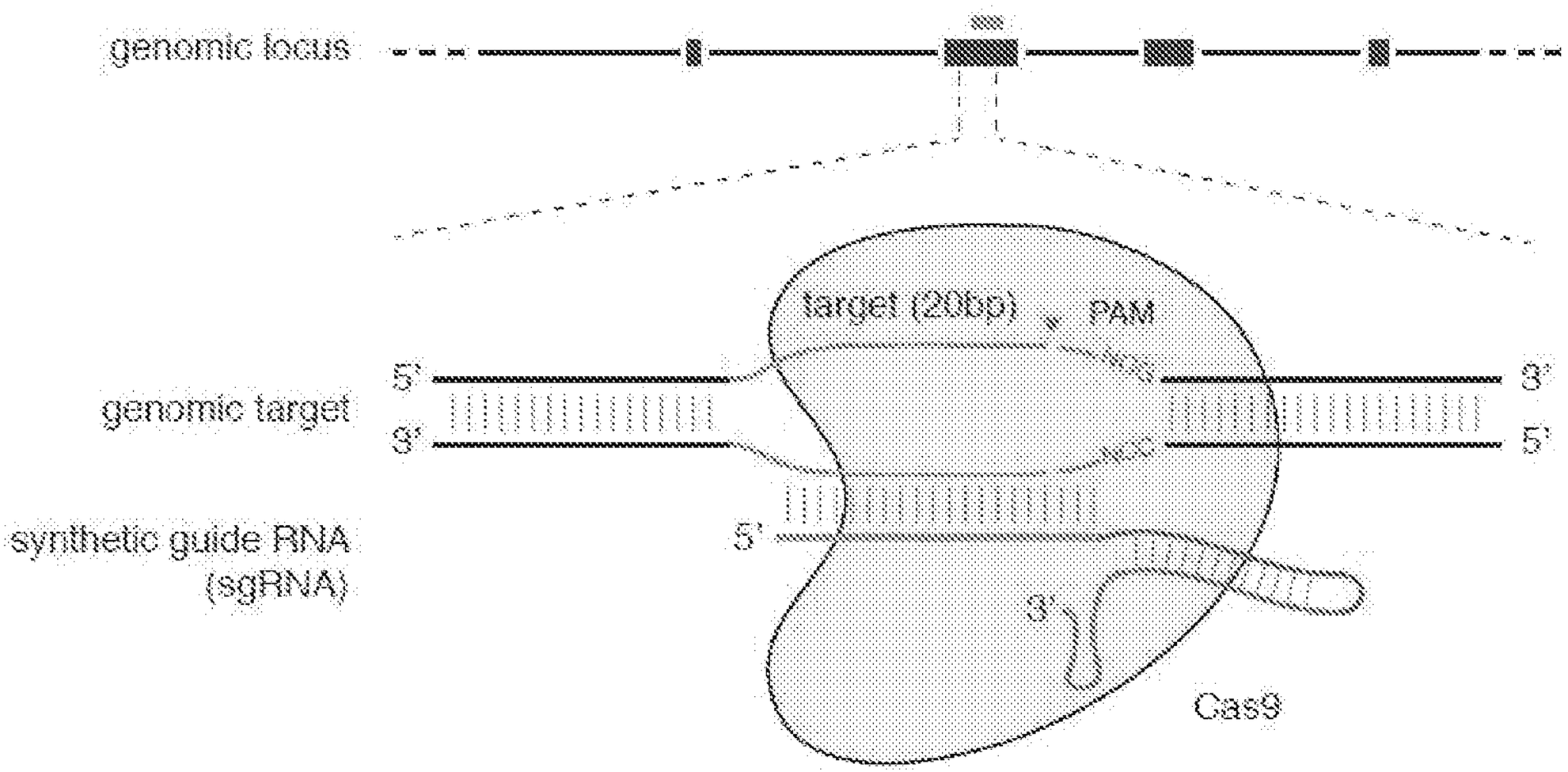

genomic locus
genomic target
synthetic guide RNA (sgRNA)
target (20bp)
PAM
5'
3'
3'
5'
5'
3'
Cas9

FIG. 1

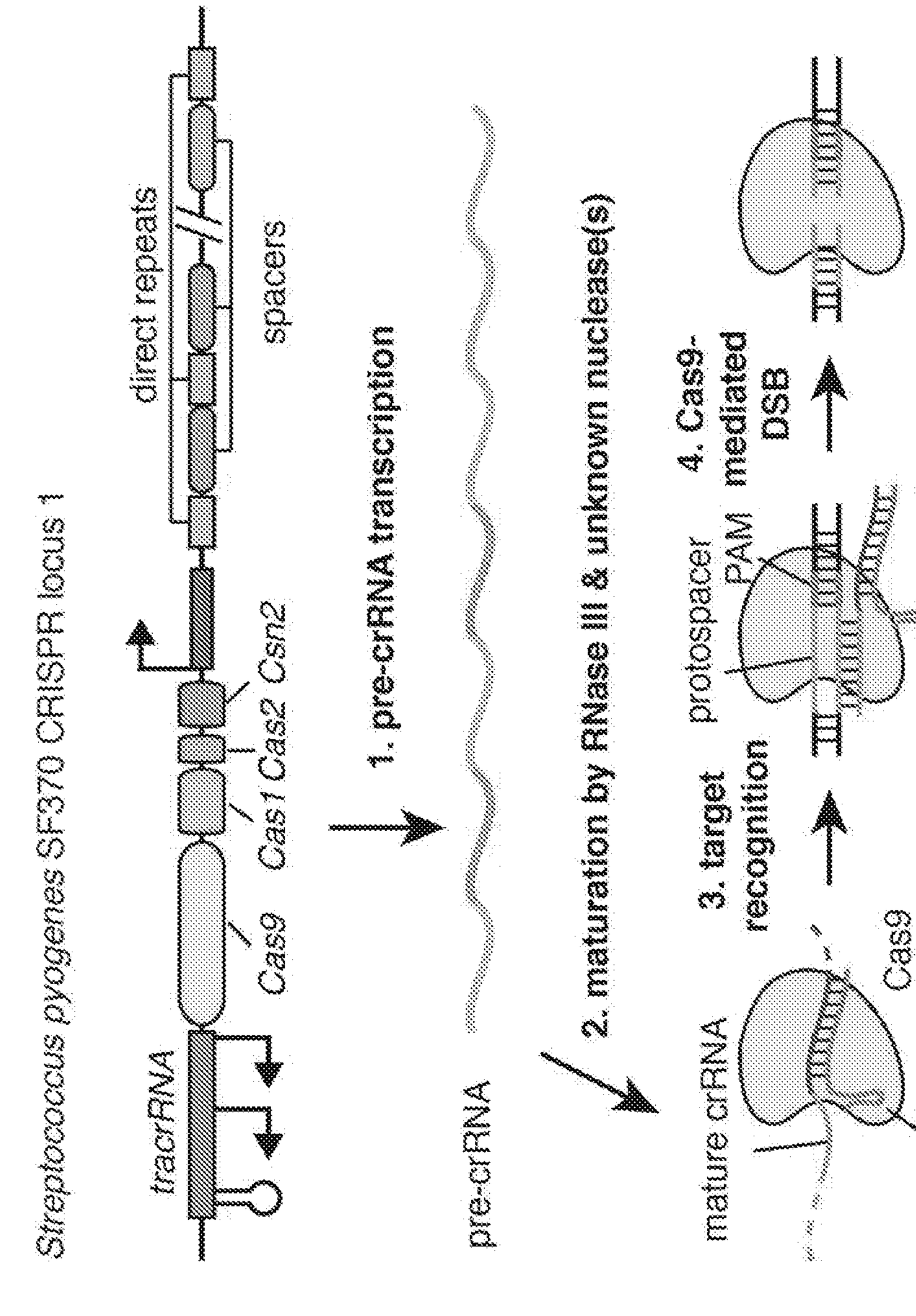

Streptococcus pyogenes SF370 CRISPR locus 1
tracrRNA
Cas9
Cas1
Cas2
Csn2
direct repeats
spacers
1. pre-crRNA transcription
pre-crRNA
2. maturation by RNase III & unknown nuclease(s)
mature crRNA
3. target recognition
protospacer
PAM
4. Cas9-mediated DSB
Cas9
processed tracrRNA

FIG. 2A

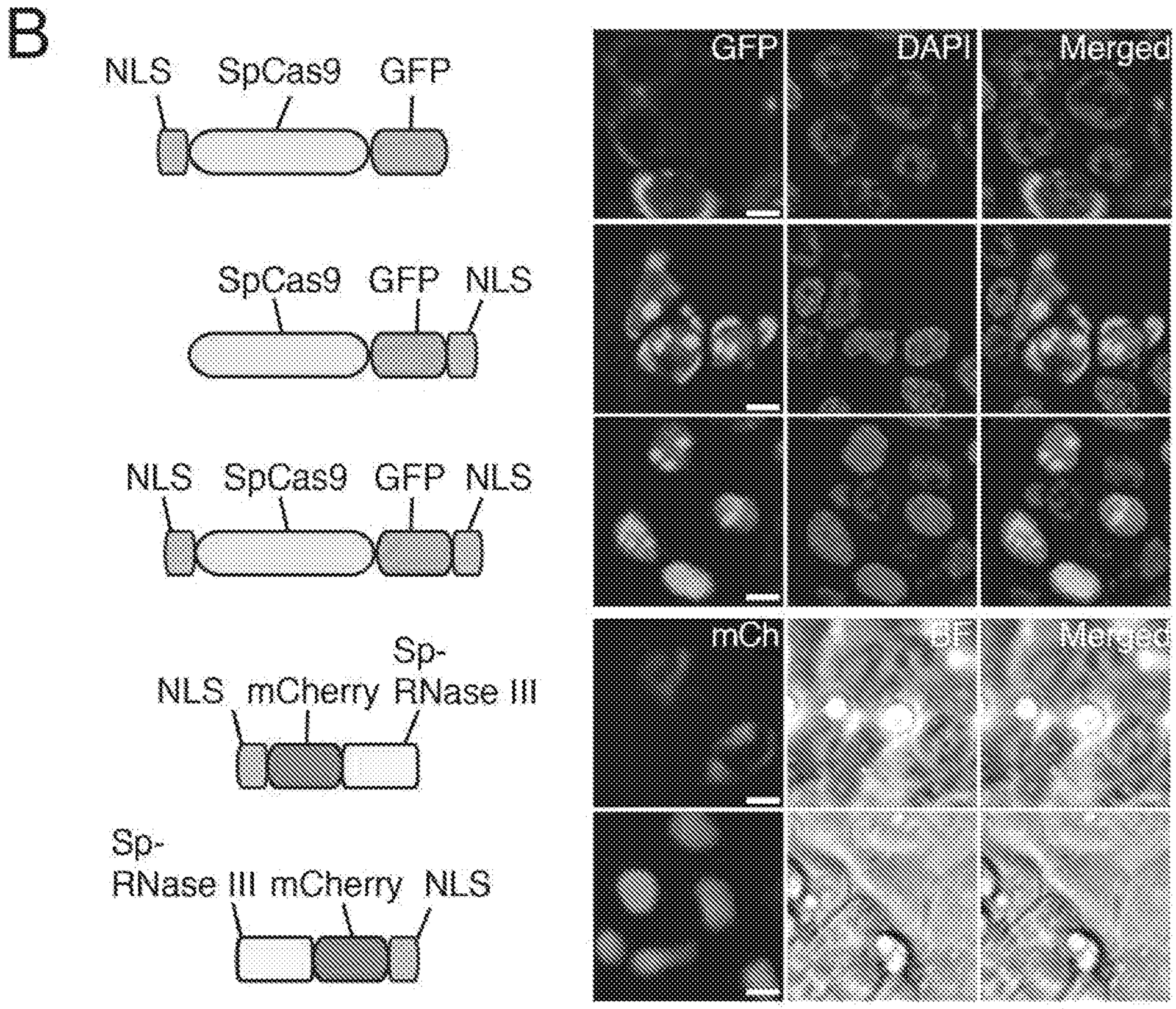
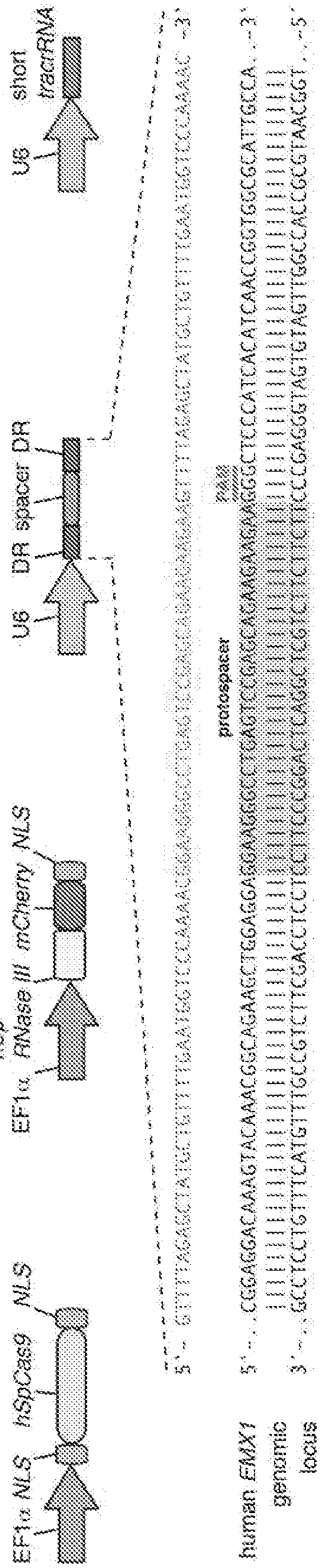

B
NLS SpCas9 GFP
SpCas9 GFP NLS
NLS SpCas9 GFP NLS
Sp-
NLS mCherry RNase III
Sp-
RNase III mCherry NLS
GFP
DAPI
Merged
mCh

FIG. 2B


U6
short tracrRNA
U6
DR spacer DR
protospacer
PAM
hSp-
EF1α hNase III mCherry NLS
EF1α NLS hSpCas9 NLS
human EMX1
genomic
locus
5'-...CGGAG
3'-...GCCTC
5'- GUUUUA

C

FIG. 2C

D

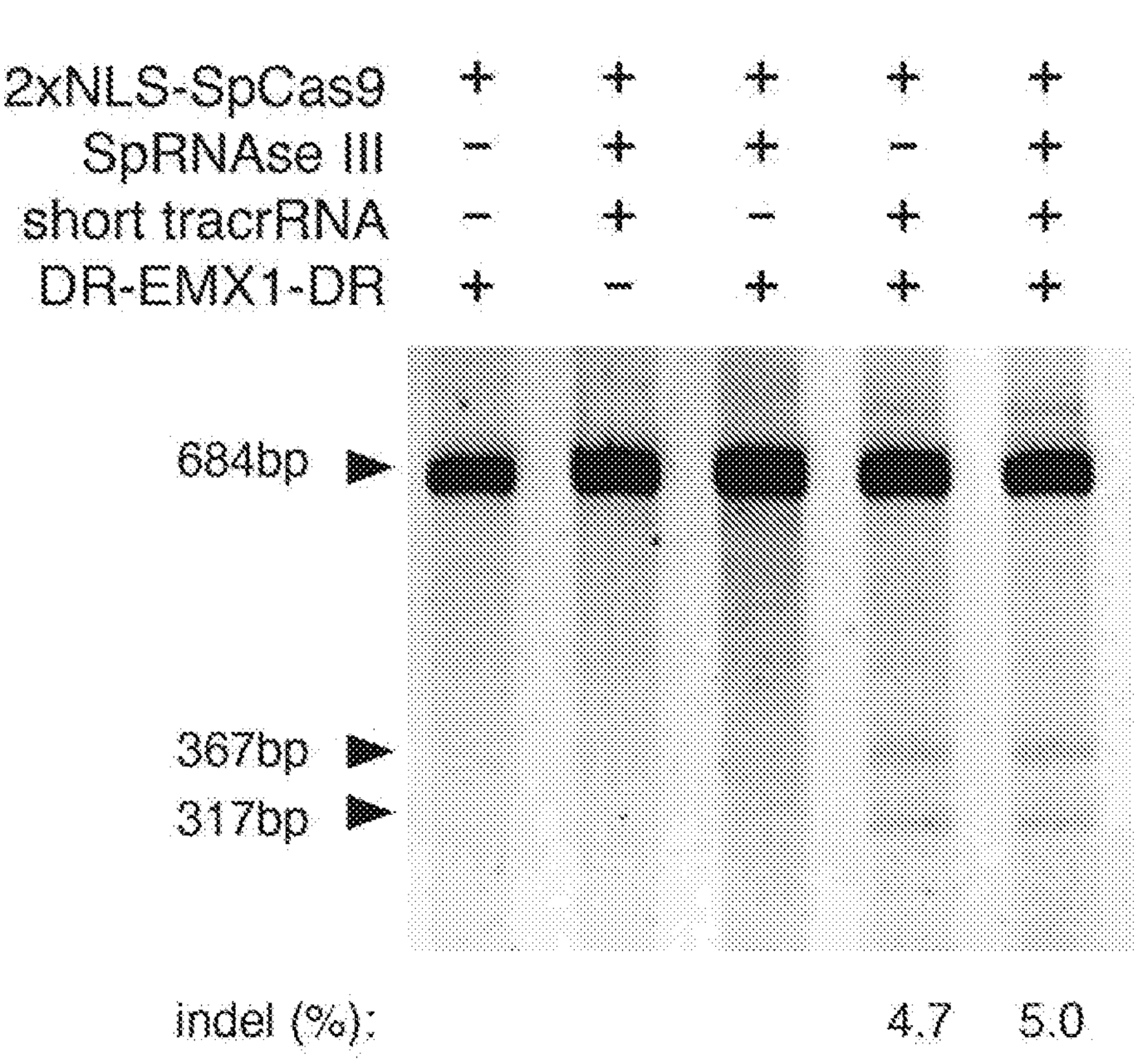

2xNLS-SpCas9 + + + + +
SpRNAse III - + + - +
short tracrRNA - + - + +
DR-EMX1-DR + - + + +
684bp
367bp
317bp
indel (%): 4.7 5.0

FIG. 2D

CA 02915845 2015-12-16

E



F

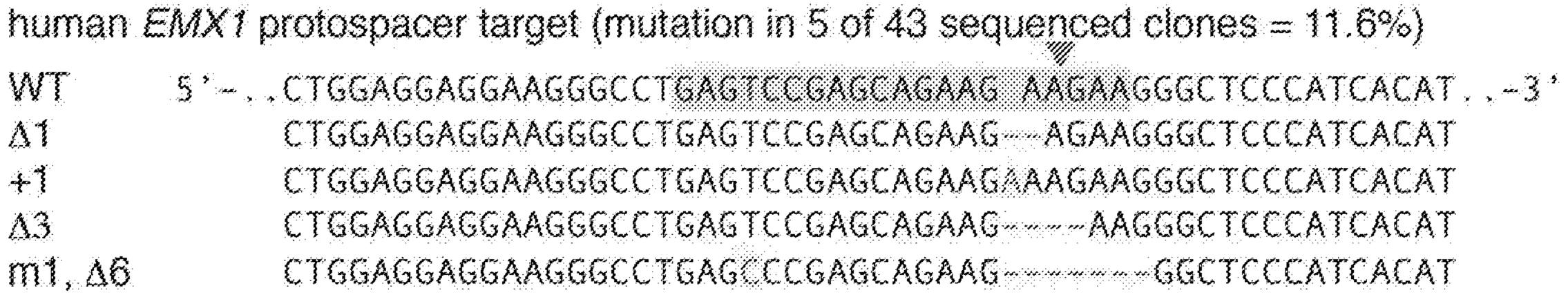


FIG. 2E-F

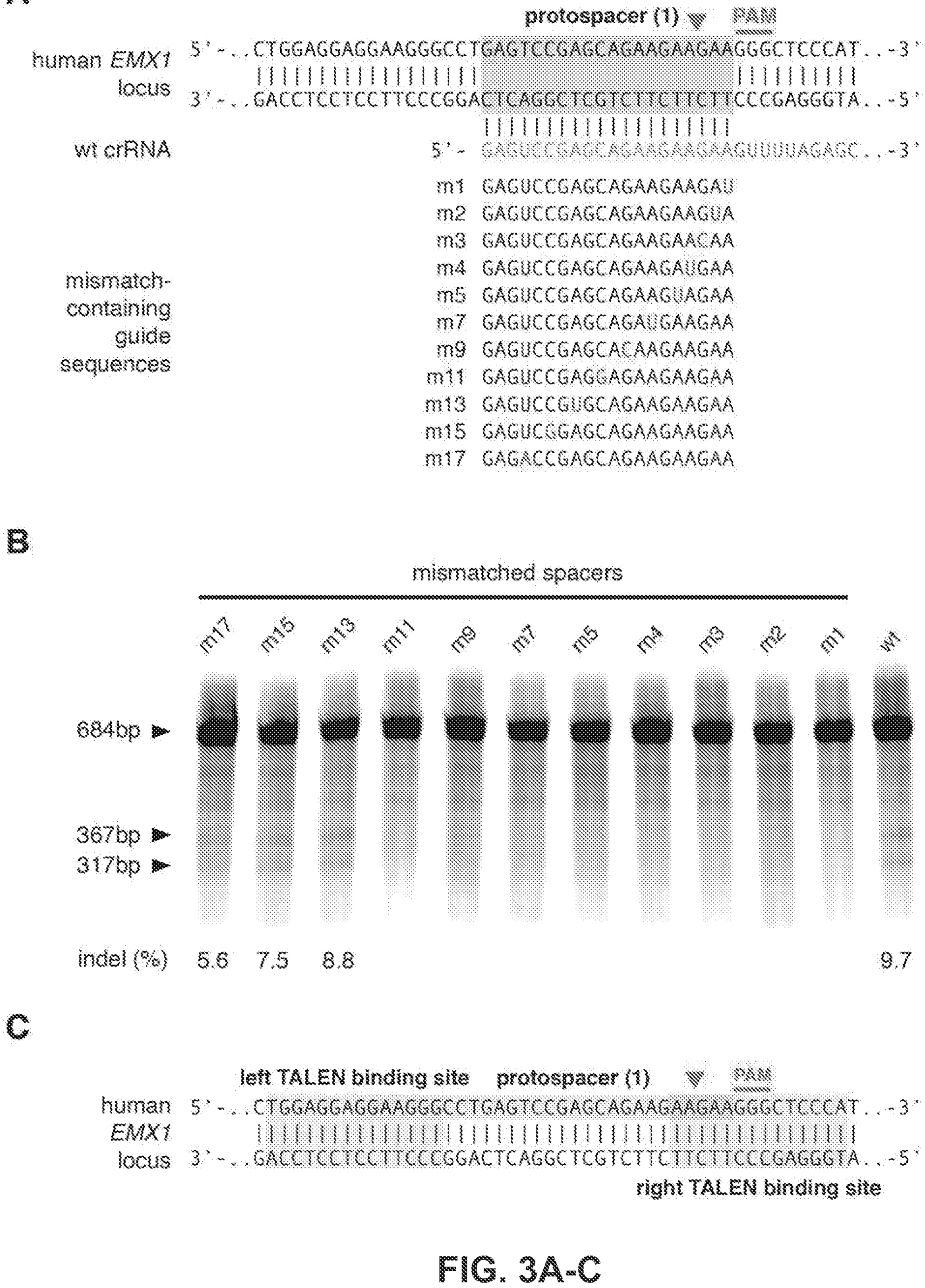

A
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
wt crRNA
5'- GAGUCCGAGCAGAAGAAGAGUUUUAGAGC..-3'
mismatch-containing guide sequences
m1 GAGUCCGAGCAGAAGAAGAU
m2 GAGUCCGAGCAGAAGAAGUA
m3 GAGUCCGAGCAGAAGAACAA
m4 GAGUCCGAGCAGAAGAUGAA
m5 GAGUCCGAGCAGAAGUAGAA
m7 GAGUCCGAGCAGAUGAAGAA
m9 GAGUCCGAGCACAAGAAGAA
m11 GAGUCCGAGGAGAAGAAGAA
m13 GAGUCCGUGCAGAAGAAGAA
m15 GAGUCGGAGCAGAAGAAGAA
m17 GAGACCGAGCAGAAGAAGAA
B
mismatched spacers
m17 m15 m13 m11 m9 m7 m5 m4 m3 m2 m1 wt
684bp
367bp
317bp
indel (%) 5.6 7.5 8.8 9.7
C
left TALEN binding site
protospacer (1)
PAM
human EMX1 locus
5'-..CTGGAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCAT..-3'
3'-..GACCTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTA..-5'
right TALEN binding site

FIG. 3A-C

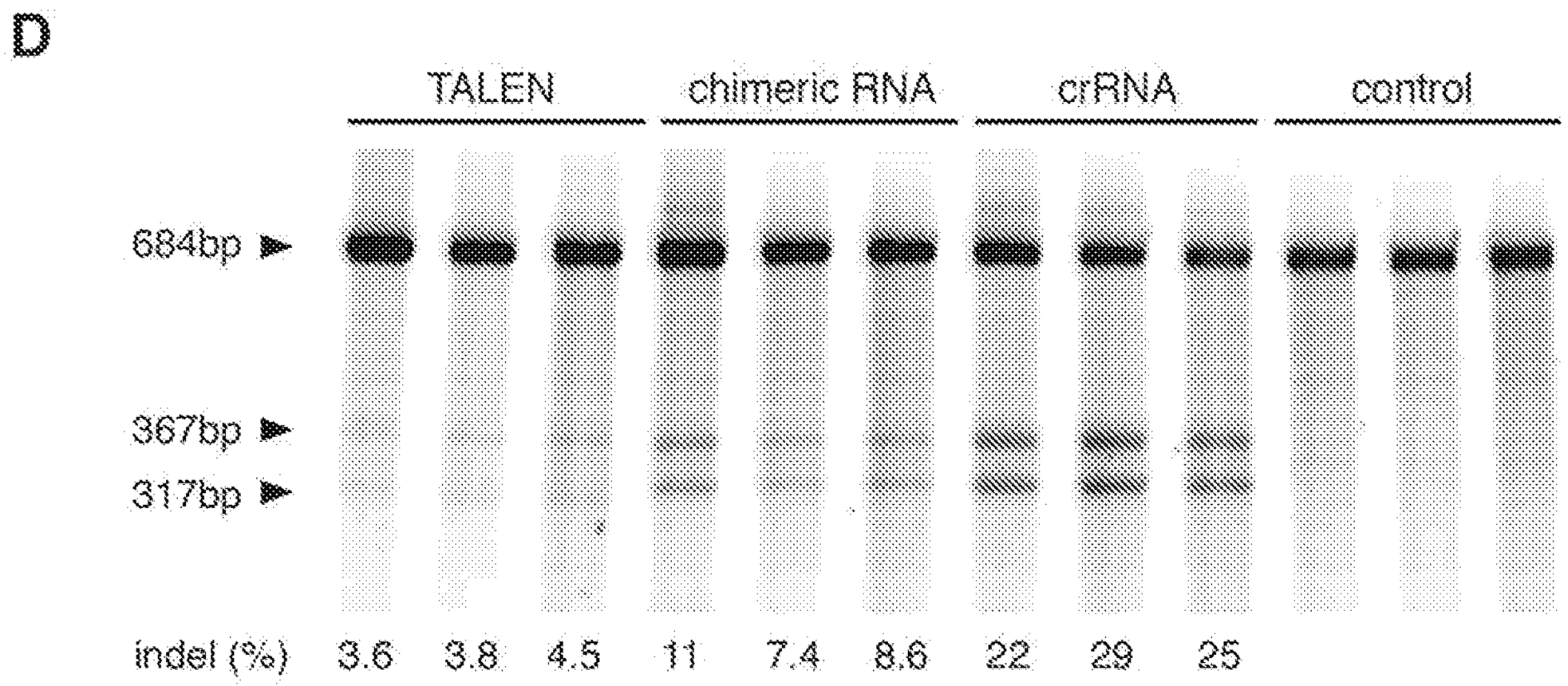
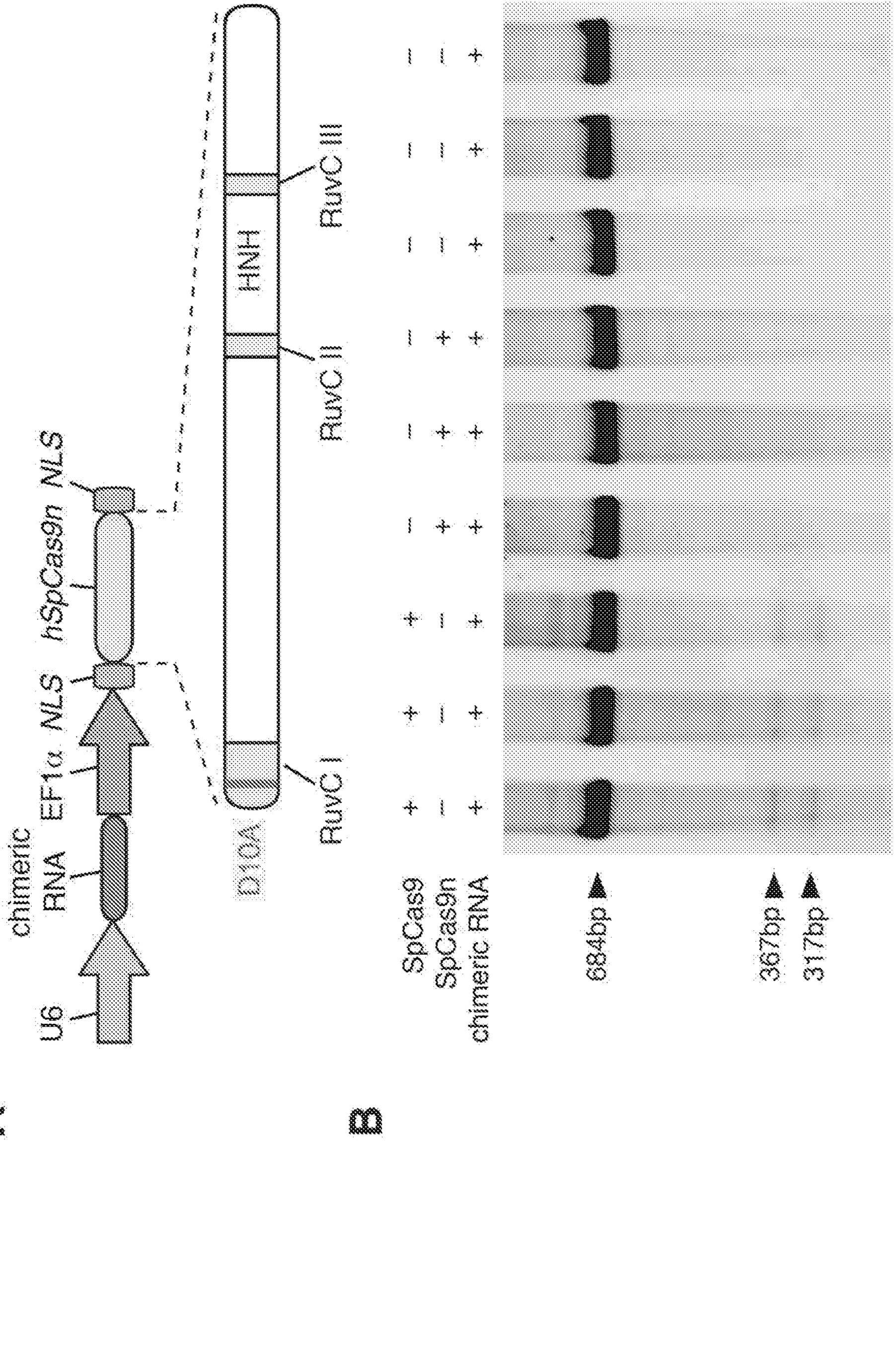

D
TALEN
chimeric RNA
crRNA
control
684bp
367bp
317bp
indel (%)
3.6
3.8
4.5
11
7.4
8.6
22
29
25

FIG. 3D

FIG. 4A-B


A
chimeric RNA
EF1α
NLS
hSpCas9n
NLS
U6
D10A
RuvC I
HNH
RuvC II
RuvC III
B
SpCas9
SpCas9n
chimeric RNA
684bp
367bp
317bp
indel (%) 5.3 4.6 3.5

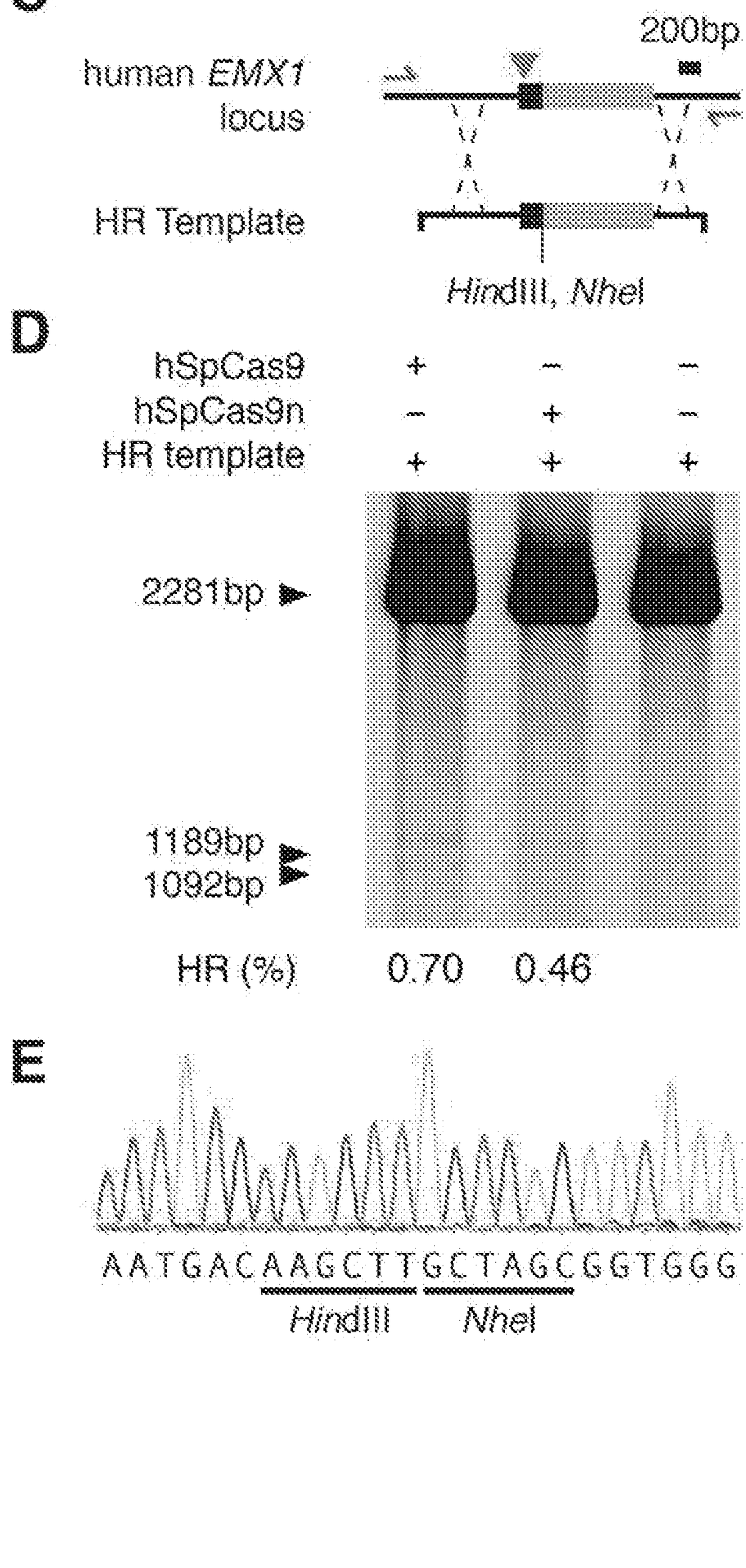

C
human EMX1 locus
200bp
HR Template
HindIII, NheI
D
hSpCas9
hSpCas9n
HR template
2281bp
1189bp
1092bp
HR (%)
0.70
0.46
E
AATGACAAGCTTGCTAGCGGTGGG
HindIII
NheI

FIG. 4C-E

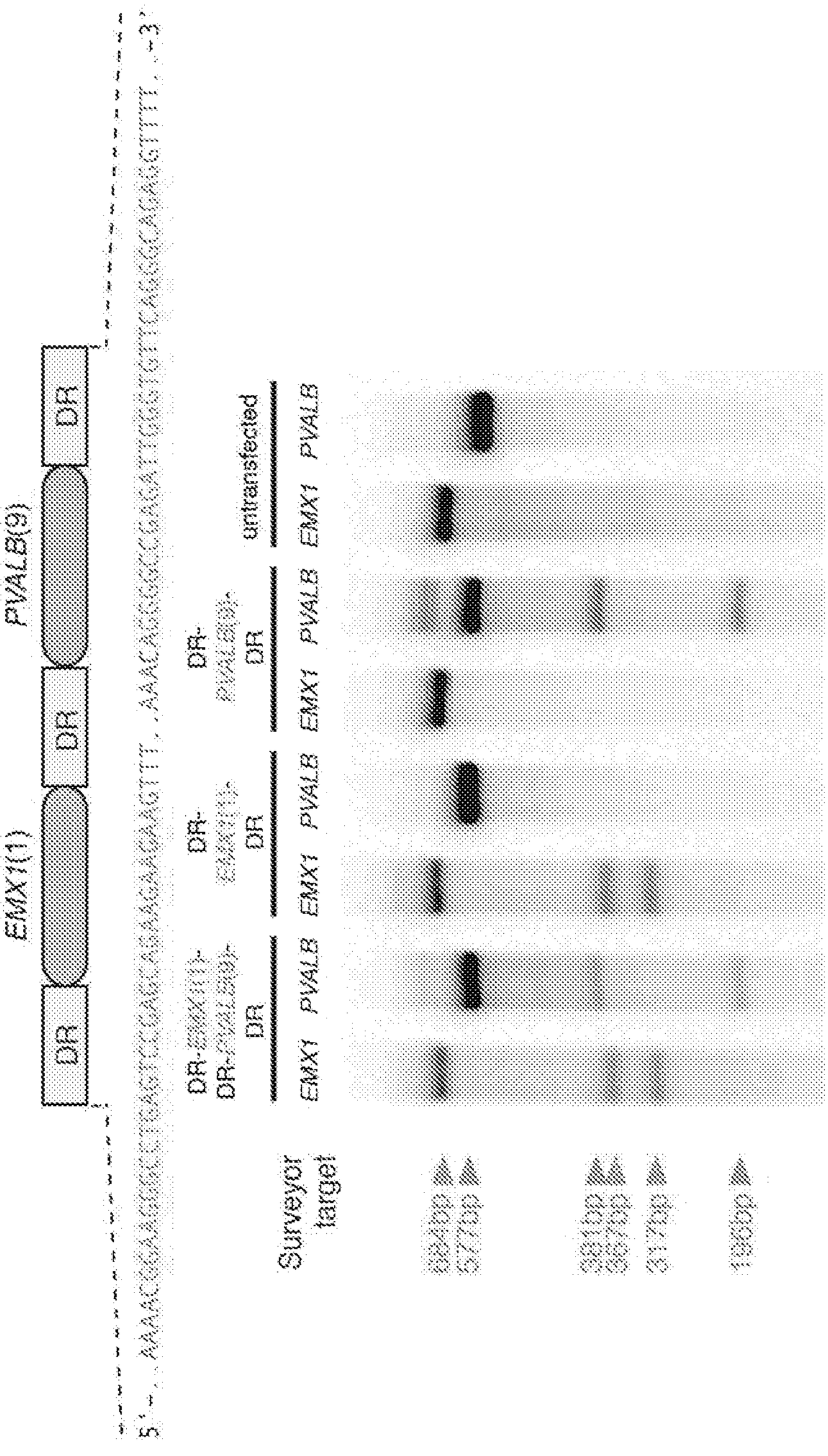

EMX1(1)
PVALB(9)
DR
DR
DR
DR-EMX1(1)-DR
DR-PVALB(9)-DR
untransfected
EMX1 PVALB EMX1 PVALB EMX1 PVALB
Surveyor target
DR-EMX1(1)-DR-PVALB(9)-DR
EMX1 PVALB
684bp
577bp
381bp
357bp
317bp
106bp
indel (%)
27 7.3
24 15

FIG. 4F

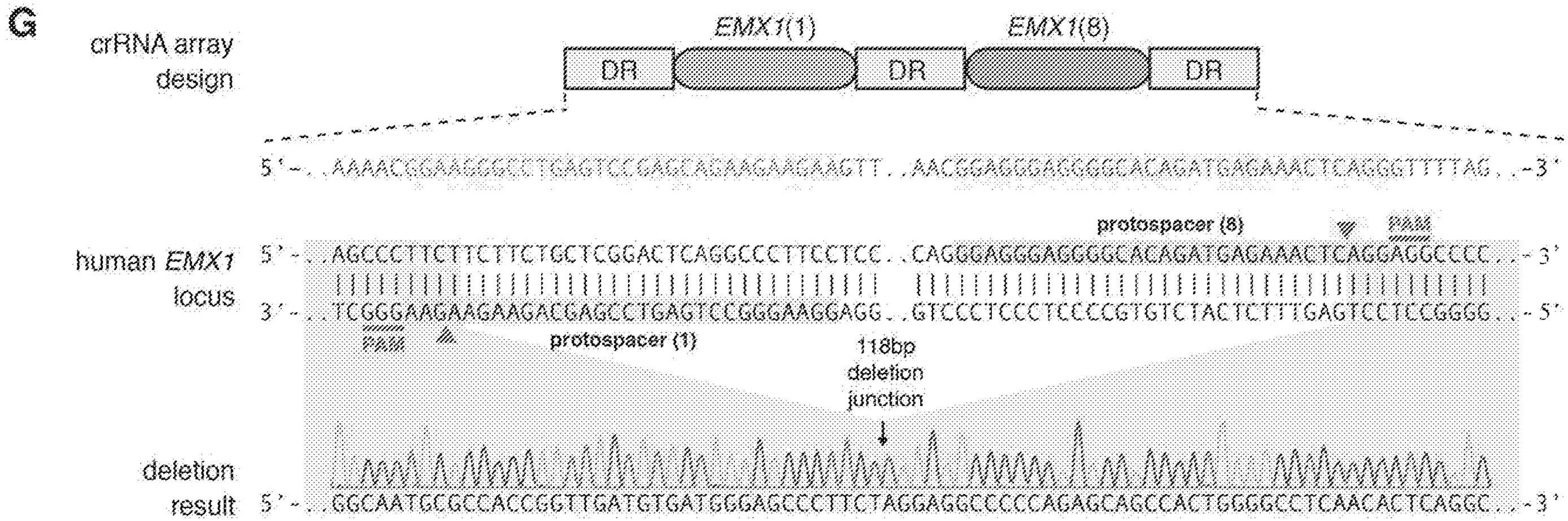
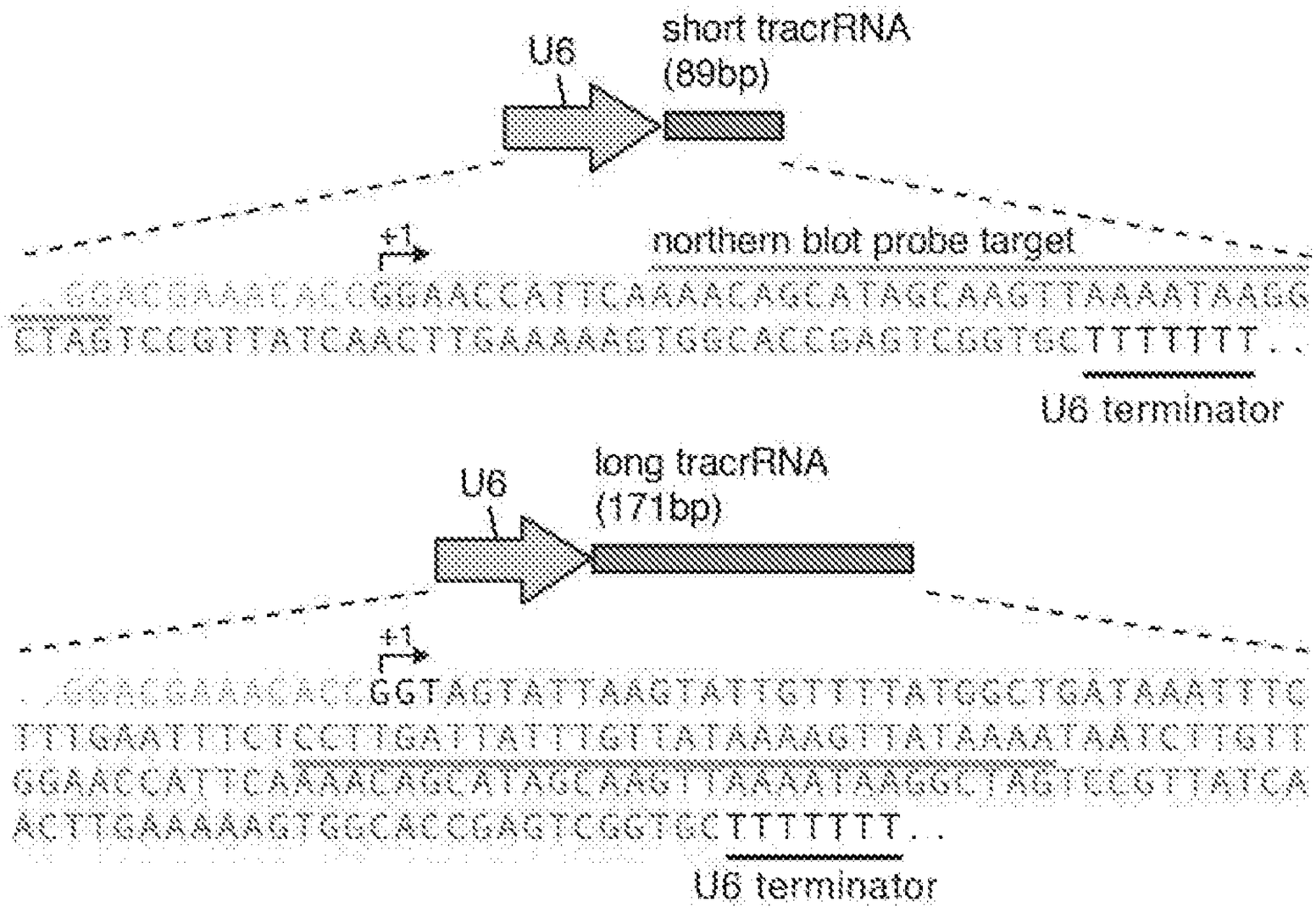

G
crRNA array design
EMX1(1)
EMX1(8)
DR
DR
DR
5'-..AAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTT..AACGGAGGGAGGGGCACAGATGAGAAACTCAGGGTTTTAG..-3'
protospacer (8)
PAM
human EMX1 locus
5'-..AGCCCTTCTTCTTCTGCTCGGACTCAGGCCCTTCCTCC..CAGGGAGGGAGGGGCACAGATGAGAAACTCAGGAGGCCCC..-3'
3'-..TCGGGAAGAAGAAGACGAGCCTGAGTCCGGGAAGGAGG..GTCCCTCCCTCCCCGTGTCTACTCTTTGAGTCCTCCGGGGG..-5'
PAM
protospacer (1)
118bp deletion junction
deletion result
5'-..GGCAATGCGCCACCGGTTGATGTGATGGGAGCCCTTCTAGGAGGCCCCCAGAGCAGCCACTGGGGCCTCAACACTCAGGC..-3'

FIG. 4G

Cas9	target species	gene	protospacer ID	protospacer sequence (5' to 3')	PAM	strand	cell line tested	% indel (pre-crRNA + tracrRNA)	% indel (chimeric RNA)
S. pyogenes SF370 type II CRISPR	*Homo sapiens*	*EMX1*	1	GGAAGGGCCTGAGTCCGAGCAGAAGAAGAA	GGG	+	293FT	20 ± 1.8	6.7 ± 0.62
		EMX1	2	CATTGGAGGTGACATCGATGTCCTCCCCAT	TGG	–	293FT	2.1 ± 0.31	N.D.
		EMX1	3	GGACATCGATGTCACCTCCAATGACTAGGG	TGG	+	293FT	14 ± 1.1	N.D.
		EMX1	4	CATCGATGTCCTCCCCATTGGCCTGCTTCG	TGG	–	293FT	11 ± 1.7	N.D.
		EMX1	5	TTCGTGGCAATGCGCCACCGGTTGATGTGA	TGG	–	293FT	4.3 ± 0.46	2.1 ± 0.51
		EMX1	6	TCGTGGCAATGCGCCACCGGTTGATGTGAT	GGG	–	293FT	4.0 ± 0.86	0.41 ± 0.25
		EMX1	7	TCCAGCTTCTGCCGTTTGTACTTTGTCCTC	CGG	–	293FT	1.5 ± 0.12	N.D.
		EMX1	8	GGAGGGAGGGGCACAGATGAGAAACTCAGG	AGG	–	293FT	7.8 ± 0.83	2.3 ± 1.2
	Homo sapiens	*PVALB*	9	AGGGGCCGAGATTGGGTGTTCAGGGCAGAG	AGG	+	293FT	21 ± 2.6	6.5 ± 0.32
		PVALB	10	ATGCAGGAGGGTGGCGAGAGGGGCCGAGAT	TGG	+	293FT	N.D.	N.D.
		PVALB	11	GGTGGCGAGAGGGGCCGAGATTGGGTGTTC	AGG	+	293FT	N.D.	N.D.
	Mus musculus	*Th*	12	CAAGCACTGAGTGCCATTAGCTAAATGCAT	AGG	–	Neuro2A	27 ± 4.3	4.1 ± 2.2
		Th	13	AATGCATAGGGTACCACCCACAGGTGCCAG	GGG	–	Neuro2A	4.8 ± 1.2	N.D.
		Th	14	ACACACATGGGAAAGCCTCTGGGCCAGGAA	AGG	+	Neuro2A	11.3 ± 1.3	N.D.
S. thermophilus LMD-9 CRISPR1	*Homo sapiens*	*EMX1*	15	GGAGGAGGTAGTATACAGAAACACAGAGAA	GTAGAAT	–	293FT	14 ± 0.88	N.T.
		EMX1	16	AGAATGTAGAGGAGTCACAGAAACTCAGCA	CTAGAAA	–	293FT	7.8 ± 0.77	N.T.

FIG. 5

A


U6
short tracrRNA
(89bp)
+1
northern blot probe target
GGACGAAACACCGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator
U6
long tracrRNA
(171bp)
+1
GGACGAAACACCGGTAGTATTAAGTATTGTTTTATGGCTGATAAATTTC
TTTGAATTTCTCCTTGATTATTTGTTATAAAAGTTATAAAATAATCTTGTT
GGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT..
U6 terminator

B

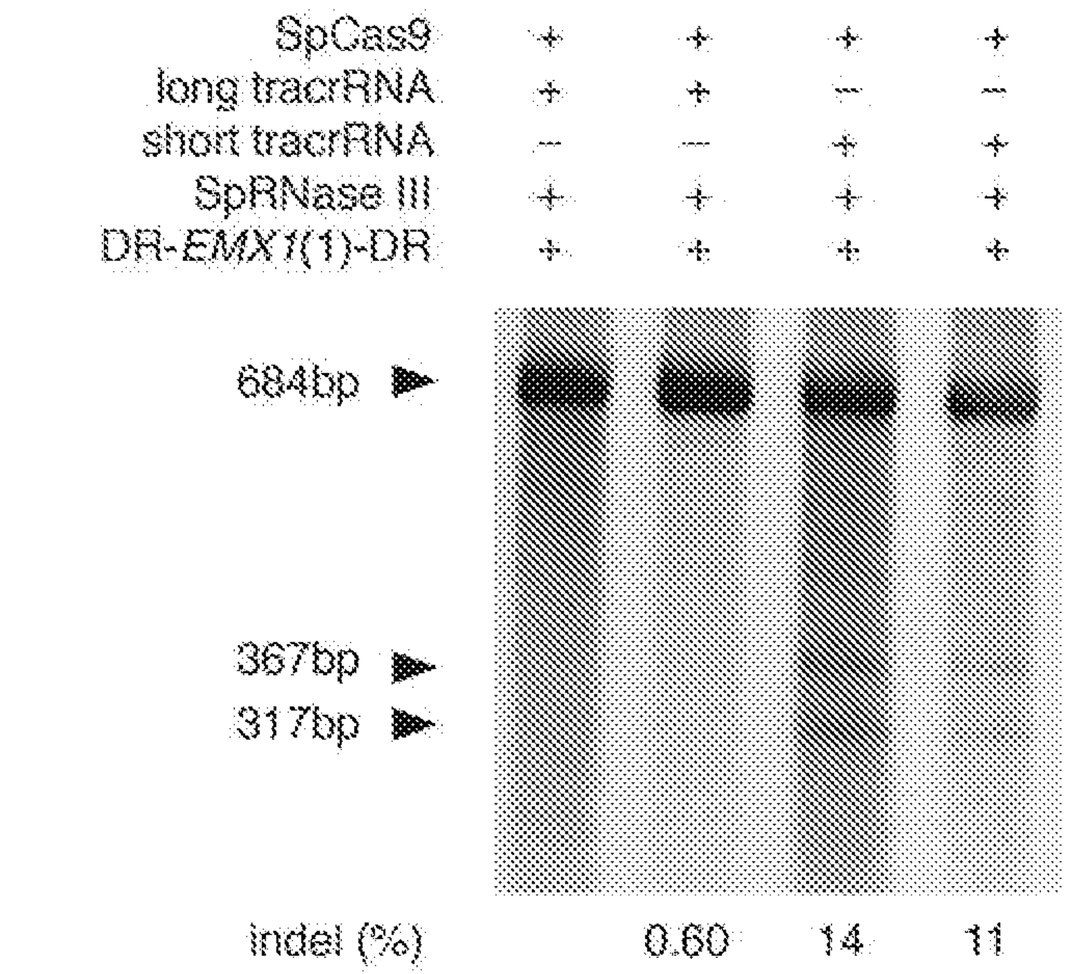

SpCas9 + + + +
long tracrRNA + + – –
short tracrRNA – – + +
SpRNase III + + + +
DR-EMX1(1)-DR + + + +
684bp
367bp
317bp
indel (%) 0.60 14 11

FIG. 6A-B

C

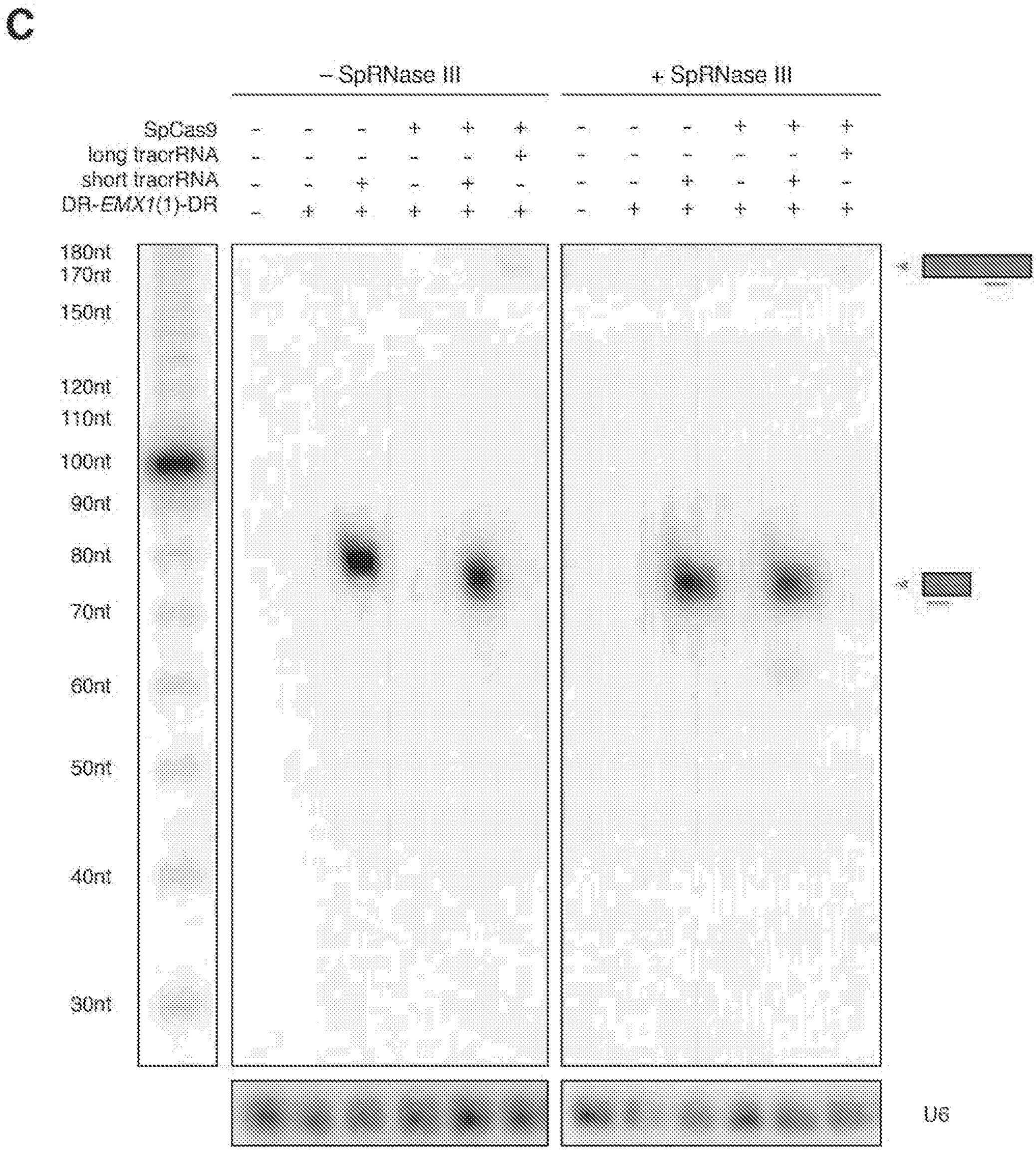

– SpRNase III
+ SpRNase III
SpCas9
long tracrRNA
short tracrRNA
DR-EMX1(1)-DR
180nt
170nt
150nt
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 6C

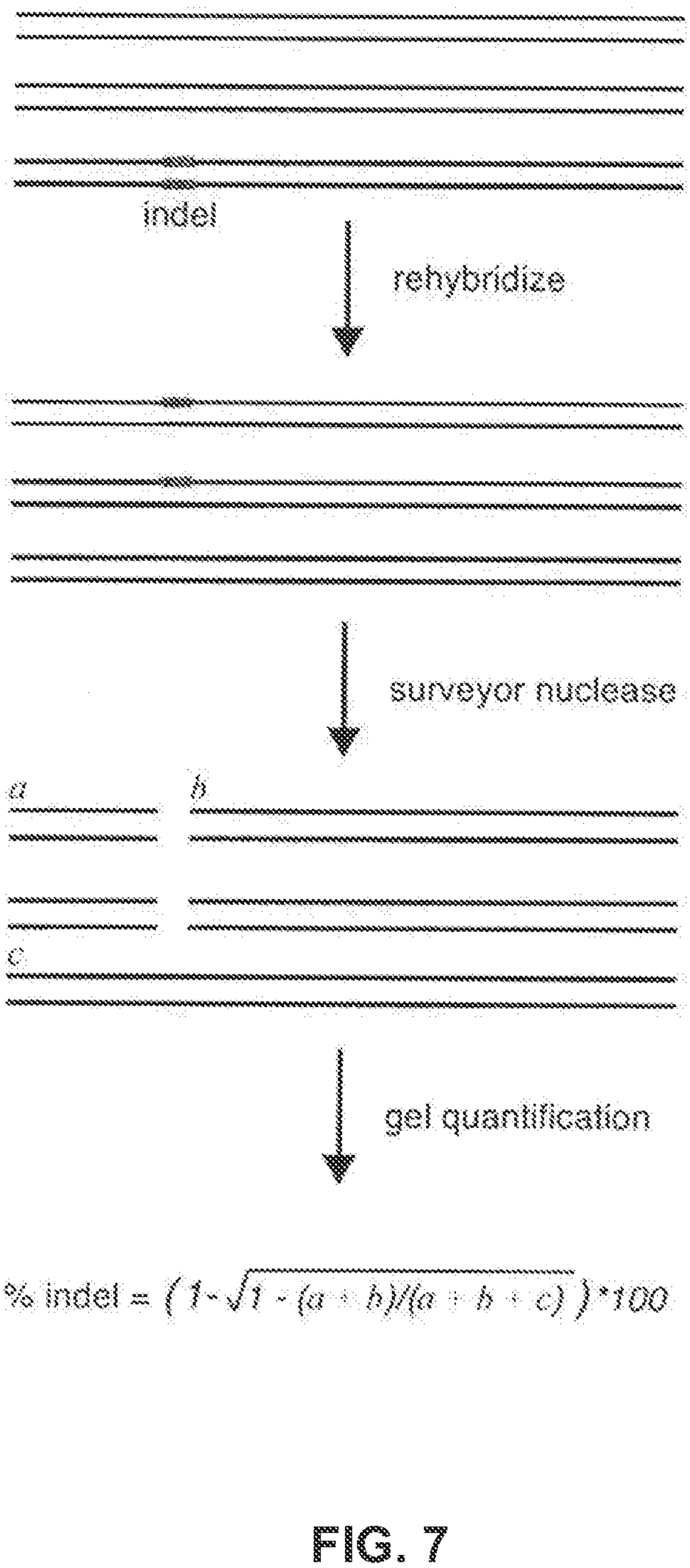

indel
rehybridize
surveyor nuclease
a
b
c
gel quantification
% indel = (1-√1 - (a + b)/(a + b + c))*100

FIG. 7

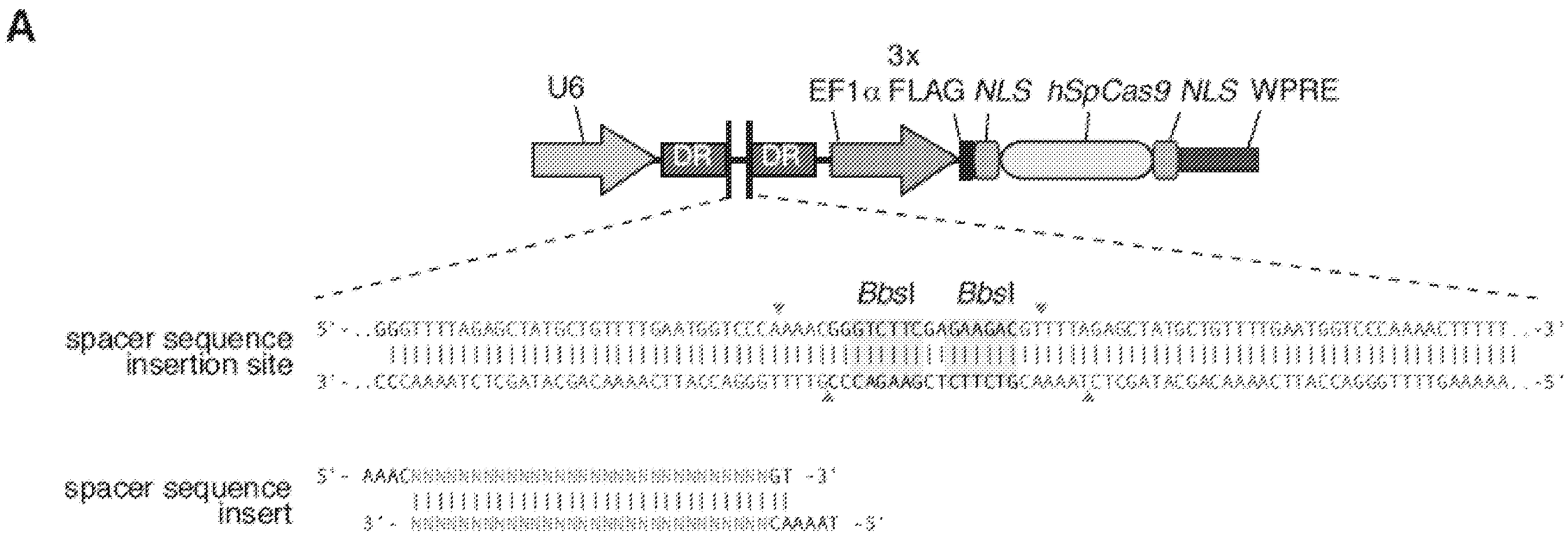
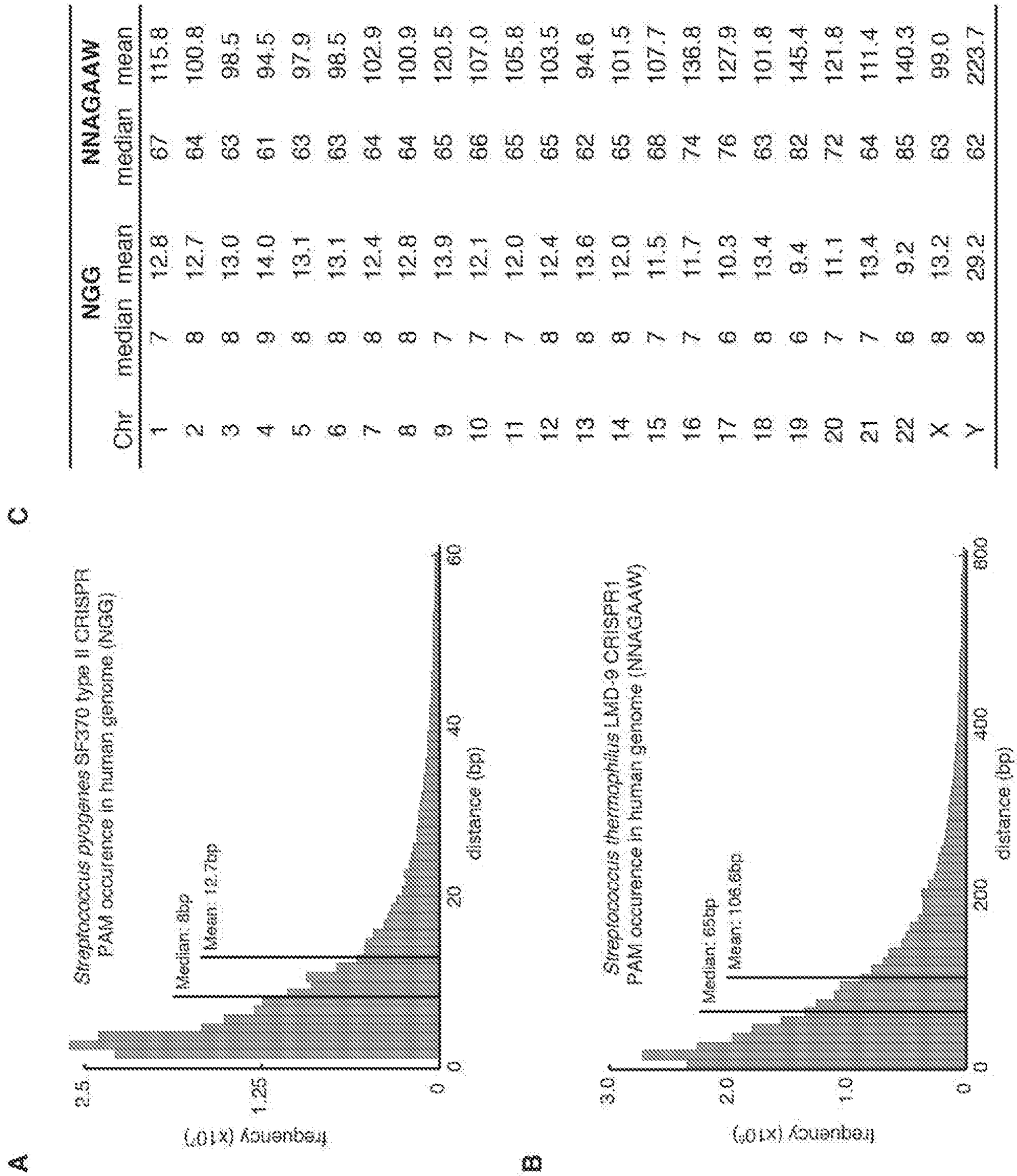

A
3x
U6
EF1α FLAG NLS hSpCas9 NLS WPRE
DR
DR
BbsI BbsI
spacer sequence insertion site
5'-..GGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGGGTCTTCGAGAAGACGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTTTT..-3'
3'-..CCCAAAATCTCGATACGACAAAACTTACCAGGGTTTTGCCCAGAAGCTCTTCTGCAAAATCTCGATACGACAAAACTTACCAGGGTTTTGAAAAA..-5'
spacer sequence insert
5'- AAACNNNNNNNNNNNNNNNNNNNNNNNNNNNNGT -3'
3'- NNNNNNNNNNNNNNNNNNNNNNNNNNNNCAAAAT -5'
B
3x
U6
EF1α FLAG NLS hSpCas9 NLS WPRE
BbsI BbsI
guide sequence insertion site
5'-..GTGGAAAGGACGAAACACCGGGTCTTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTT..-3'
3'-..CACCTTTCCTGCTTTGTGCCCAGAAGCTCTTCTGGACAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCAGGCAAAAA..-5'
guide sequence insert
5'- CACCGNNNNNNNNNNNNNNNNNNN -3'
3'- CNNNNNNNNNNNNNNNNNNNCAAA -5'

FIG. 8A-B

FIG. 9A-C

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



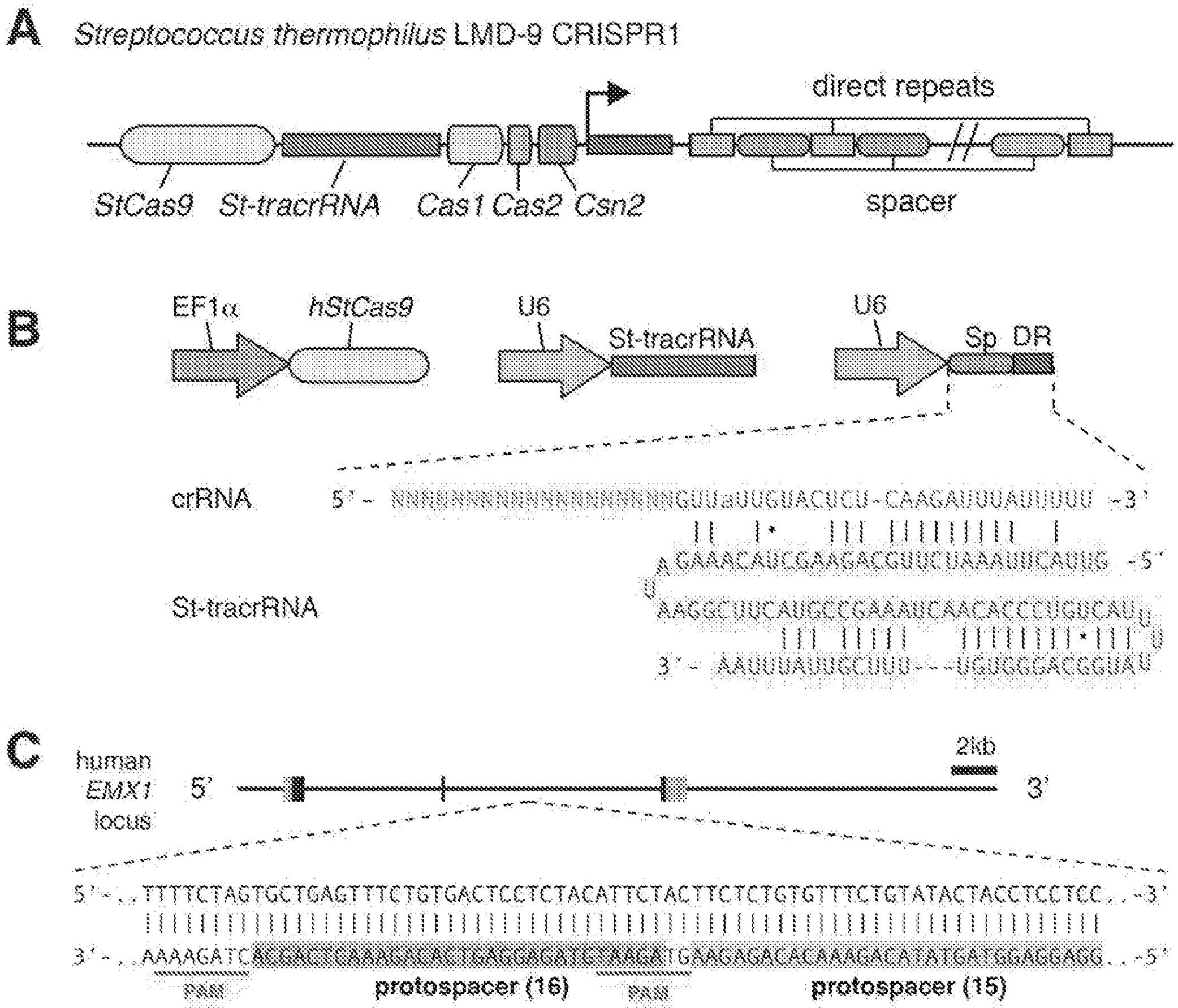

A
Streptococcus thermophilus LMD-9 CRISPR1
direct repeats
StCas9
St-tracrRNA
Cas1
Cas2
Csn2
spacer
B
EF1α
hStCas9
U6
St-tracrRNA
U6
Sp
DR
crRNA
St-tracrRNA
C
human EMX1 locus
5'
2kb
3'
protospacer (16)
PAM
protospacer (15)

FIG. 10A-C

D

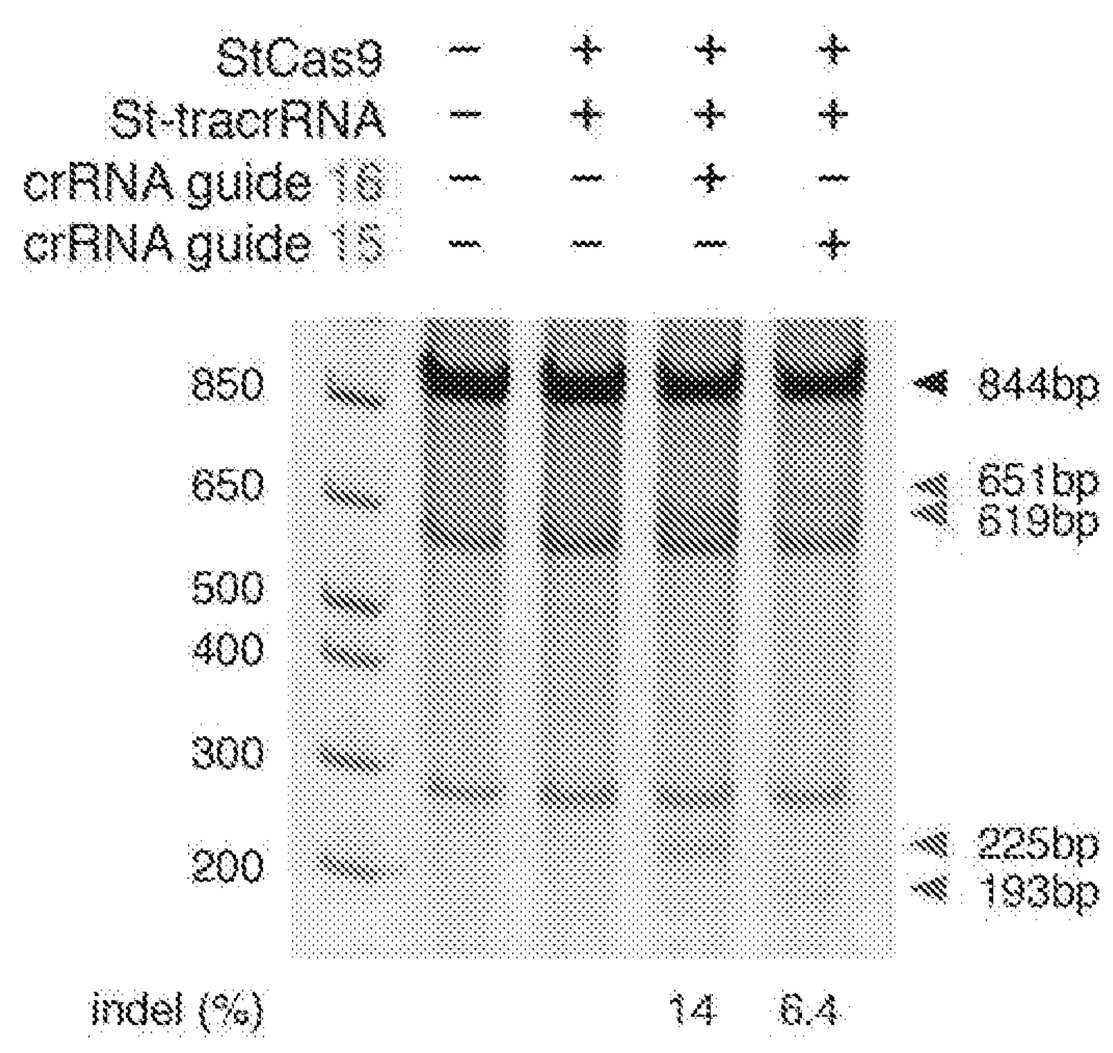

StCas9 − + + +
St-tracrRNA − + + +
crRNA guide 18 − − + −
crRNA guide 15 − − − +
850
650
500
400
300
200
844bp
651bp
619bp
225bp
193bp
indel (%) 14 6.4

FIG. 10D

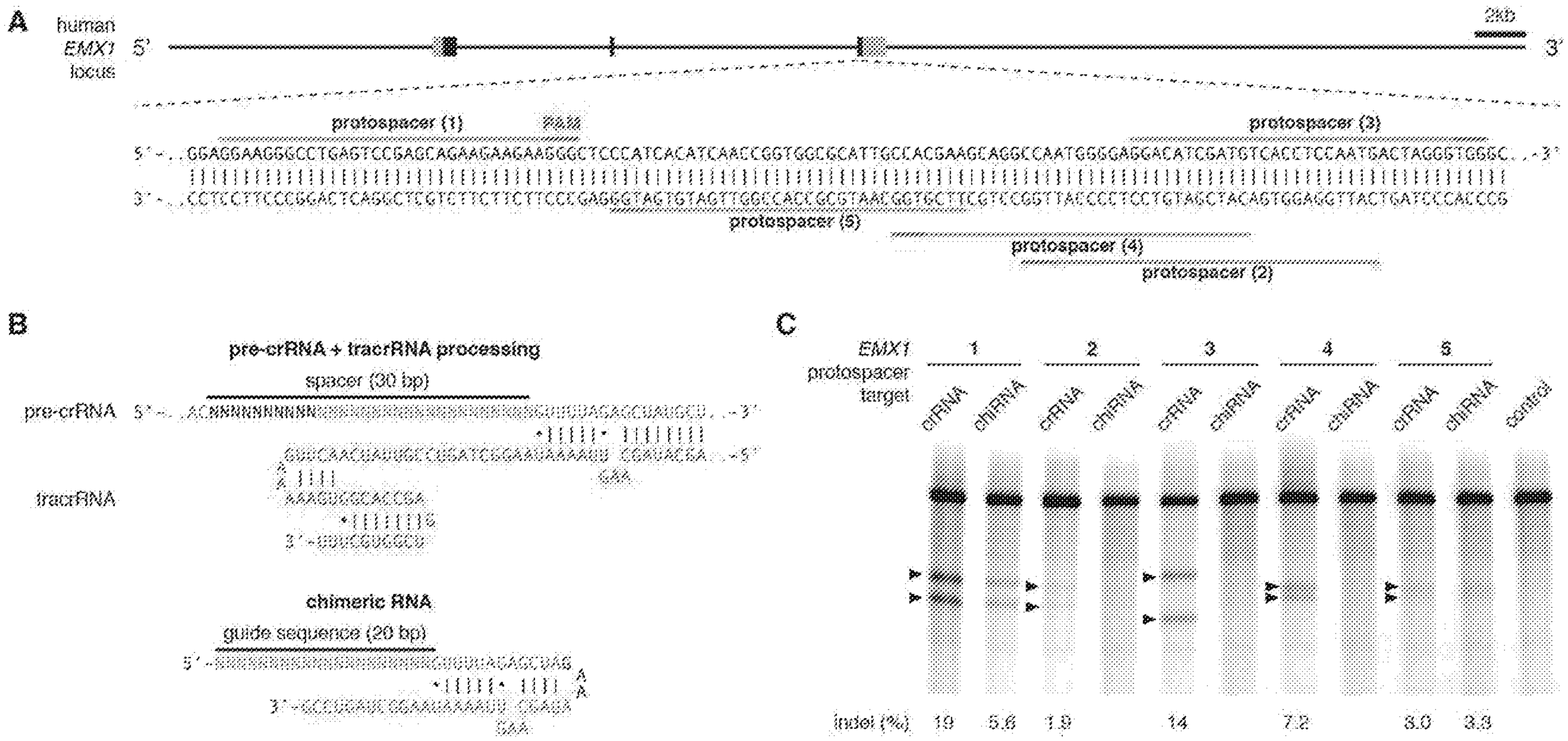
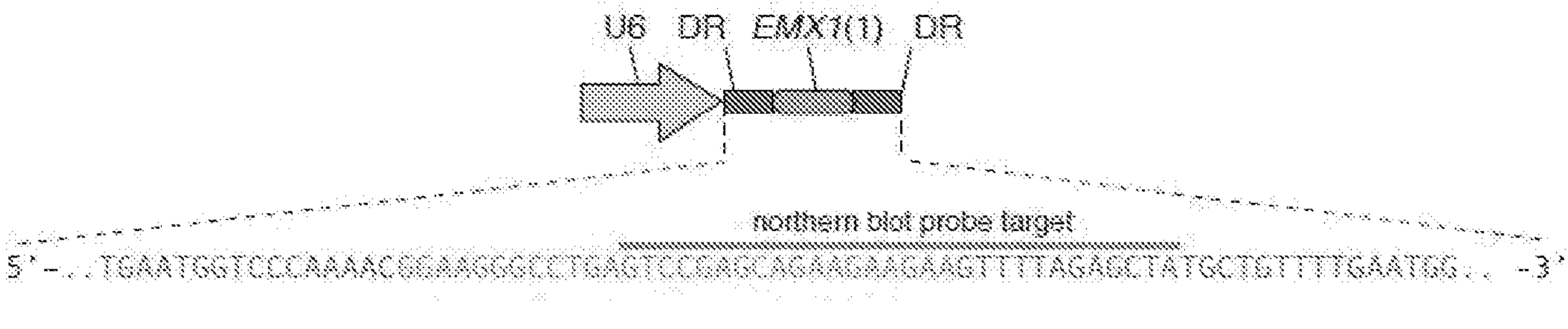

A
human EMX1 locus
5'
3'
2kb
protospacer (1)
protospacer (3)
protospacer (5)
protospacer (4)
protospacer (2)
B
pre-crRNA + tracrRNA processing
spacer (30 bp)
pre-crRNA
tracrRNA
chimeric RNA
guide sequence (20 bp)
C
EMX1 protospacer target
1
2
3
4
5
crRNA
chiRNA
control
indel (%)
19
5.6
1.9
14
7.2
8.0
3.3

FIG. 11A-C

A


U6
DR
EMX1(1)
DR
northern blot probe target
5'-..TGAATGGTCCCAAAACGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTATGCTGTTTTGAATGG..-3'

B

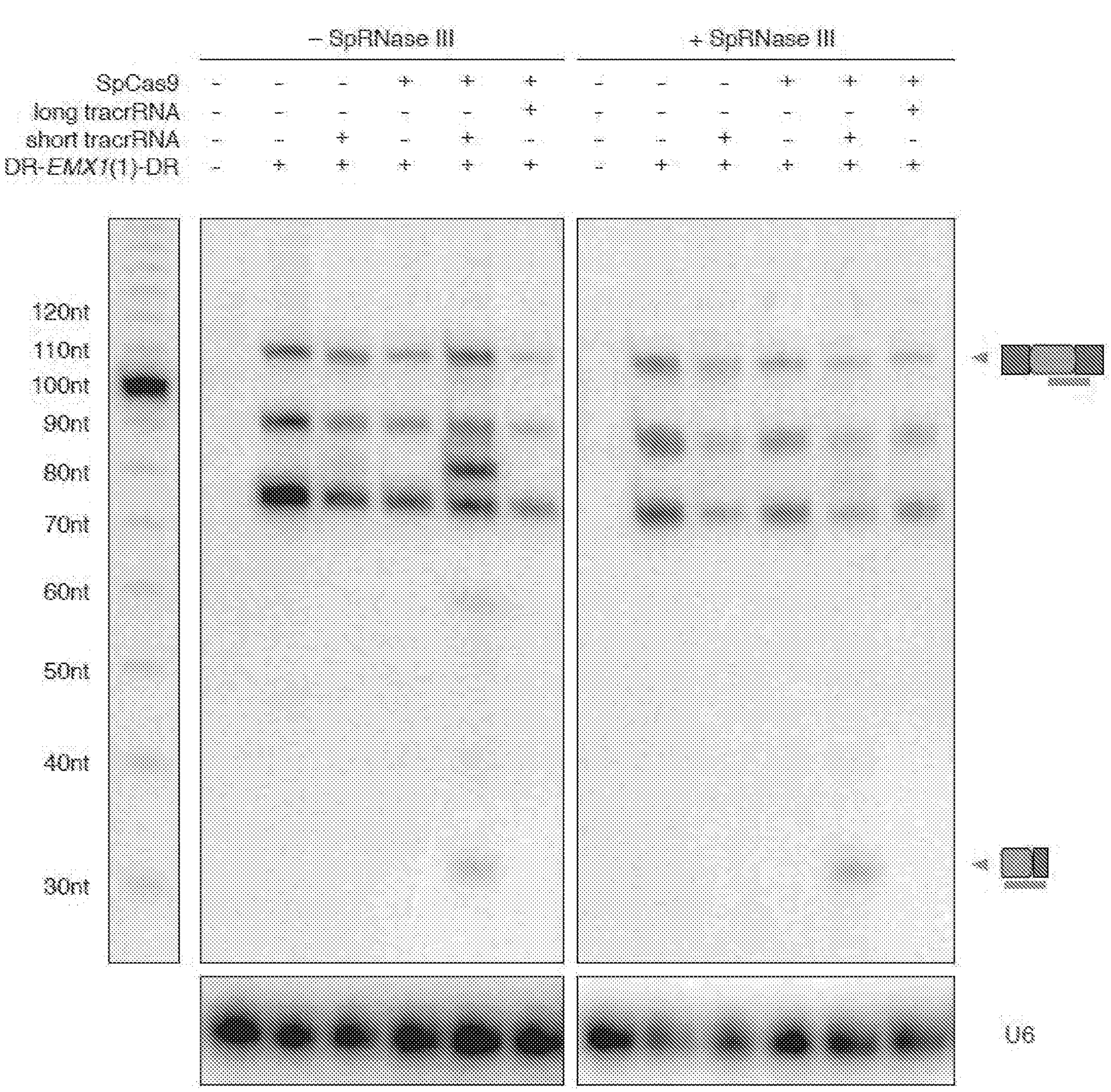

– SpRNase III
+ SpRNase III
SpCas9 – – – + + + – – – + + +
long tracrRNA – – – – – + – – – – – +
short tracrRNA – – + – + – – – + – + –
DR-EMX1(1)-DR – + + + + + – + + + + +
120nt
110nt
100nt
90nt
80nt
70nt
60nt
50nt
40nt
30nt
U6

FIG. 12A-B

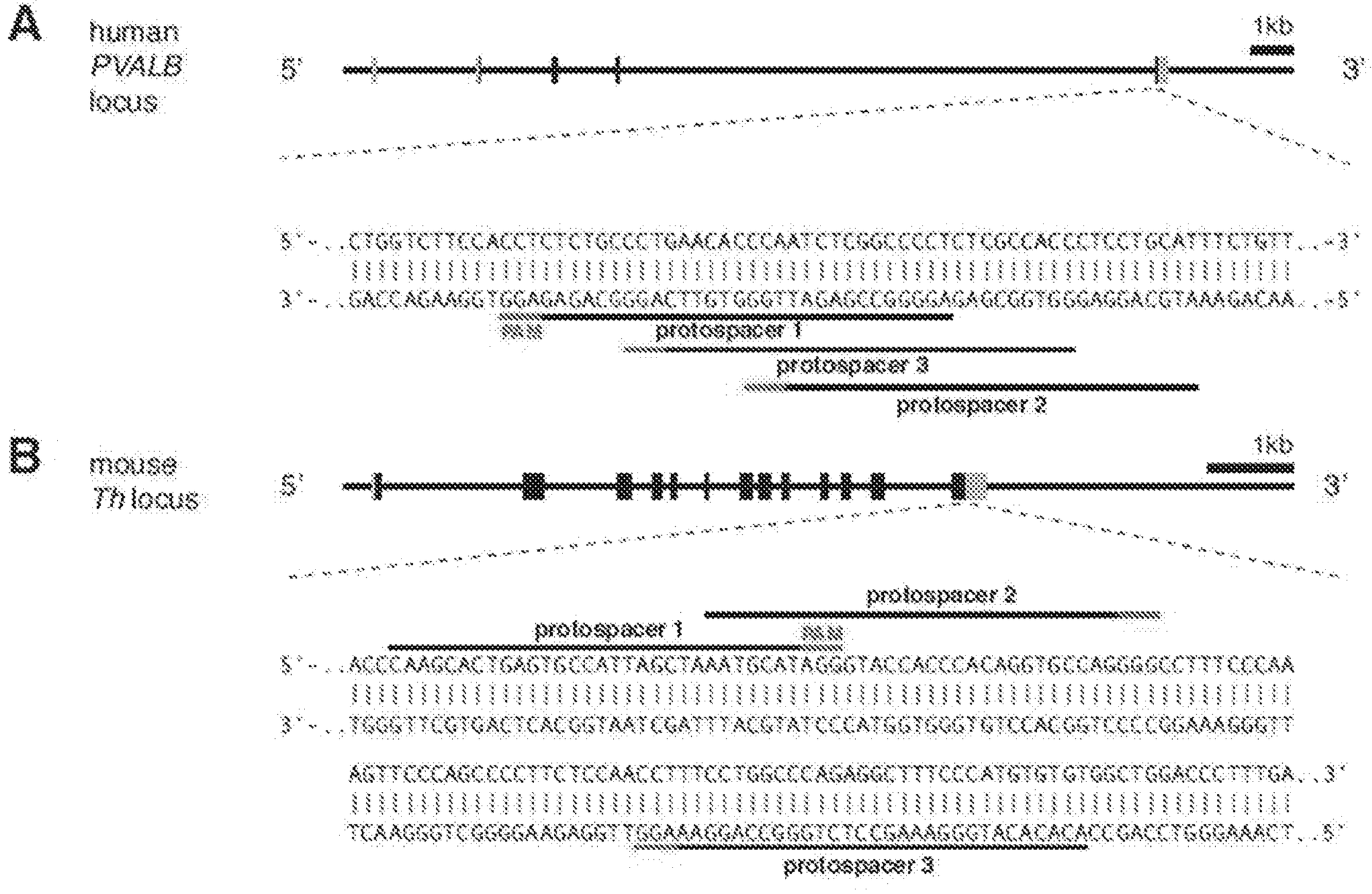

A
human
PVALB
locus
5'
1kb
3'
5'...CTGGTCTTCCACCTCTCTGCCCTGAACACCCAATCTGGCCCCTCTCGCCACCCTCCTGCATTTCTGTT...3'
3'...GACCAGAAGGTGGAGAGACGGGACTTGTGGGTTAGACCCGGGGAGAGCGGTGGGAGGACGTAAAGACAA...5'
protospacer 1
protospacer 3
protospacer 2
B
mouse
Th locus
5'
1kb
3'
protospacer 2
protospacer 1
5'...ACCCAAGCACTGAGTGCCATTAGCTAAATGCATAGGGTACCACCCACAGGTGCCAGGGGCCTTTCCCAA
3'...TGGGTTCGTGACTCACGGTAATCGATTTACGTATCCCATGGTGGGTGTCCACGGTCCCCGGAAAGGGTT
AGTTCCCAGCCCCTTCTCCAACCTTTCCTGGCCCAGAGGCTTTCCCATGTGTGTGGCTGGACCCTTTGA...3'
TCAAGGGTCGGGGAAGAGGTTGGAAAGGACCGGGTCTCCGAAAGGGTACACACACCGACCTGGGAAACT...5'
protospacer 3

FIG. 13A-B

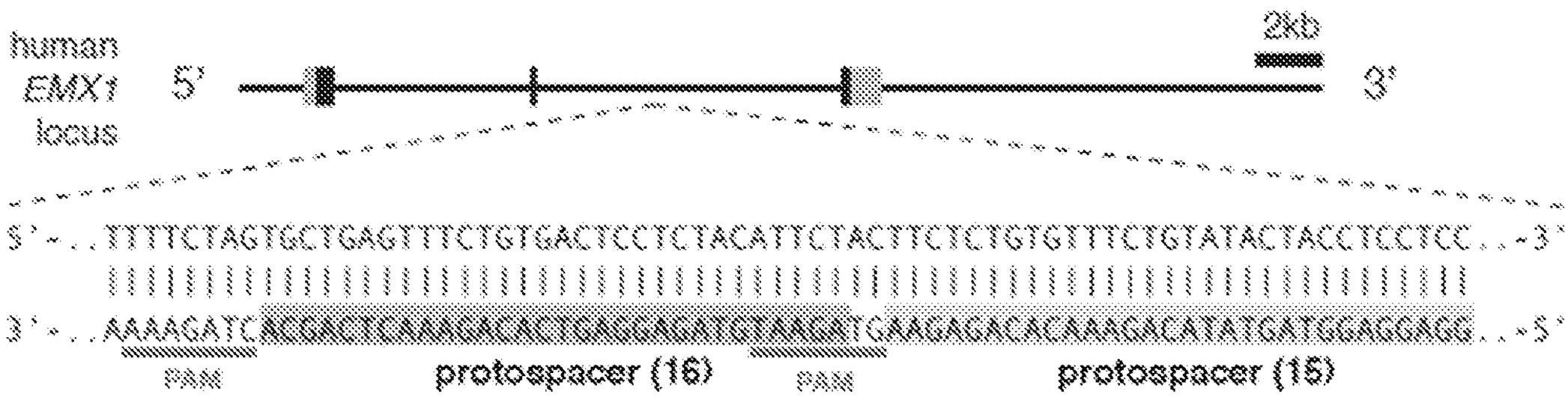

human
EMX1
locus
5'
2kb
3'
5' ...TTTTCTAGTGCTGAGTTTCTGTGACTCCTCTACATTCTACTTCTCTGTGTTTCTGTATACTACCTCCTCC...3'
3' ...AAAAGATCACGACTCAAAGACACTGACGACATGTAAGATGAAGAGACACAAAGACATATGATGGAGGAGG...5'
PAM
protospacer (16)
PAM
protospacer (15)

FIG. 14

Primer name	Assay	Genomic Target	Primer sequence
Sp-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	AAAACCACCCTTCTCTCTGGC
Sp-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	GGAGATTGGAGACACGGAGAG
Sp-PVALB-F	SURVEYOR assay, sequencing	*PVALB*	CTGGAAAGCCAATGCCTGAC
Sp-PVALB-R	SURVEYOR assay, sequencing	*PVALB*	GGCAGCAAACTCCTTGTCCT
Sp-Th-F	SURVEYOR assay, sequencing	*Th*	GTGCTTTGCAGAGGCCTACC
Sp-Th-R	SURVEYOR assay, sequencing	*Th*	CCTGGAGCGCATGCAGTAGT
St-EMX1-F	SURVEYOR assay, sequencing	*EMX1*	ACCTTCTGTGTTTCCACCATTC
St-EMX1-R	SURVEYOR assay, sequencing	*EMX1*	TTGGGGAGTGCACAGACTTC
Sp-EMX1-RFLP-F	RFLP, sequencing	*EMX1*	GGCTCCCTGGGTTCAAAGTA
Sp-EMX1-RFLP-R	RFLP, sequencing	*EMX1*	AGAGGGGTCTGGATGTCGTAA
Pb_EMX1_sp1	Northern Blot Probe	Not applicable	TAGCTCTAAAACTTCTTCTTCTGCTCGGAC
Pb_tracrRNA	Northern Blot Probe	Not applicable	CTAGCCTTATTTTAACTTGCTATGCTGTTT

FIG. 15

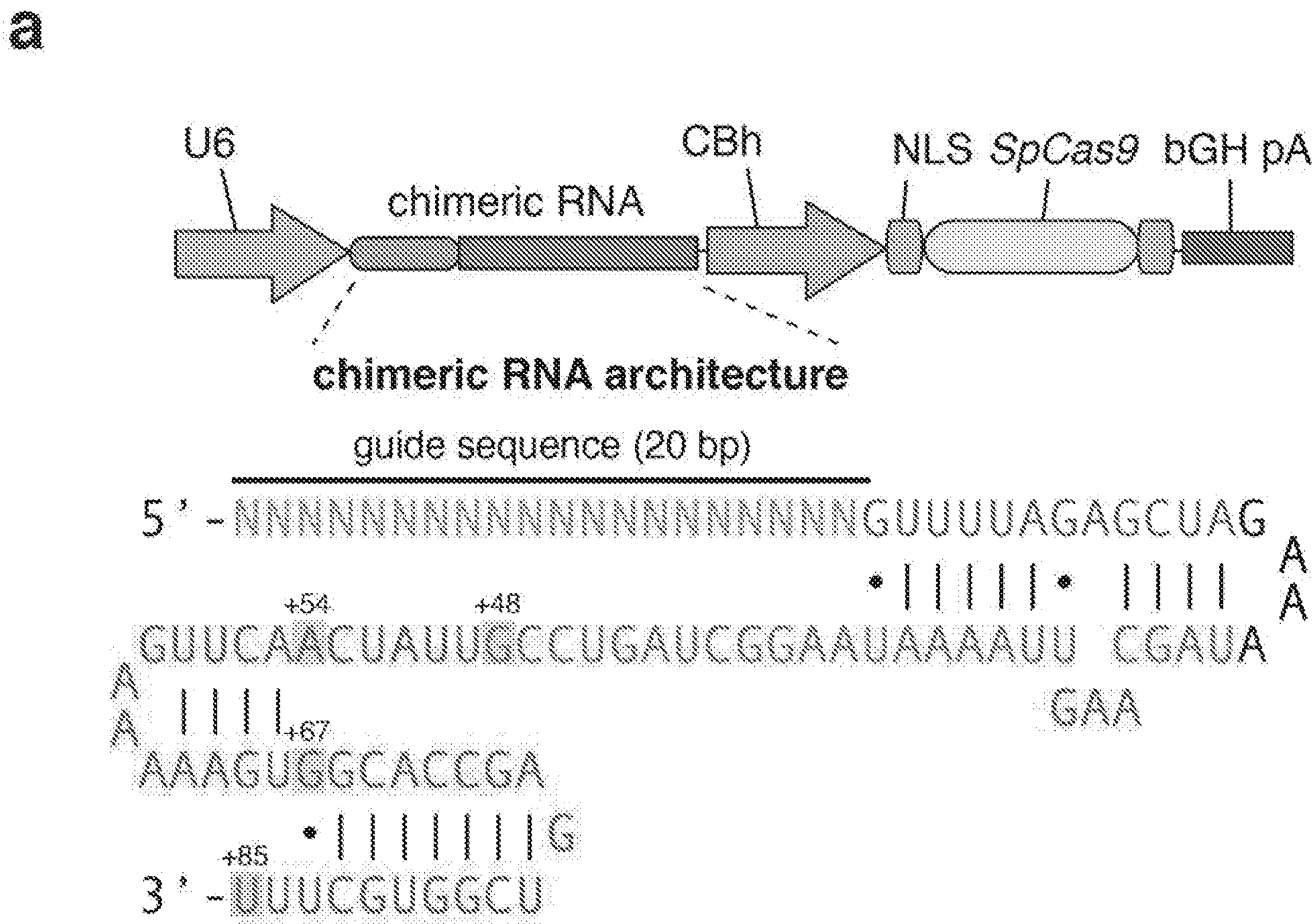

a
U6
chimeric RNA
CBh
NLS SpCas9 bGH pA
chimeric RNA architecture
guide sequence (20 bp)
5' - NNNNNNNNNNNNNNNNNNNN GUUUUAGAGCUAG
A
A
+54
+48
GUUCAACUAUUGCCUGAUCGGAAUAAAAAUU CGAUA
A
A
+67
AAAGUGGCACCGA
+85
G
3' - UUUCGUGGCU

FIG. 16A

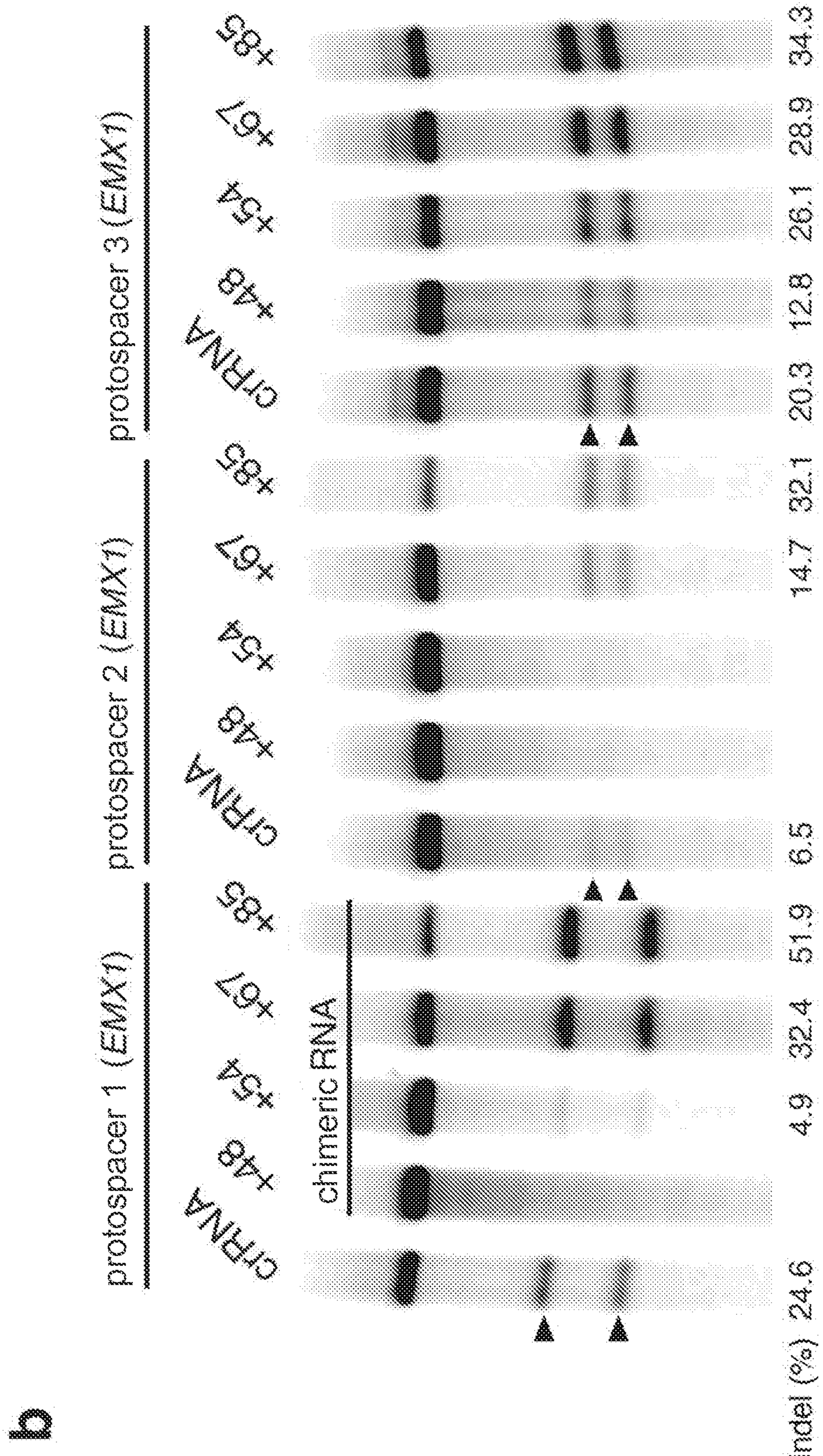

b
protospacer 1 (EMX1)
protospacer 2 (EMX1)
protospacer 3 (EMX1)
chimeric RNA
crRNA +48
+54
+67
+85
indel (%)
4.9
32.4
51.9
6.5
14.7
32.1
20.3
12.8
26.1
28.9
34.3

FIG. 16B

C

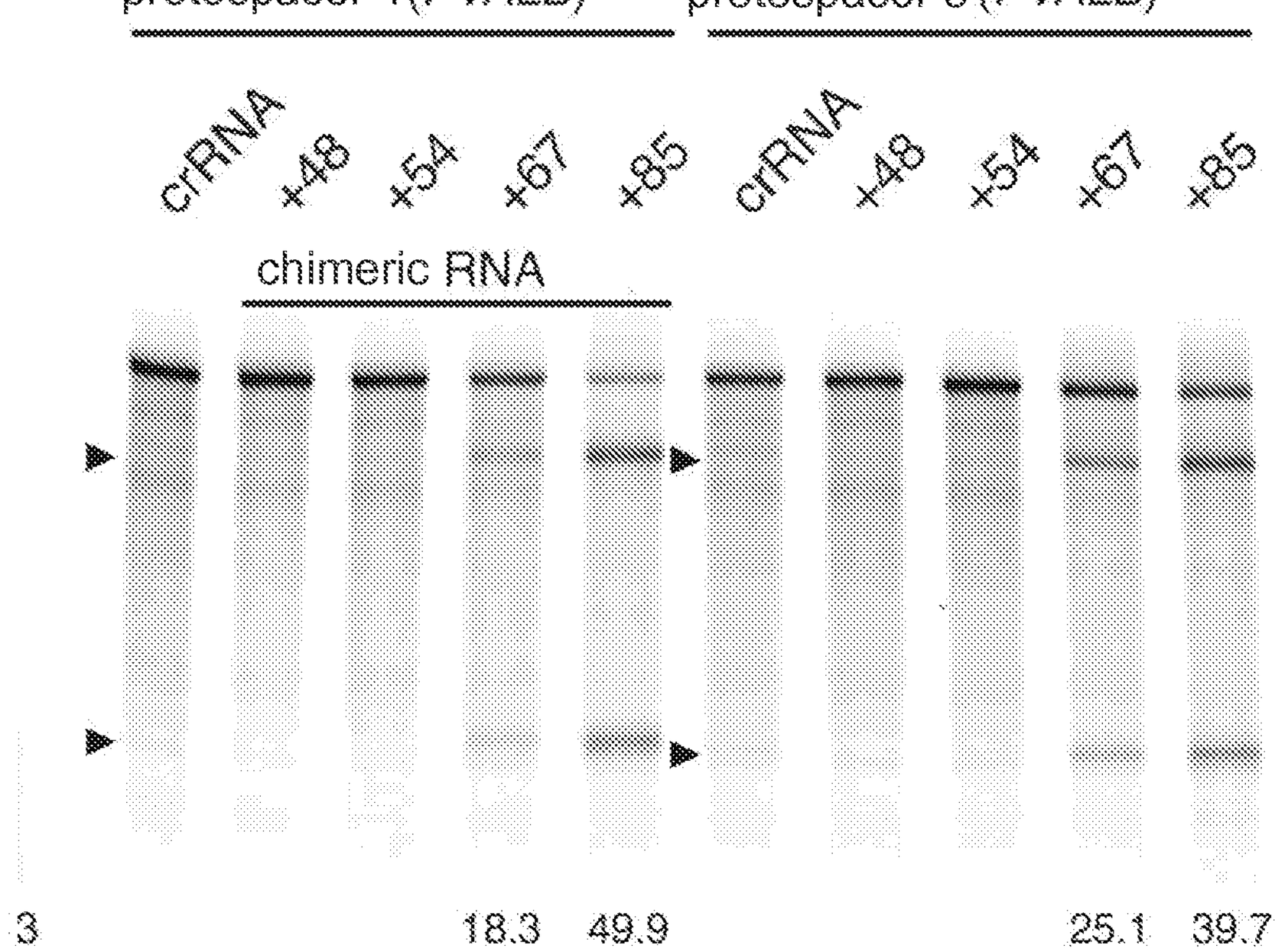

protospacer 4 (PVALB)
protospacer 5 (PVALB)
crRNA
+48
+54
+67
+85
crRNA
+48
+54
+67
+85
chimeric RNA
3
18.3
49.9
25.1
39.7

FIG. 16C

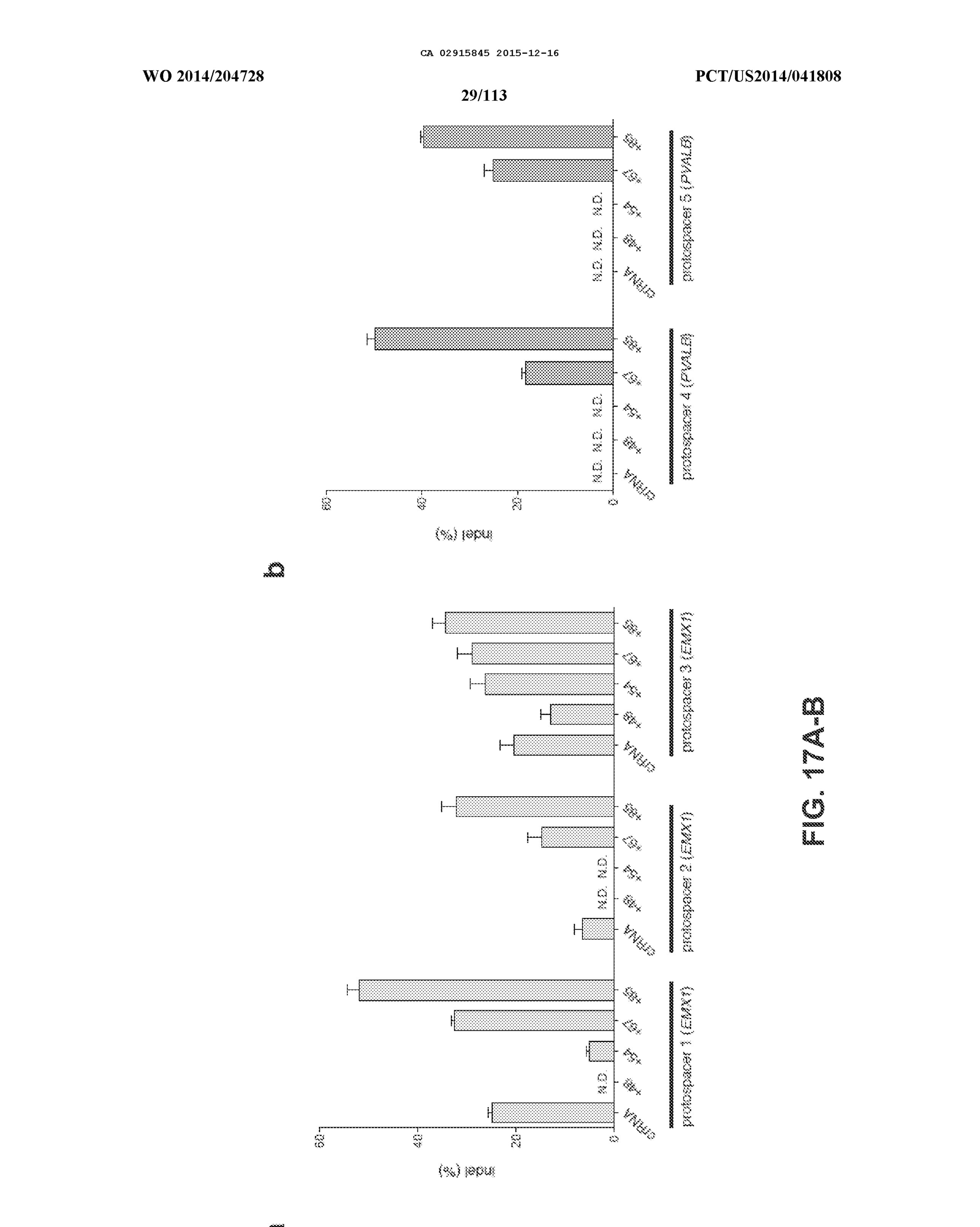

B
protospacer 4 (PVALB)
protospacer 3 (EMX1)
protospacer 2 (EMX1)
protospacer 1 (EMX1)
protospacer 5 (PVALB)
sgRNA
gRNA
N.D.
indel (%)
0
20
40
60

FIG. 14A-B

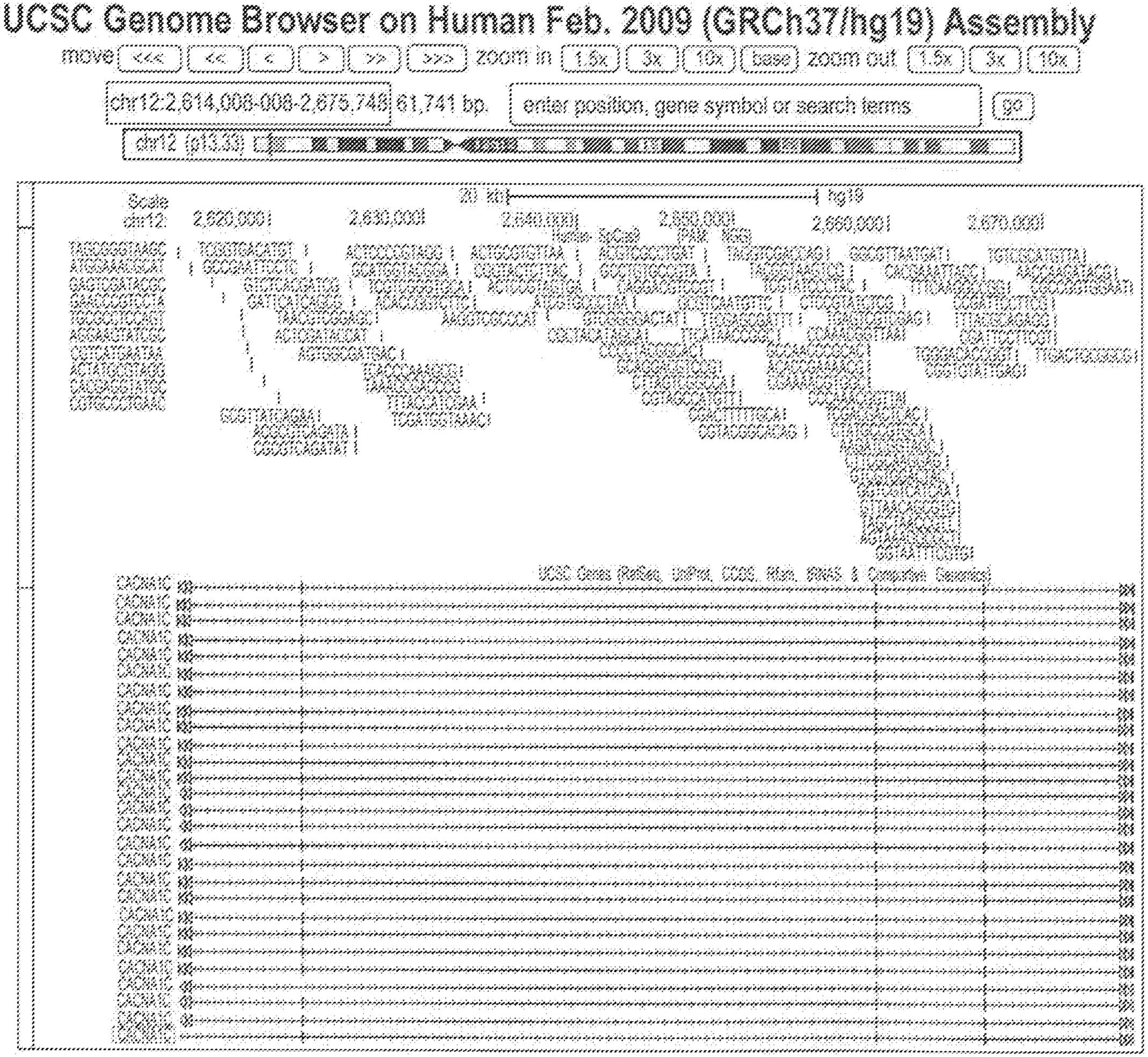

UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly
move
zoom in
zoom out
chr12:2,614,008-008-2,675,748
61,741 bp.
enter position, gene symbol or search terms
go
chr12 (p13.33)
20 kb
hg19
Scale
chr12:
2,620,000
2,630,000
2,640,000
2,650,000
2,660,000
2,670,000
UCSC Genes (RefSeq, UniProt, CCDS, Rfam, tRNAs & Comparative Genomics)

FIG. 18

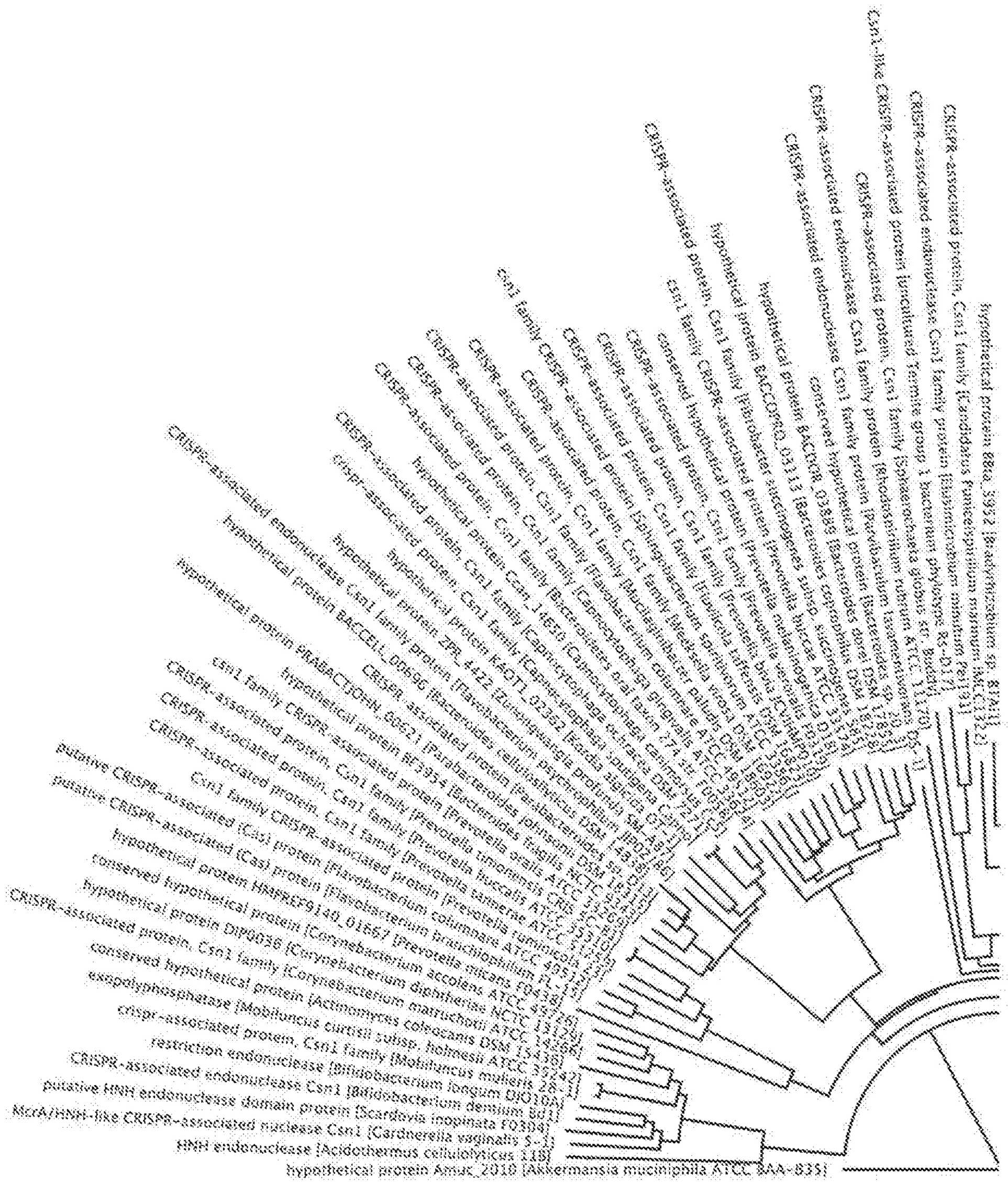
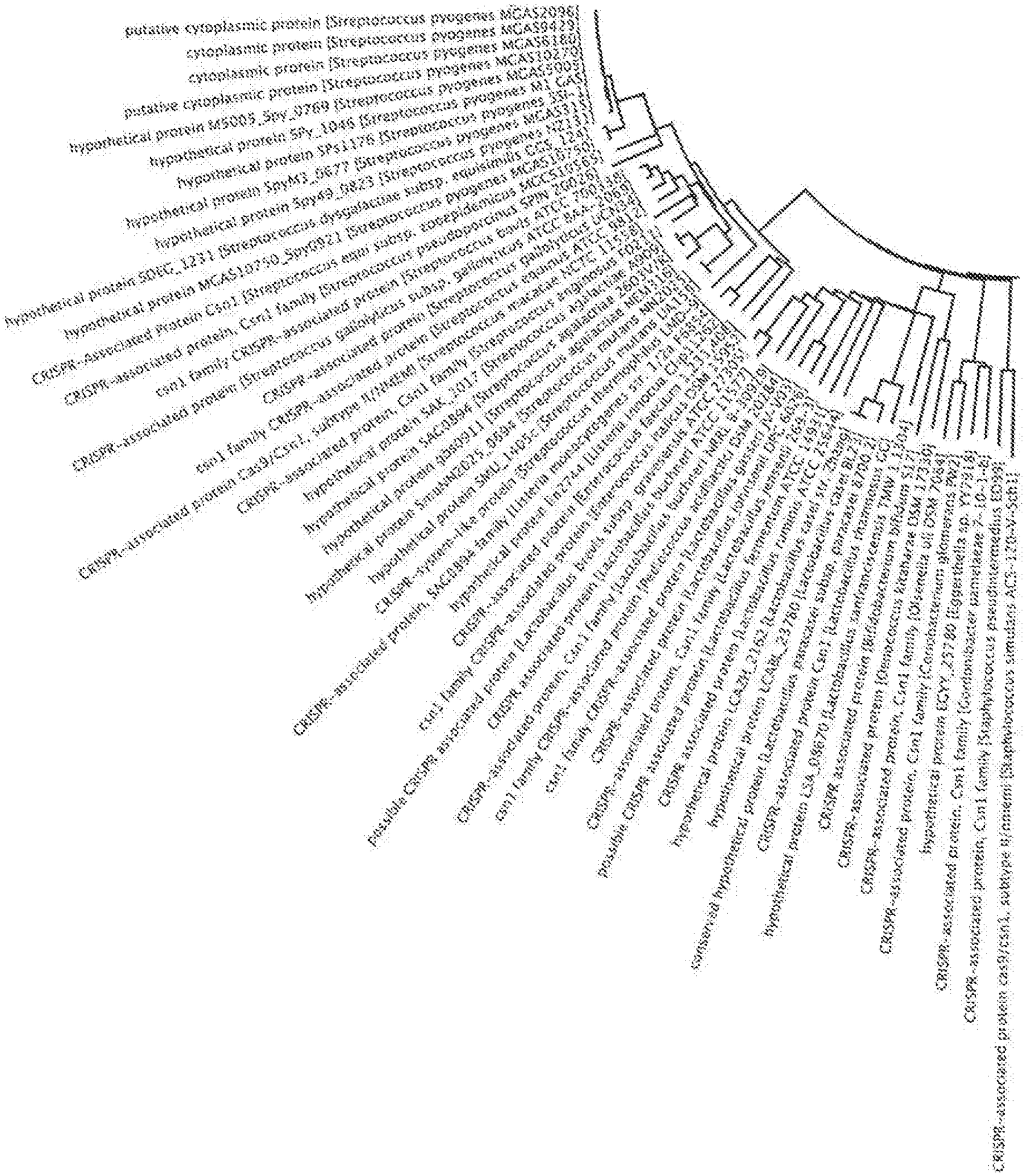

hypothetical protein BBta_3952 [Bradyrhizobium sp. BTAi1]
CRISPR-associated protein, Csn1 family [Candidatus Puniceispirillum marinum IMCC1322]
CRISPR-associated endonuclease Csn1 family protein [Ruminiclostridium thermocellum DSM 1313]
CRISPR-associated protein [uncultured Termite group 1 bacterium phylotype Rs-D17]
CRISPR-associated protein, Csn1 family [Spirosoma linguale DSM 74]
CRISPR-associated protein Csn1 family protein [Rhodospirillum rubrum ATCC 11170]
CRISPR-associated endonuclease Csn1 family protein [Parvibaculum lavamentivorans DS-1]
CRISPR-associated endonuclease Csn1 family protein [Bacteroides coprocola DSM 17136]
hypothetical protein BACCOPRO_03113 [Bacteroides coprocola DSM 17136]
hypothetical protein [Prevotella melaninogenica ATCC 25845]
conserved hypothetical protein BACCAP_03889 [Bacteroides capillosus ATCC 29799]
CRISPR-associated protein, Csn1 family [Prevotella buccae ATCC 33574]
CRISPR-associated endonuclease Csn1 family protein [Bacteroides sp. 2_2]
hypothetical protein BACSTE_00196 [Bacteroides stercoris ATCC 43183]
hypothetical protein BACOVA_03138 [Bacteroides ovatus ATCC 8483]
conserved hypothetical protein [Bacteroides sp. 4_3_47FAA]
CRISPR-associated protein, Csn1 family [Prevotella oralis ATCC 33269]
CRISPR-associated protein, Csn1 family [Prevotella tannerae ATCC 51259]
Csn1 family CRISPR-associated protein [Prevotella ruminicola 23]
CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
CRISPR-associated protein, Csn1 family [Prevotella timonensis CRIS 5-D1-B]
hypothetical protein BACCELL_00696 [Bacteroides cellulosilyticus DSM 14838]
CRISPR-associated protein, Csn1 family [Parabacteroides johnsonii DSM 18315]
hypothetical protein ZPR_4422 [Zunongwangia profunda SM-A87]
Csn1 family protein [Mariniphaga sediminis DSM 19839]
hypothetical protein BACCELL_00696 [Bacteroides cellulosilyticus DSM 14838]
CRISPR-associated protein, Csn1 family [Parabacteroides distasonis ATCC 8503]
hypothetical protein BF3954 [Bacteroides fragilis NCTC 9343]
CRISPR-associated protein, Csn1 family [Prevotella oralis ATCC 33269]
CRISPR-associated protein, Csn1 family [Prevotella tannerae ATCC 51259]
Csn1 family CRISPR-associated protein [Prevotella ruminicola 23]
putative CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
CRISPR-associated (Cas) protein [Flavobacterium columnare ATCC 49512]
hypothetical protein HMPREF9140_01667 [Prevotella micans F0438]
conserved hypothetical protein [Capnocytophaga ochracea DSM 7271]
hypothetical protein DIP0036 [Corynebacterium diphtheriae NCTC 13129]
CRISPR-associated protein, Csn1 family [Corynebacterium matruchotii ATCC 14266]
exopolyphosphatase [Mobiluncus curtisii subsp. holmesii ATCC 35242]
conserved hypothetical protein [Actinomyces coleocanis DSM 15436]
crispr-associated protein, Csn1 family [Mobiluncus mulieris 28-1]
restriction endonuclease [Bifidobacterium longum DJO10A]
CRISPR-associated endonuclease Csn1 [Bifidobacterium dentium Bd1]
putative HNH endonuclease domain protein [Scardovia inopinata F0304]
McrA/HNH-like CRISPR-associated nuclease Csn1 [Cardinerella vaginalis 5-1]
HNH endonuclease [Acidothermus cellulolyticus 11B]
hypothetical protein Amuc_2010 [Akkermansia muciniphila ATCC BAA-835]

FIG. 19A


hypothetical protein KKC_1488 [Kingella kingae ATCC 23330]
hypothetical protein NMCC_0397 [Neisseria meningitidis 8249]
hypothetical protein NMA_1760 [Neisseria meningitidis alpha14]
hypothetical protein NGK_0631 [Neisseria meningitidis 053442]
putative CRISPR-associated protein [Neisseria lactamica 020-06]
hypothetical protein NMVQ1_0320 [Neisseria cinerea ATCC 14685]
CRISPR-associated endonuclease Cas1 family protein [Actinobacillus minor NM305]
hypothetical protein HMPREF9123_0020 [Neisseria bacilliformis ATCC BAA-1200]
CRISPR-associated endonuclease Cas1 family protein [Pasteurella multocida subsp. multocida str. Pm70]
hypothetical protein PM1127 [Pasteurella multocida subsp. multocida str. Pm70]
CRISPR-associated endonuclease Cas1 family protein [Actinobacillus pleuropneumoniae serovar 10 str. D13039]
hypothetical protein PARA_18570 [Haemophilus parainfluenzae T3T1]
CRISPR-associated protein, Csn1 family [Actinobacillus succinogenes 130Z]
CRISPR-associated endonuclease Cas1 family protein [Ruminococcus albus 8]
CRISPR-associated protein, Csn1 family [Nitratifractor salsuginis DSM 16511]
CRISPR-associated protein, csn1 family [Mycoplasma mobile 163K]
hypothetical protein MMOB0330 [Mycoplasma mobile 163K]
CRISPR-associated protein, Csn1 family [Haemophilus parainfluenzae T3T1]
CRISPR-associated protein, Csn1 family [Haemophilus parainfluenzae HK262


putative cytoplasmic protein [Streptococcus pyogenes MGAS2096]
cytoplasmic protein [Streptococcus pyogenes MGAS9429]
cytoplasmic protein [Streptococcus pyogenes MGAS6180]
putative cytoplasmic protein [Streptococcus pyogenes MGAS10270]
hypothetical protein M5005_Spy_0760 [Streptococcus pyogenes MGAS5005]
hypothetical protein SPy_1046 [Streptococcus pyogenes M1 GAS]
hypothetical protein SPs1176 [Streptococcus pyogenes MGAS315]
hypothetical protein Spy49_0677 [Streptococcus pyogenes NZ131]
hypothetical protein Spy49_0823 [Streptococcus pyogenes MGAS10750]
hypothetical protein SDEG_1231 [Streptococcus dysgalactiae subsp. equisimilis GGS_124]
hypothetical protein MGAS10750_Spy0823 [Streptococcus pyogenes MGAS10750]
CRISPR-associated protein, Csn1 family [Streptococcus agalactiae 2603V/R]
CRISPR-associated protein, Csn1 family [Streptococcus agalactiae NEM316]
csn1 family CRISPR-associated protein [Streptococcus gallolyticus UCN34]
CRISPR-associated protein [Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069]
CRISPR-associated protein [Streptococcus equinus ATCC 9812]
csn1 family CRISPR-associated protein [Streptococcus bovis ATCC 700338]
CRISPR-associated protein [Streptococcus pseudoporcinus SPIN 20026]
CRISPR-associated protein [Streptococcus equinus NCTC 11538]
CRISPR-associated protein [Streptococcus infantarius subsp. infantarius CJ18]
CRISPR-associated protein [Streptococcus agalactiae ATCC 27591]
hypothetical protein SAK_2017 [Streptococcus agalactiae 2603V/R]
hypothetical protein SAG3894 [Streptococcus agalactiae NEM316]
hypothetical protein SAN2GNE0275_05910 [Streptococcus agalactiae GD201008-001]
hypothetical protein SAUSA300_1405 [Staphylococcus aureus subsp. aureus USA300_TCH1516]
CRISPR-system-like protein [Streptococcus pneumoniae GA17545]
CRISPR family [Listeria monocytogenes str. 1/2a F6854]
hypothetical protein lin2744 [Listeria monocytogenes str. 1/2a F6854]
CRISPR-associated protein [Enterococcus faecium DO]
CRISPR-associated protein [Lactobacillus gasseri JV-V03]
CRISPR-associated protein [Lactobacillus gasseri ATCC 33323]
CRISPR-associated protein [Lactobacillus gasseri MV-22]
CRISPR-associated protein [Lactobacillus gasseri ATCC 33323]
CRISPR-associated protein, Csn1 family [Lactobacillus helveticus DPC 4571]
CRISPR-associated protein [Lactobacillus helveticus H10]
CRISPR-associated protein [Lactobacillus acidophilus ATCC 4796]
CRISPR-associated protein [Lactobacillus gasseri ATCC 33323]
CRISPR-associated protein [Lactobacillus johnsonii DPC 6026]
CRISPR-associated protein [Lactobacillus johnsonii FI9785]
CRISPR-associated protein, Csn1 family [Lactobacillus fermentum ATCC 14931]
CRISPR-associated protein [Lactobacillus rhamnosus ATCC 53103]
CRISPR-associated protein [Lactobacillus rhamnosus ATCC 8530]
CRISPR-associated protein [Lactobacillus rhamnosus GG]
CRISPR-associated protein [Lactobacillus rhamnosus LMS2-1]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP2]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP3]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP4]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP5]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP6]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP7]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP8]
CRISPR-associated protein [Lactobacillus rhamnosus LRHMDP9]
CRISPR-associated protein [Lactobacillus rhamnosus


hypothetical protein MovS_000710 [Mycoplasma ovipneumoniae SC01]
Cas1 family CRISPR-associated protein [Mycoplasma canis PG 14]
hypothetical protein MS53_0582 [Mycoplasma synoviae 53]
Cas1 family CRISPR-associated protein [Mycoplasma gallisepticum str. F]
Cas1 family CRISPR-associated protein [Mycoplasma gallisepticum str. R(low)]
CRISPR-associated protein Cas9/Csn1 [Staphylococcus aureus subsp. aureus]
conserved hypothetical protein [Staphylococcus lugdunensis M23590]
hypothetical protein EUBSOL_01797 [Eubacterium saburreum DSM 3986]
hypothetical protein CLOSPI_00488 [Clostridium spiroforme DSM 1552]
CRISPR-associated protein, Csn1 family [Enterococcus faecalis TX0012]
hypothetical protein RUMVEN_00141 [Eubacterium ventriosum ATCC 27560]
CRISPR-system related protein [Eubacterium rectale ATCC 33656]
CRISPR-associated endonuclease Csn1 family [Lactobacillus farciminis KCTC 3681]
CRISPR-associated protein, Csn1 family protein [Streptococcus parauberis KCTC 11537]
CRISPR-system family protein [Streptococcus pseudoporcinus SPIN 20026]
hypothetical protein stu0657 [Streptococcus thermophilus LMG 18311]
CRISPR-system-like protein stu0657 [Streptococcus thermophilus CNRZ1066]
hypothetical protein stu0657 [Streptococcus thermophilus LMD-9]
CRISPR-associated protein, Csn1 family [Streptococcus suis 89/1591]
hypothetical protein SSUST1_01721 [Streptococcus suis ST3]
CRISPR-associated protein, Csn1 family [Streptococcus pasteurianus ATCC 43144]
hypothetical protein spb0160 [Streptococcus infantarius subsp. infantarius ATCC BAA-102]
membrane protein WS1613 [Wolinella succinogenes DSM 1740]
hypothetical protein [Francisella novicida U112]
CRISPR-associated large protein [Francisella philomiragia subsp. philomiragia ATCC 25017]
putative CRISPR-associated protein, Csn1 family [Haemophilus parainfluenzae T3T1]
CRISPR-associated protein (Csn1 family) [Haemophilus sputorum HK 2154]
hypothetical protein HMPREF0454_01128 [Actinobacillus pleuropneumoniae serovar 7 str. AP76]
CRISPR-associated protein, Csn1 family [Actinobacillus suis H91-0380]
CRISPR-associated protein, Csn1 family [Actinobacillus minor NM305]
CRISPR-associated protein, Csn1 family [Pasteurella dagmatis ATCC 43325]
CRISPR-associated protein, Csn1 family [Haemophilus sp. oral taxon 851 str. F0397]
CRISPR-associated protein, Csn1 family [Haemophilus parahaemolyticus ATCC 33392]
CRISPR-associated protein, Csn1 family [Haemophilus parainfluenzae ATCC 33392]
CRISPR-associated protein, Csn1 family [Haemophilus sp. oral taxon 851 str. F0397

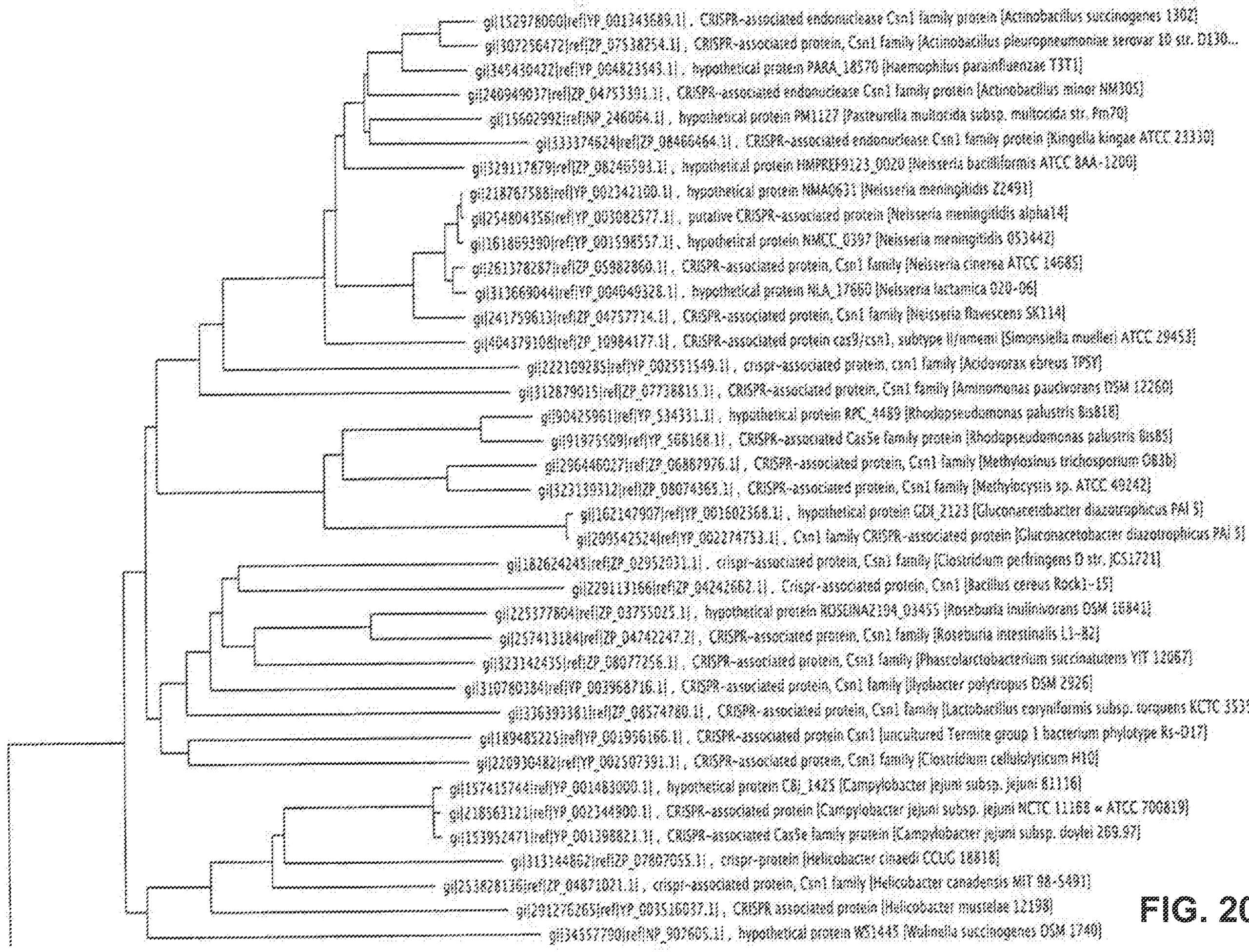

gi|152978060|ref|YP_001343689.1| , CRISPR-associated endonuclease Csn1 family protein [Actinobacillus succinogenes 130Z]
gi|307259472|ref|ZP_07538254.1| , CRISPR-associated protein, Csn1 family [Actinobacillus pleuropneumoniae serovar 10 str. D130...
gi|345439422|ref|YP_004823543.1| , hypothetical protein PARA_18570 [Haemophilus parainfluenzae T3T1]
gi|240949037|ref|ZP_04753391.1| , CRISPR-associated endonuclease Csn1 family protein [Actinobacillus minor NM305]
gi|15602993|ref|NP_246064.1| , hypothetical protein PM1127 [Pasteurella multocida subsp. multocida str. Pm70]
gi|333374624|ref|ZP_08466464.1| , CRISPR-associated endonuclease Csn1 family protein [Kingella kingae ATCC 23330]
gi|329117879|ref|ZP_08246591.1| , hypothetical protein HMPREF9123_0020 [Neisseria bacilliformis ATCC BAA-1200]
gi|218767588|ref|YP_002342100.1| , hypothetical protein NMA0631 [Neisseria meningitidis Z2491]
gi|254804356|ref|YP_003082577.1| , putative CRISPR-associated protein [Neisseria meningitidis alpha14]
gi|161869390|ref|YP_001598557.1| , hypothetical protein NMCC_0397 [Neisseria meningitidis 053442]
gi|261378287|ref|ZP_05982860.1| , CRISPR-associated protein, Csn1 family [Neisseria cinerea ATCC 14685]
gi|313668044|ref|YP_004049328.1| , hypothetical protein NLA_17660 [Neisseria lactamica 020-06]
gi|241759613|ref|ZP_04757714.1| , CRISPR-associated protein, Csn1 family [Neisseria flavescens SK114]
gi|404379108|ref|ZP_10984177.1| , CRISPR-associated protein cas9/csn1, subtype II/nmeni [Simonsiella muelleri ATCC 29453]
gi|222109285|ref|YP_002551549.1| , crispr-associated protein, csn1 family [Acidovorax ebreus TPSY]
gi|312879015|ref|ZP_07738815.1| , CRISPR-associated protein, Csn1 family [Aminomonas paucivorans DSM 12260]
gi|90425961|ref|YP_534331.1| , hypothetical protein RPC_4489 [Rhodopseudomonas palustris BisB18]
gi|91975589|ref|YP_568168.1| , CRISPR-associated Cas5e family protein [Rhodopseudomonas palustris BisB5]
gi|290446017|ref|ZP_06887976.1| , CRISPR-associated protein, Csn1 family [Methyloxinus trichosporium OB3b]
gi|323139312|ref|ZP_08074365.1| , CRISPR-associated protein, Csn1 family [Methylocystis sp. ATCC 49242]
gi|162147907|ref|YP_001602368.1| , hypothetical protein GDI_2123 [Gluconacetobacter diazotrophicus PAl 5]
gi|209542524|ref|YP_002274753.1| , Csn1 family CRISPR-associated protein [Gluconacetobacter diazotrophicus PAl 5]
gi|182624243|ref|ZP_02952031.1| , crispr-associated protein, Csn1 family [Clostridium perfringens D str. JCS1721]
gi|229113166|ref|ZP_04242662.1| , Crispr-associated protein, Csn1 [Bacillus cereus Rock1-15]
gi|225377804|ref|ZP_03755025.1| , hypothetical protein ROSEINA2194_03455 [Roseburia inulinivorans DSM 16841]
gi|257413184|ref|ZP_04742247.1| , CRISPR-associated protein, Csn1 family [Roseburia intestinalis L1-82]
gi|323142435|ref|ZP_08077256.1| , CRISPR-associated protein, Csn1 family [Phascolarctobacterium succinatutens YIT 12067]
gi|310780184|ref|YP_003968516.1| , CRISPR-associated protein, Csn1 family [Ilyobacter polytropus DSM 2926]
gi|336393381|ref|ZP_08574780.1| , CRISPR-associated protein, Csn1 family [Lactobacillus coryniformis subsp. torquens KCTC 3535...
gi|189485223|ref|YP_001956166.1| , CRISPR-associated protein Csn1 [uncultured Termite group 1 bacterium phylotype Rs-D17]
gi|220930482|ref|YP_002507391.1| , CRISPR-associated protein, Csn1 family [Clostridium cellulolyticum H10]
gi|157415744|ref|YP_001483000.1| , hypothetical protein C8J_1425 [Campylobacter jejuni subsp. jejuni 81116]
gi|218563121|ref|YP_002344900.1| , CRISPR-associated protein [Campylobacter jejuni subsp. jejuni NCTC 11168 = ATCC 700819]
gi|153952471|ref|YP_001398821.1| , CRISPR-associated Cas5e family protein [Campylobacter jejuni subsp. doylei 269.97]
gi|313144862|ref|ZP_07807055.1| , crispr-protein [Helicobacter cinaedi CCUG 18818]
gi|213828136|ref|ZP_04871021.1| , crispr-associated protein, Csn1 family [Helicobacter canadensis MIT 98-5491]
gi|291276265|ref|YP_003516037.1| , CRISPR associated protein [Helicobacter mustelae 12198]
gi|34557790|ref|NP_907605.1| , hypothetical protein WS1445 [Wolinella succinogenes DSM 1740]

FIG. 20A

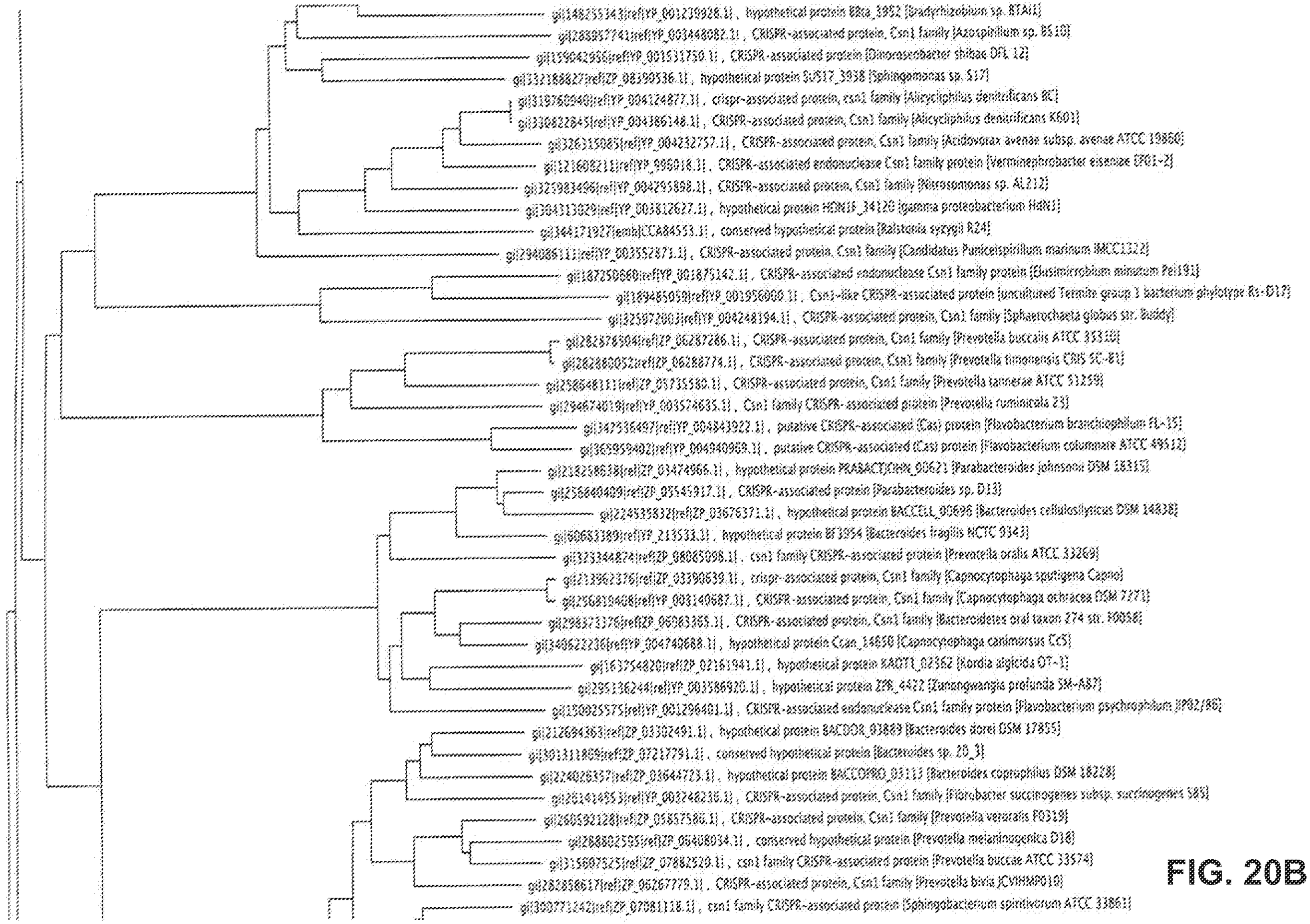

gi|148255143|ref|YP_001239928.1| , hypothetical protein Bbta_3952 [Bradyrhizobium sp. BTAi1]
gi|288957741|ref|YP_003448082.1| , CRISPR-associated protein, Csn1 family [Azospirillum sp. B510]
gi|159042956|ref|YP_001531750.1| , CRISPR-associated protein [Dinoroseobacter shibae DFL 12]
gi|332188827|ref|ZP_08190536.1| , hypothetical protein SUS17_3938 [Sphingomonas sp. S17]
gi|319760940|ref|YP_004124877.1| , crispr-associated protein, csn1 family [Alicyclobacillus denitrificans BC]
gi|330822845|ref|YP_004386148.1| , CRISPR-associated protein, Csn1 family [Alicyclobacillus denitrificans K601]
gi|326315085|ref|YP_004232757.1| , CRISPR-associated protein, Csn1 family [Acidovorax avenae subsp. avenae ATCC 19860]
gi|121608211|ref|YP_996018.1| , CRISPR-associated endonuclease Csn1 family protein [Verminephrobacter eiseniae EF01-2]
gi|322983496|ref|YP_004295898.1| , CRISPR-associated protein, Csn1 family [Nitrosomonas sp. AL212]
gi|304313029|ref|YP_003812627.1| , hypothetical protein HDN1F_34120 [gamma proteobacterium HdN1]
gi|344171927|emb|CCA84553.1| , conserved hypothetical protein [Ralstonia syzygii R24]
gi|294086111|ref|YP_003552871.1| , CRISPR-associated protein, Csn1 family [Candidatus Puniceispirillum marinum IMCC1322]
gi|187250660|ref|YP_001875142.1| , CRISPR-associated endonuclease Csn1 family protein [Elusimicrobium minutum Pei191]
gi|189485019|ref|YP_001956960.1| , Csn1-like CRISPR-associated protein [uncultured Termite group 1 bacterium phylotype Rs-D17]
gi|325972003|ref|YP_004248194.1| , CRISPR-associated protein, Csn1 family [Spherochaeta globus str. Buddy]
gi|282878504|ref|ZP_06287286.1| , CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
gi|282880052|ref|ZP_06288774.1| , CRISPR-associated protein, Csn1 family [Prevotella timonensis CRIS 5C-B1]
gi|258648111|ref|ZP_05735580.1| , CRISPR-associated protein, Csn1 family [Prevotella tannerae ATCC 51259]
gi|294674019|ref|YP_003574635.1| , Csn1 family CRISPR-associated protein [Prevotella ruminicola 23]
gi|342531649|ref|YP_004843922.1| , putative CRISPR-associated (Cas) protein [Flavobacterium branchiophilum FL-15]
gi|363959402|ref|YP_004940969.1| , putative CRISPR-associated (Cas) protein [Flavobacterium columnare ATCC 49512]
gi|218238638|ref|ZP_03474966.1| , hypothetical protein PRABACTJOHN_00621 [Parabacteroides johnsonii DSM 18315]
gi|256660409|ref|ZP_05545917.1| , CRISPR-associated protein [Parabacteroides sp. D13]
gi|224535832|ref|ZP_03676371.1| , hypothetical protein BACCELL_00696 [Bacteroides cellulosilyticus DSM 14838]
gi|60683389|ref|YP_213533.1| , hypothetical protein BF3954 [Bacteroides fragilis NCTC 9343]
gi|323344824|ref|ZP_08085098.1| , csn1 family CRISPR-associated protein [Prevotella oralis ATCC 33269]
gi|213962376|ref|ZP_03390639.1| , crispr-associated protein, Csn1 family [Capnocytophaga sputigena Capno]
gi|256819408|ref|YP_003140687.1| , CRISPR-associated protein, Csn1 family [Capnocytophaga ochracea DSM 7271]
gi|298373376|ref|ZP_06983365.1| , CRISPR-associated protein, Csn1 family [Bacteroidetes oral taxon 274 str. F0058]
gi|340622236|ref|YP_004740688.1| , hypothetical protein Csan_14650 [Capnocytophaga canimorsus Cc5]
gi|163754820|ref|ZP_02161941.1| , hypothetical protein KAOT1_02362 [Kordia algicida OT-1]
gi|295136244|ref|YP_003586920.1| , hypothetical protein ZPR_4422 [Zunongwangia profunda SM-A87]
gi|150025575|ref|YP_001296401.1| , CRISPR-associated endonuclease Csn1 family protein [Flavobacterium psychrophilum JIP02/86]
gi|212694363|ref|ZP_03302491.1| , hypothetical protein BACDOR_03889 [Bacteroides dorei DSM 17855]
gi|301311869|ref|ZP_07217791.1| , conserved hypothetical protein [Bacteroides sp. 20_3]
gi|224026357|ref|ZP_03644723.1| , hypothetical protein BACCOPRO_03113 [Bacteroides coprophilus DSM 18228]
gi|261414553|ref|YP_003248235.1| , CRISPR-associated protein, Csn1 family [Fibrobacter succinogenes subsp. succinogenes S85]
gi|260592128|ref|ZP_05857586.1| , CRISPR-associated protein, Csn1 family [Prevotella veroralis F0319]
gi|288803595|ref|ZP_06408034.1| , conserved hypothetical protein [Prevotella melaninogenica D18]
gi|315607525|ref|ZP_07882529.1| , csn1 family CRISPR-associated protein [Prevotella buccae ATCC 33574]
gi|282838617|ref|ZP_06267779.1| , CRISPR-associated protein, Csn1 family [Prevotella bivia JCVIHMP010]
gi|300771242|ref|ZP_07081118.1| , csn1 family CRISPR-associated protein [Sphingobacterium spiritivorum ATCC 33861]

FIG. 20B

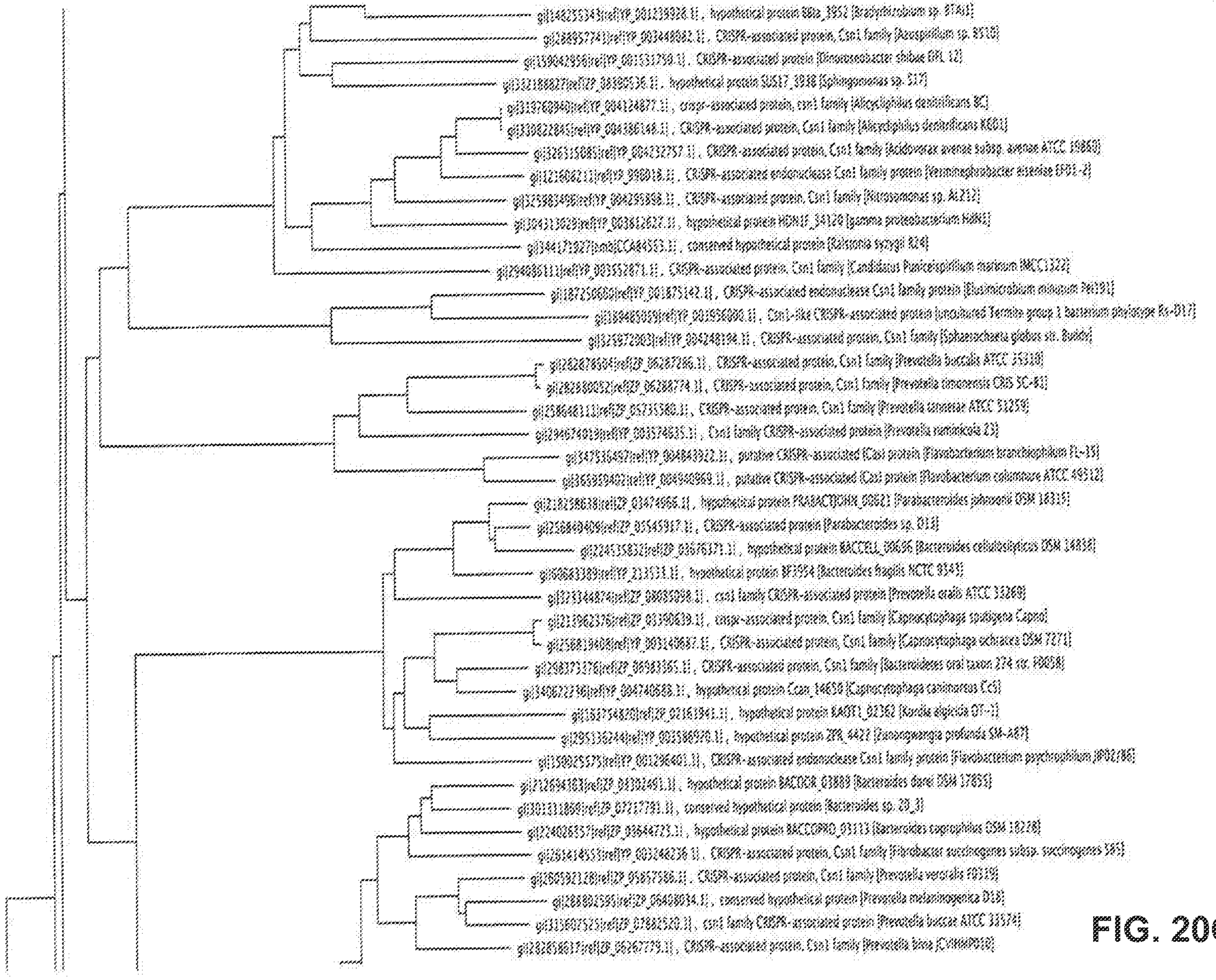

gi|148253343|ref|YP_001239928.1|, hypothetical protein Bbta_3951 [Bradyrhizobium sp. BTAi1]
gi|288957741|ref|YP_003448082.1|, CRISPR-associated protein, Csn1 family [Azospirillum sp. B510]
gi|139042956|ref|YP_001331790.1|, CRISPR-associated protein [Rhodopseudomonas palustris BisA53]
gi|332188827|ref|ZP_08045536.1|, hypothetical protein SJAG_3938 [Sphingomonas sp. A1]
gi|313678940|ref|YP_004124877.1|, cmr3-associated protein, csm1 family [Alkaliphilus oremlandii OhILAs]
gi|330822284|ref|YP_004386148.1|, CRISPR-associated protein, Csn1 family [Alkaliphilus oremlandii KH001]
gi|326515085|ref|YP_004232757.1|, CRISPR-associated protein, Csn1 family [Acidovorax avenae subsp. avenae ATCC 19860]
gi|121604021|ref|YP_998018.1|, CRISPR-associated endonuclease Csn1 family protein [Verminephrobacter eiseniae EF01-2]
gi|325983496|ref|YP_004259968.1|, CRISPR-associated protein, Csn1 family [Nitrosomonas sp. Is79A3]
gi|294139629|ref|YP_003612622.1|, hypothetical protein HDN1F_34120 [gamma proteobacterium HdN1]
gi|344171927|emb|CCA84513.1|, conserved hypothetical protein [Ralstonia syzygii R24]
gi|294086111|ref|YP_003552871.1|, CRISPR-associated protein, Csn1 family [Candidatus Puniceispirillum marinum IMCC1322]
gi|187735600|ref|YP_001875142.1|, CRISPR-associated endonuclease Csn1 family protein [Burkholderia mallei PRL-20]
gi|189485059|ref|YP_001956000.1|, Csn1-like CRISPR-associated protein [uncultured Termite group 1 bacterium phylotype Rs-D17]
gi|312197290|ref|YP_004248194.1|, CRISPR-associated protein, Csn1 family [Ruthia magnifica str. Cpropsm]
gi|282878604|ref|ZP_06287286.1|, CRISPR-associated protein, Csn1 family [Prevotella buccalis ATCC 35310]
gi|282880052|ref|ZP_06288774.1|, CRISPR-associated protein, Csn1 family [Prevotella timonensis CRIS 5C-B1]
gi|238648111|ref|ZP_05375580.1|, CRISPR-associated protein, Csn1 family [Prevotella ruminicola ATCC 51925]
gi|294674019|ref|YP_003574635.1|, Csn1 family CRISPR-associated protein [Prevotella ruminicola 23]
gi|347736492|ref|YP_004843942.1|, putative CRISPR-associated Csn1 protein [Flavobacterium branchiophilum FL-15]
gi|336910402|ref|YP_004950969.1|, putative CRISPR-associated Csn1 protein [Flavobacterium columnare ATCC 49512]
gi|218258838|ref|ZP_03474966.1|, hypothetical protein PRABACTJOHN_00621 [Parabacteroides johnsonii DSM 18315]
gi|256840409|ref|ZP_05545917.1|, CRISPR-associated protein [Parabacteroides sp. D13]
gi|224533832|ref|ZP_03674371.1|, hypothetical protein BACCELL_00636 [Bacteroides cellulosilyticus DSM 14838]
gi|60683189|ref|YP_213333.1|, hypothetical protein BF1994 [Bacteroides fragilis NCTC 9343]
gi|323144874|gb|EZZ25098.1|, csn1 family CRISPR-associated protein [Prevotella oralis ATCC 33269]
gi|311062376|ref|ZP_07190439.1|, cmr3-associated protein, Csn1 family [Capnocytophaga ochracea Capno]
gi|256819408|ref|YP_003140687.1|, CRISPR-associated protein, Csn1 family [Capnocytophaga ochracea DSM 7271]
gi|298373178|ref|ZP_06983165.1|, CRISPR-associated protein, Csn1 family [Bacteroidetes oral taxon 274 str. F0058]
gi|340622298|ref|YP_004740688.1|, hypothetical protein Ccan_14650 [Capnocytophaga canimorsus Cc5]
gi|152754620|ref|ZP_02161941.1|, hypothetical protein BAGM1_02362 [Burkholderia algorifa GT--1]
gi|295133624|ref|YP_003586300.1|, hypothetical protein ZPR_4427 [Zunongwangia profunda SM-A87]
gi|159902527|ref|YP_001236401.1|, CRISPR-associated endonuclease Csn1 family protein [Flavobacterium psychrophilum JIP02/86]
gi|212694183|ref|ZP_03302491.1|, hypothetical protein BACDOR_03889 [Bacteroides dorei DSM 17855]
gi|301311866|ref|ZP_07217781.1|, conserved hypothetical protein [Bacteroides sp. 20_3]
gi|224025837|ref|ZP_03644203.1|, hypothetical protein BACCOPRO_01113 [Bacteroides coprophilus DSM 18228]
gi|258614553|ref|YP_003240236.1|, CRISPR-associated protein, Csn1 family [Flavobacterium succinicans subsp. succinicans 5B5]
gi|260592128|ref|ZP_05857586.1|, CRISPR-associated protein, Csn1 family [Prevotella veroralis F0319]
gi|288801295|ref|ZP_06408034.1|, conserved hypothetical protein [Prevotella melaninogenica D18]
gi|313607525|ref|ZP_07802520.1|, csn1 family CRISPR-associated protein [Prevotella buccae ATCC 33574]
gi|282561617|ref|ZP_06267279.1|, CRISPR-associated protein, Csn1 family [Prevotella bivia JCVIHMP010]

FIG. 20C

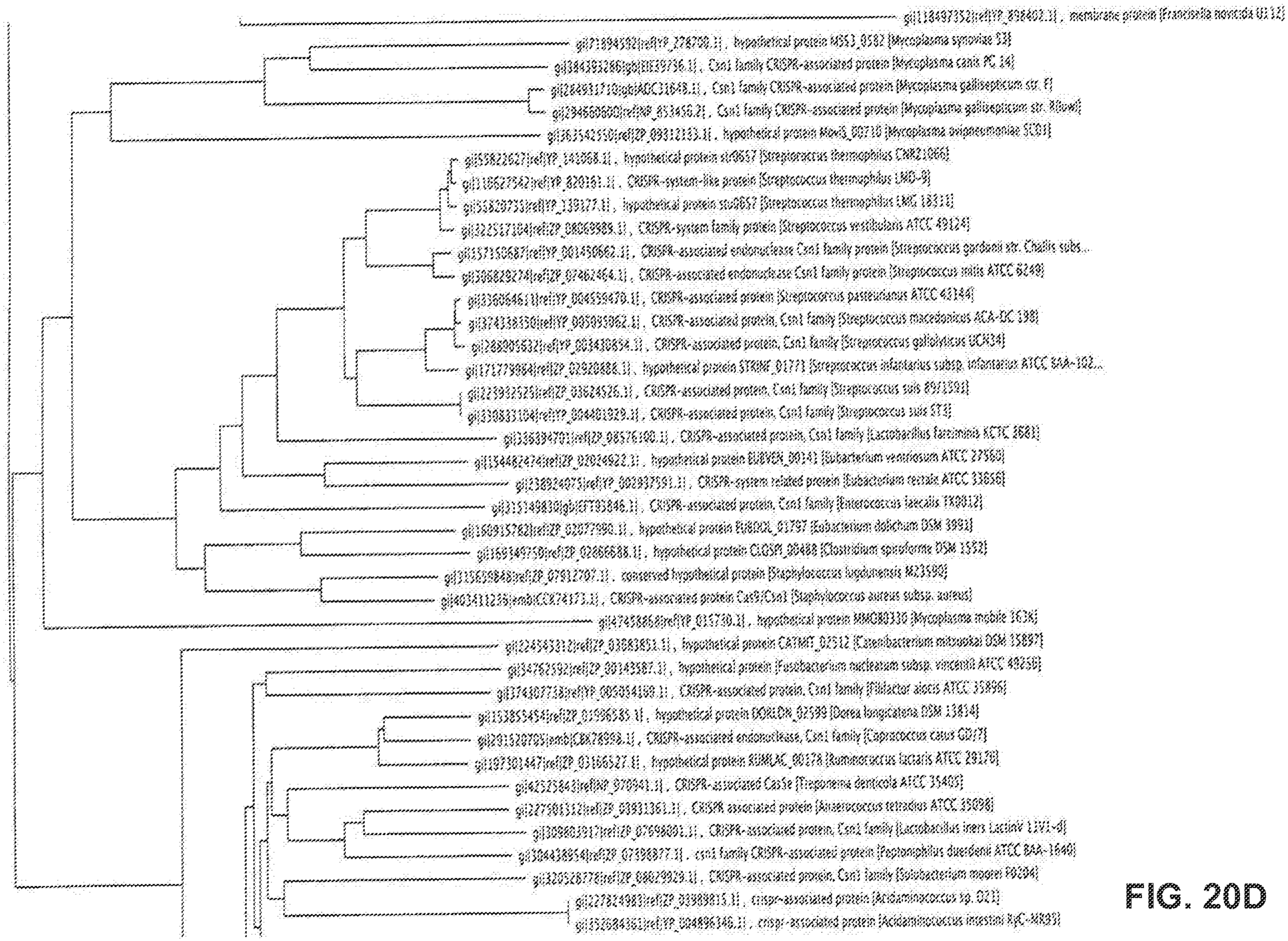

gi|118497352|ref|YP_898402.1| , membrane protein [Francisella novicida U112]
gi|71894593|ref|YP_278700.1| , hypothetical protein MSS3_0582 [Mycoplasma synoviae 53]
gi|384393286|gb|EIE39736.1| , Cas1 family CRISPR-associated protein [Mycoplasma canis PG 14]
gi|284931710|gb|ADC31648.1| , Cas1 family CRISPR-associated protein [Mycoplasma gallisepticum str. F]
gi|294680800|ref|YP_003456.2| , Cas1 family CRISPR-associated protein [Mycoplasma gallisepticum str. R(low)]
gi|363542150|ref|ZP_09312193.1| , hypothetical protein MoviS_00710 [Mycoplasma pneumoniae 5C01]
gi|55822627|ref|YP_141068.1| , hypothetical protein stu0657 [Streptococcus thermophilus CNRZ1066]
gi|116627542|ref|YP_820161.1| , CRISPR-system-like protein [Streptococcus thermophilus LMD-9]
gi|55820733|ref|YP_139177.1| , hypothetical protein stu0657 [Streptococcus thermophilus LMG 18311]
gi|225171841|ref|ZP_08069589.1| , CRISPR-system family protein [Streptococcus vestibularis ATCC 49124]
gi|157150687|ref|YP_001450662.1| , CRISPR-associated endonuclease Cas1 family protein [Streptococcus gordonii str. Challis subs...
gi|306828278|ref|ZP_07462464.1| , CRISPR-associated endonuclease Cas1 family protein [Streptococcus mitis ATCC 6249]
gi|3360646|gb|AAC69470.1| , CRISPR-associated protein [Streptococcus pasteurianus ATCC 43144]
gi|374338350|ref|YP_005093062.1| , CRISPR-associated protein, Cas1 family [Streptococcus macedonicus ACA-DC 198]
gi|288905632|ref|YP_003430854.1| , CRISPR-associated protein, Cas1 family [Streptococcus gallolyticus UCN34]
gi|171779964|ref|ZP_02920886.1| , hypothetical protein STRINF_01771 [Streptococcus infantarius subsp. infantarius ATCC BAA-102...
gi|223932525|ref|ZP_03624526.1| , CRISPR-associated protein, Cas1 family [Streptococcus suis 89/1591]
gi|330833104|ref|YP_004401929.1| , CRISPR-associated protein, Cas1 family [Streptococcus suis ST3]
gi|336394701|ref|ZP_08576100.1| , CRISPR-associated protein, Cas1 family [Lactobacillus farciminis KCTC 3681]
gi|154482474|ref|ZP_02024922.1| , hypothetical protein EUBVEN_00141 [Eubacterium ventriosum ATCC 27560]
gi|238924075|ref|YP_002937591.1| , CRISPR-system related protein [Eubacterium rectale ATCC 33656]
gi|315149830|gb|EFT93846.1| , CRISPR-associated protein, Cas1 family [Enterococcus faecalis TX0012]
gi|160915782|ref|ZP_02077990.1| , hypothetical protein EUBDOL_01797 [Eubacterium dolichum DSM 3991]
gi|169349758|ref|ZP_02866638.1| , hypothetical protein CLOSPI_00488 [Clostridium spiroforme DSM 1552]
gi|119659848|ref|ZP_07912797.1| , conserved hypothetical protein [Staphylococcus lugdunensis M23590]
gi|401411236|emb|CCK74173.1| , CRISPR-associated protein Cas9/Csn1 [Staphylococcus aureus subsp. aureus]
gi|47458686|ref|YP_015730.1| , hypothetical protein MMCB0330 [Mycoplasma mobile 163K]
gi|224543213|ref|ZP_03683852.1| , hypothetical protein CATMIT_02512 [Catenibacterium mitsuokai DSM 15897]
gi|347623592|ref|ZP_08143587.1| , hypothetical protein [Fusobacterium nucleatum subsp. vincentii ATCC 49256]
gi|374307738|ref|YP_005054169.1| , CRISPR-associated protein, Cas1 family [Filifactor alocis ATCC 35896]
gi|153855454|ref|ZP_01996585.1| , hypothetical protein DORLON_02599 [Dorea longicatena DSM 13814]
gi|291520705|emb|CBK78998.1| , CRISPR-associated endonuclease, Cas1 family [Coprococcus catus GD/7]
gi|187301447|ref|ZP_03166527.1| , hypothetical protein RUMLAC_00178 [Ruminococcus lactaris ATCC 29176]
gi|42525844|ref|NP_970041.1| , CRISPR-associated Cas5e [Treponema denticola ATCC 35405]
gi|227981312|ref|ZP_03931361.1| , CRISPR associated protein [Anaerococcus tetradius ATCC 35098]
gi|330961917|ref|ZP_07596801.1| , CRISPR-associated protein, Cas1 family [Lactobacillus iners LactinV 11V1-d]
gi|304438954|ref|ZP_07198877.1| , cas1 family CRISPR-associated protein [Peptoniphilus duerdenii ATCC BAA-1640]
gi|320526778|ref|ZP_08029929.1| , CRISPR-associated protein, Cas1 family [Solobacterium moorei F0204]
gi|227824983|ref|ZP_03989815.1| , crispr-associated protein [Acidaminococcus sp. D21]
gi|152684361|ref|YP_004896146.1| , crispr-associated protein [Acidaminococcus intestini RyC-MR95]

FIG. 20D

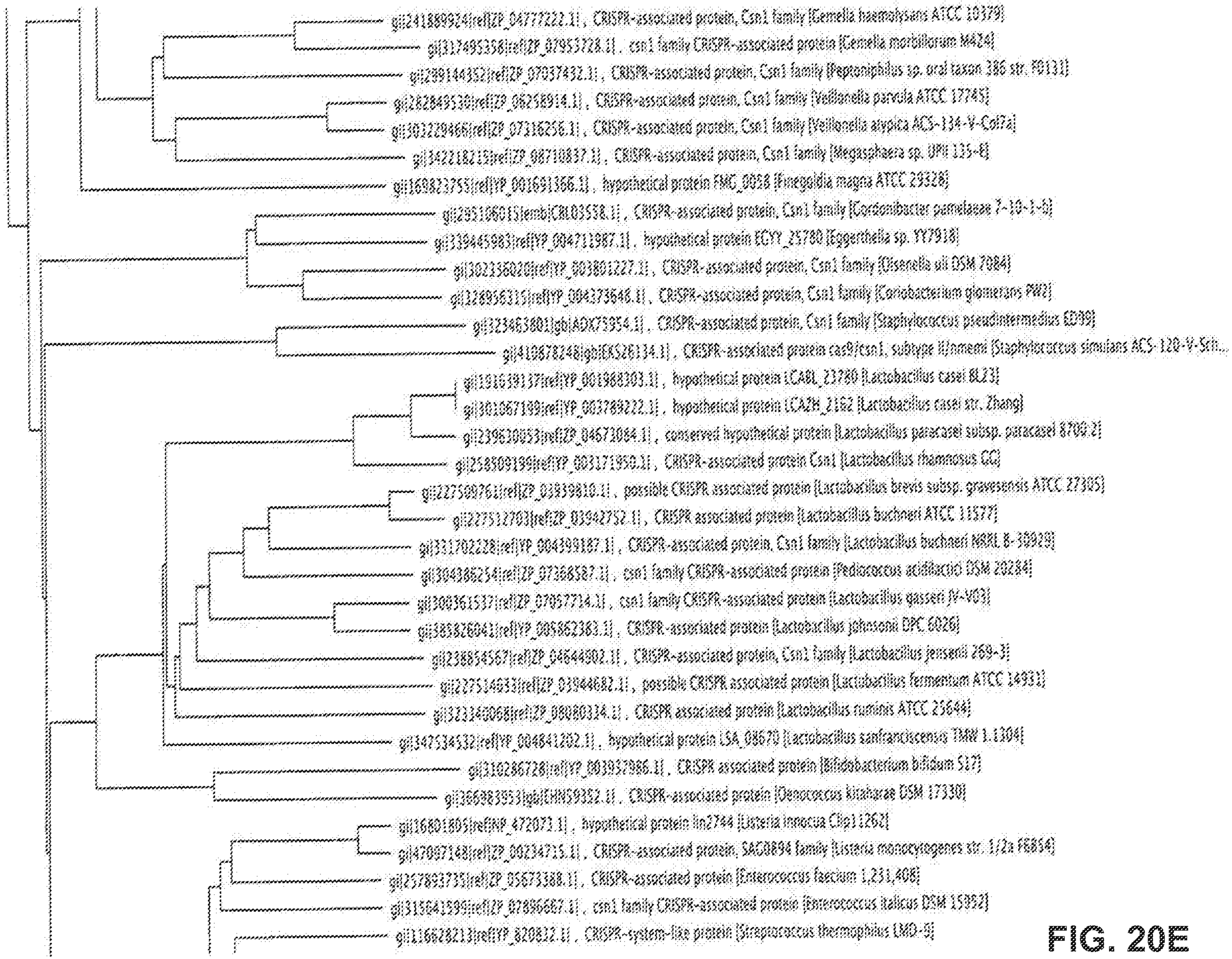

gi|241889924|ref|ZP_04777222.1| , CRISPR-associated protein, Csn1 family [Gemella haemolysans ATCC 10379]
gi|317495356|ref|ZP_07953728.1| , csn1 family CRISPR-associated protein [Gemella morbillorum M424]
gi|289144352|ref|ZP_07037432.1| , CRISPR-associated protein, Csn1 family [Peptoniphilus sp. oral taxon 386 str. F0131]
gi|282849530|ref|ZP_06258914.1| , CRISPR-associated protein, Csn1 family [Veillonella parvula ATCC 17745]
gi|302329466|ref|ZP_07316256.1| , CRISPR-associated protein, Csn1 family [Veillonella atypica ACS-134-V-Col7a]
gi|342218219|ref|ZP_08710837.1| , CRISPR-associated protein, Csn1 family [Megasphaera sp. UPII 135-E]
gi|169823755|ref|YP_001691366.1| , hypothetical protein FMG_0058 [Finegoldia magna ATCC 29328]
gi|295106015|emb|CBL03558.1| , CRISPR-associated protein, Csn1 family [Gordonibacter pamelaeae 7-10-1-b]
gi|239945983|ref|YP_004711987.1| , hypothetical protein EGYY_25780 [Eggerthella sp. YY7918]
gi|302358020|ref|YP_003801227.1| , CRISPR-associated protein, Csn1 family [Olsenella uli DSM 7084]
gi|328956315|ref|YP_004373646.1| , CRISPR-associated protein, Csn1 family [Coriobacterium glomerans PW2]
gi|323463601|gb|ADX75954.1| , CRISPR-associated protein, Csn1 family [Staphylococcus pseudintermedius ED99]
gi|410678248|gb|EKS26134.1| , CRISPR-associated protein cas9/csn1, subtype II/nmeni [Staphylococcus simulans ACS-120-V-Sch...
gi|191639137|ref|YP_001988303.1| , hypothetical protein LCABL_23780 [Lactobacillus casei BL23]
gi|301067199|ref|YP_003798222.1| , hypothetical protein LCAZH_2162 [Lactobacillus casei str. Zhang]
gi|239630053|ref|ZP_04673084.1| , conserved hypothetical protein [Lactobacillus paracasei subsp. paracasei 8700:2]
gi|258509199|ref|YP_003171950.1| , CRISPR-associated protein Csn1 [Lactobacillus rhamnosus GG]
gi|227509761|ref|ZP_03839810.1| , possible CRISPR associated protein [Lactobacillus brevis subsp. gravesensis ATCC 27305]
gi|227512703|ref|ZP_03942752.1| , CRISPR associated protein [Lactobacillus buchneri ATCC 11577]
gi|331702228|ref|YP_004399187.1| , CRISPR-associated protein, Csn1 family [Lactobacillus buchneri NRRL B-30929]
gi|304386254|ref|ZP_07368587.1| , csn1 family CRISPR-associated protein [Pediococcus acidilactici DSM 20284]
gi|300361537|ref|ZP_07057714.1| , csn1 family CRISPR-associated protein [Lactobacillus gasseri JV-V03]
gi|385826041|ref|YP_005862383.1| , CRISPR-associated protein [Lactobacillus johnsonii DPC 6026]
gi|238854567|ref|ZP_04644902.1| , CRISPR-associated protein, Csn1 family [Lactobacillus jensenii 269-3]
gi|227514833|ref|ZP_03944682.1| , possible CRISPR associated protein [Lactobacillus fermentum ATCC 14931]
gi|323340068|ref|ZP_08080314.1| , CRISPR associated protein [Lactobacillus ruminis ATCC 25644]
gi|347534332|ref|YP_004841202.1| , hypothetical protein LSA_08670 [Lactobacillus sanfranciscensis TMW 1.1304]
gi|310286728|ref|YP_003937986.1| , CRISPR associated protein [Bifidobacterium bifidum S17]
gi|366983553|gb|EHN59352.1| , CRISPR-associated protein [Dehalococcoides mccartyi DSM 17330]
gi|16801805|ref|NP_472073.1| , hypothetical protein lin2744 [Listeria innocua Clip11262]
gi|47097148|ref|ZP_00234715.1| , CRISPR-associated protein, SAG0894 family [Listeria monocytogenes str. 1/2a F6854]
gi|257893735|ref|ZP_05673388.1| , CRISPR-associated protein [Enterococcus faecium 1,231,408]
gi|315041399|ref|ZP_07886687.1| , csn1 family CRISPR-associated protein [Enterococcus italicus DSM 15952]
gi|116628213|ref|YP_820832.1| , CRISPR-system-like protein [Streptococcus thermophilus LMD-9]

FIG. 20E

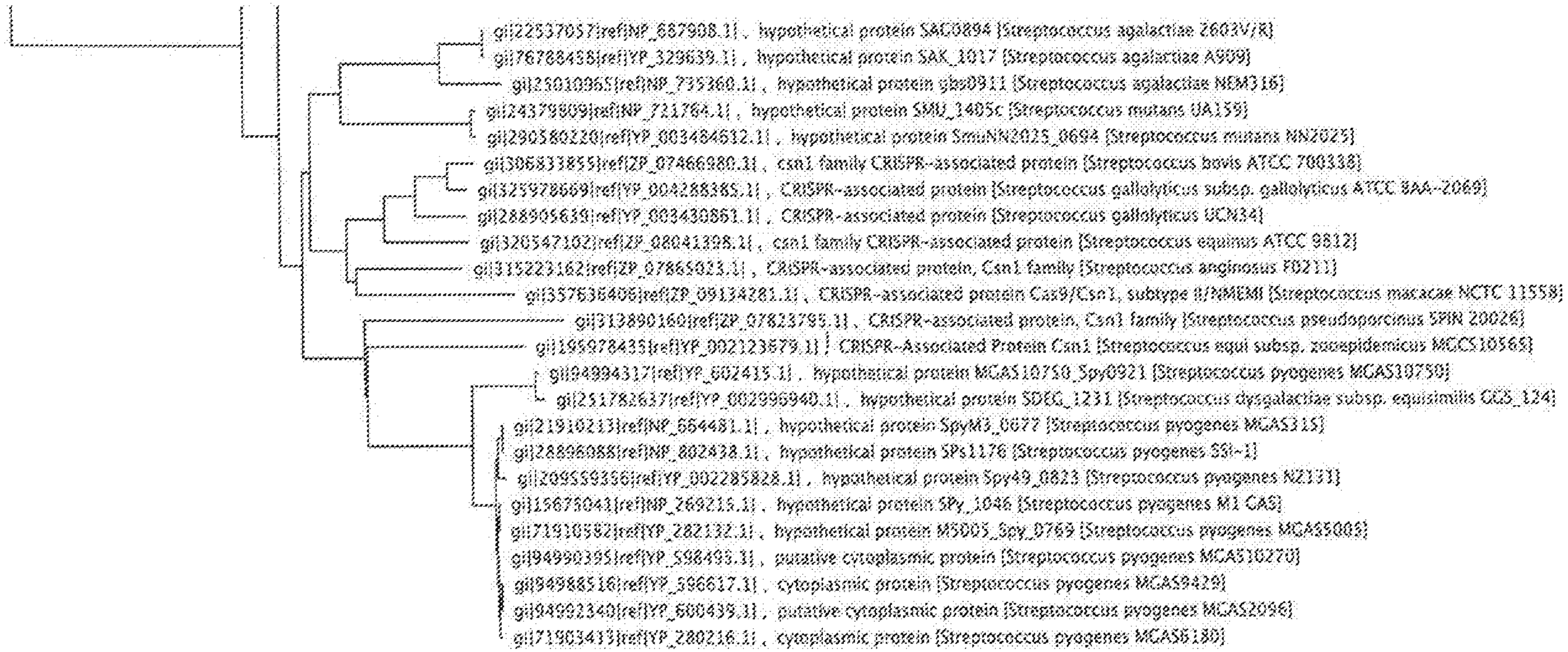

gi|22537057|ref|NP_687908.1| , hypothetical protein SAG0894 [Streptococcus agalactiae 2603V/R]
gi|76788438|ref|YP_329639.1| , hypothetical protein SAK_1017 [Streptococcus agalactiae A909]
gi|25010065|ref|NP_735260.1| , hypothetical protein gbs0911 [Streptococcus agalactiae NEM316]
gi|24379809|ref|NP_721764.1| , hypothetical protein SMU_1405c [Streptococcus mutans UA159]
gi|290580220|ref|YP_003484612.1| , hypothetical protein SmuNN2025_0694 [Streptococcus mutans NN2025]
gi|306833855|ref|ZP_07466980.1| , csn1 family CRISPR-associated protein [Streptococcus bovis ATCC 700338]
gi|325978669|ref|YP_004288385.1| , CRISPR-associated protein [Streptococcus gallolyticus subsp. gallolyticus ATCC BAA-2069]
gi|288905639|ref|YP_003430861.1| , CRISPR-associated protein [Streptococcus gallolyticus UCN34]
gi|320547102|ref|ZP_08041398.1| , csn1 family CRISPR-associated protein [Streptococcus equinus ATCC 9812]
gi|315223162|ref|ZP_07865023.1| , CRISPR-associated protein, Csn1 family [Streptococcus anginosus F0211]
gi|357636406|ref|ZP_09134281.1| , CRISPR-associated protein Cas9/Csn1, subtype II/NMEMI [Streptococcus macacae NCTC 11558]
gi|313890160|ref|ZP_07823795.1| , CRISPR-associated protein, Csn1 family [Streptococcus pseudoporcinus SPIN 20026]
gi|199978435|ref|YP_002123679.1| , CRISPR-Associated Protein Csn1 [Streptococcus equi subsp. zooepidemicus MGCS10565]
gi|94994317|ref|YP_602415.1| , hypothetical protein MGAS10750_Spy0921 [Streptococcus pyogenes MGAS10750]
gi|251782637|ref|YP_002996940.1| , hypothetical protein SDEG_1231 [Streptococcus dysgalactiae subsp. equisimilis GGS_124]
gi|21910213|ref|NP_664481.1| , hypothetical protein SpyM3_0677 [Streptococcus pyogenes MGAS315]
gi|28896088|ref|NP_802438.1| , hypothetical protein SPs1176 [Streptococcus pyogenes SSI-1]
gi|209559336|ref|YP_002285828.1| , hypothetical protein Spy49_0823 [Streptococcus pyogenes NZ131]
gi|15675041|ref|NP_269215.1| , hypothetical protein SPy_1046 [Streptococcus pyogenes M1 GAS]
gi|71910582|ref|YP_282132.1| , hypothetical protein M5005_Spy_0769 [Streptococcus pyogenes MGAS5005]
gi|94990395|ref|YP_598495.1| , putative cytoplasmic protein [Streptococcus pyogenes MGAS10270]
gi|94988516|ref|YP_596617.1| , cytoplasmic protein [Streptococcus pyogenes MGAS9429]
gi|94992340|ref|YP_600439.1| , putative cytoplasmic protein [Streptococcus pyogenes MGAS2096]
gi|71903413|ref|YP_280216.1| , cytoplasmic protein [Streptococcus pyogenes MGAS6180]

FIG. 20F

a

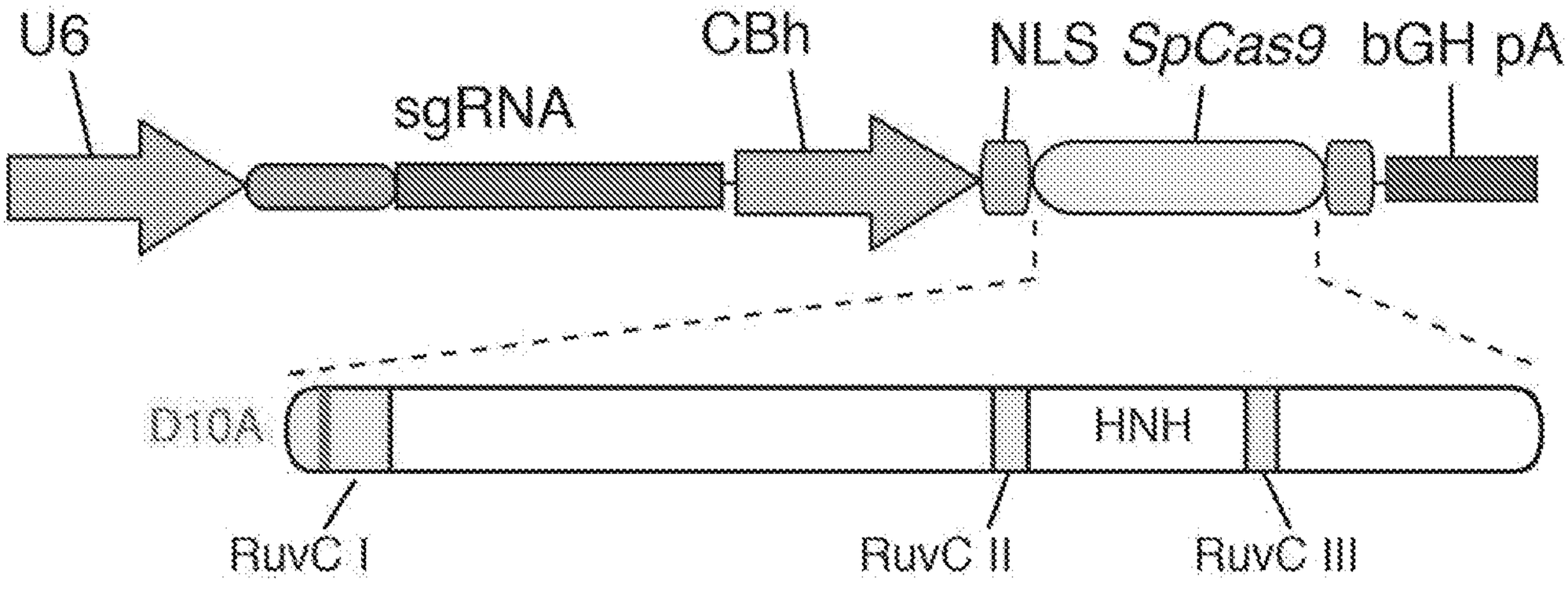


FIG. 21A

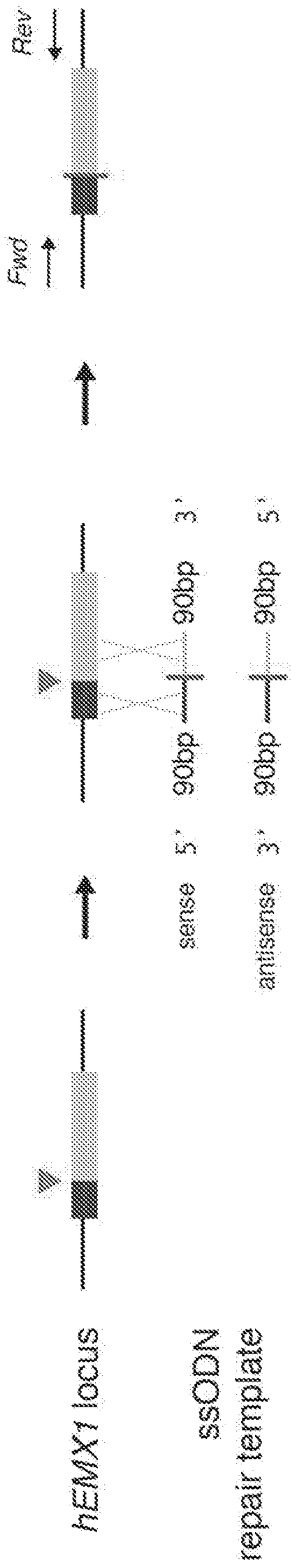

Rev
Fwd
90bp
3'
90bp
90bp
5'
90bp
HindIII, NheI
5'
sense
3'
antisense
b
hEMX1 locus
ssODN
repair template

FIG. 21B

C

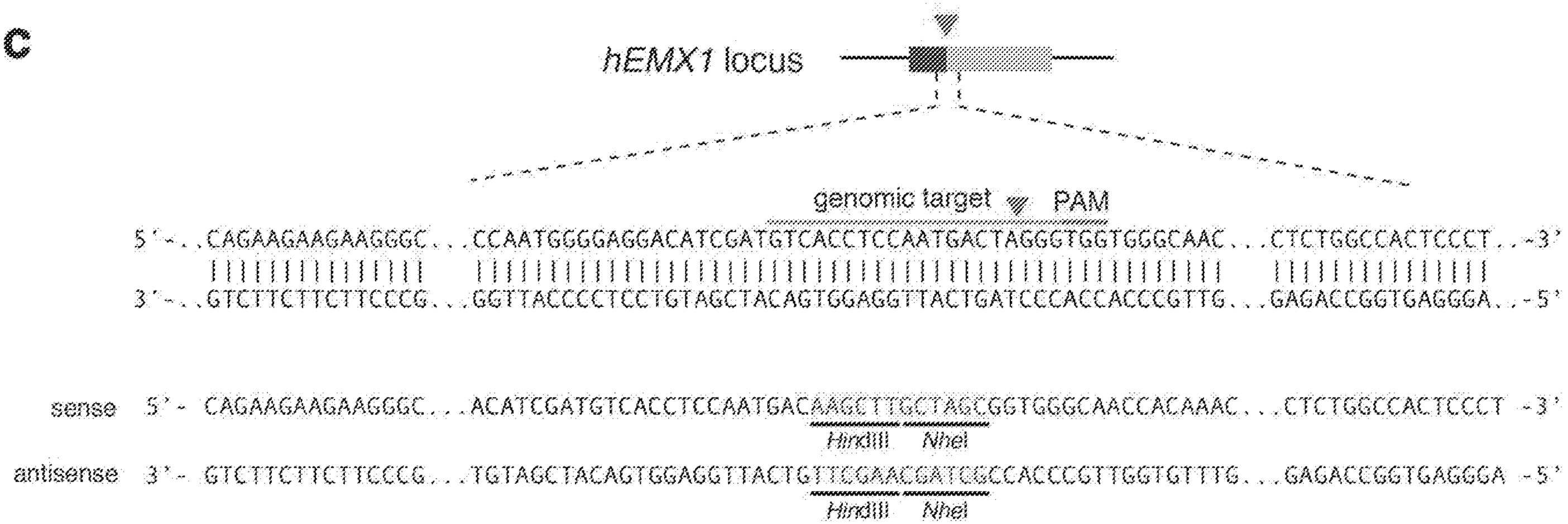

hEMX1 locus
genomic target
PAM
5'-..CAGAAGAAGAAGGGC...CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGTGGGCAAC...CTCTGGCCACTCCCT..-3'
3'-..GTCTTCTTCTTCCCG...GGTTACCCCTCCTGTAGCTACAGTGGAGGTTACTGATCCCACCACCCGTTG...GAGACCGGTGAGGGA..-5'
sense 5'- CAGAAGAAGAAGGGC...ACATCGATGTCACCTCCAATGACAAGCTTGCTAGCGGTGGGCAACCACAAAC...CTCTGGCCACTCCCT -3'
HindIII NheI
antisense 3'- GTCTTCTTCTTCCCG...TGTAGCTACAGTGGAGGTTACTGTTCGAACGATCGCCACCCGTTGGTGTTTG...GAGACCGGTGAGGGA -5'
HindIII NheI

FIG. 21C

d

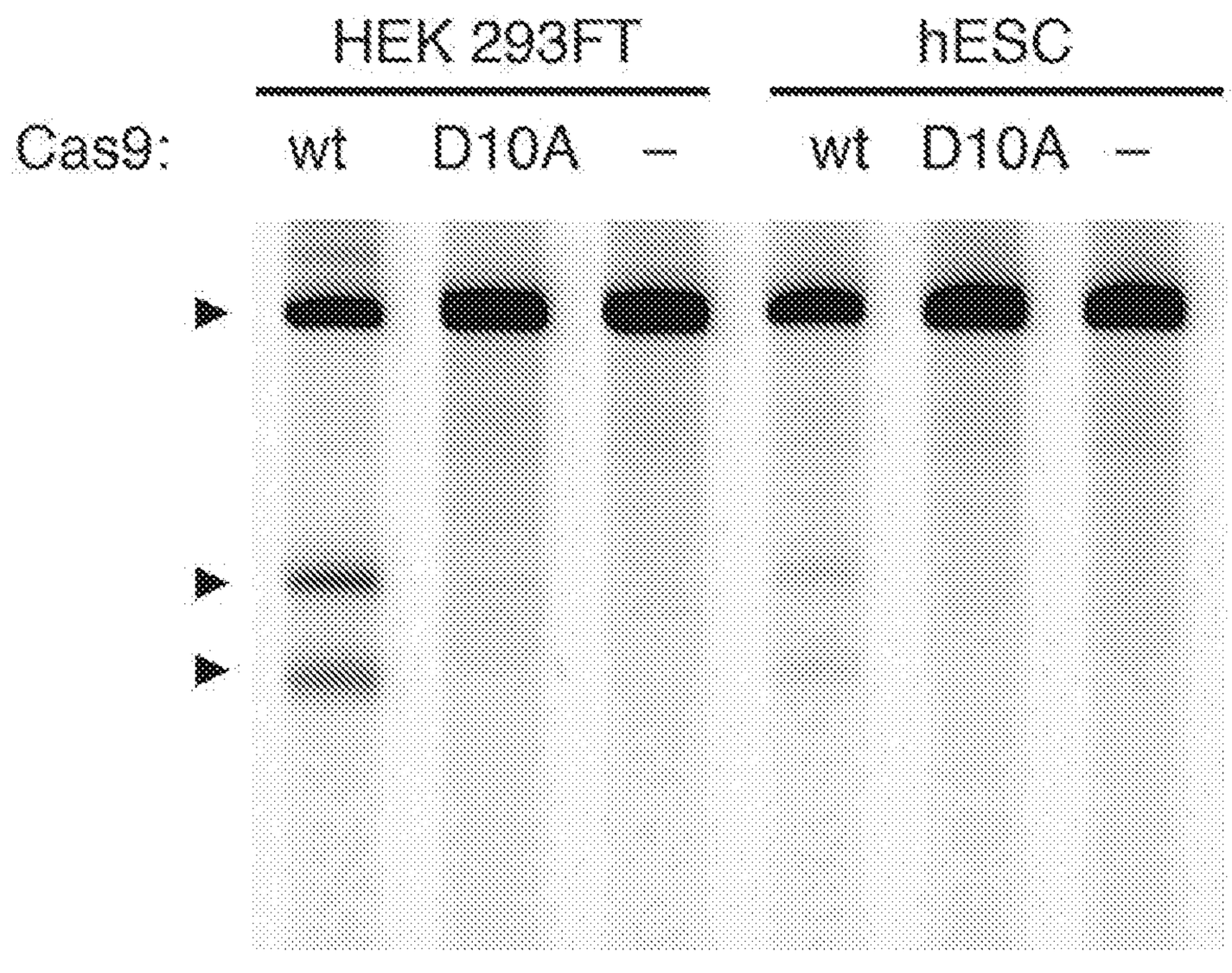


FIG. 21D

a

guide oligo (top) 5'- CACCnnnnnnnnnnnnnnnnnnn -3'

(bottom) 3'- nnnnnnnnnnnnnnnnnnnCAAA -5'

guide oligo insert

PX330

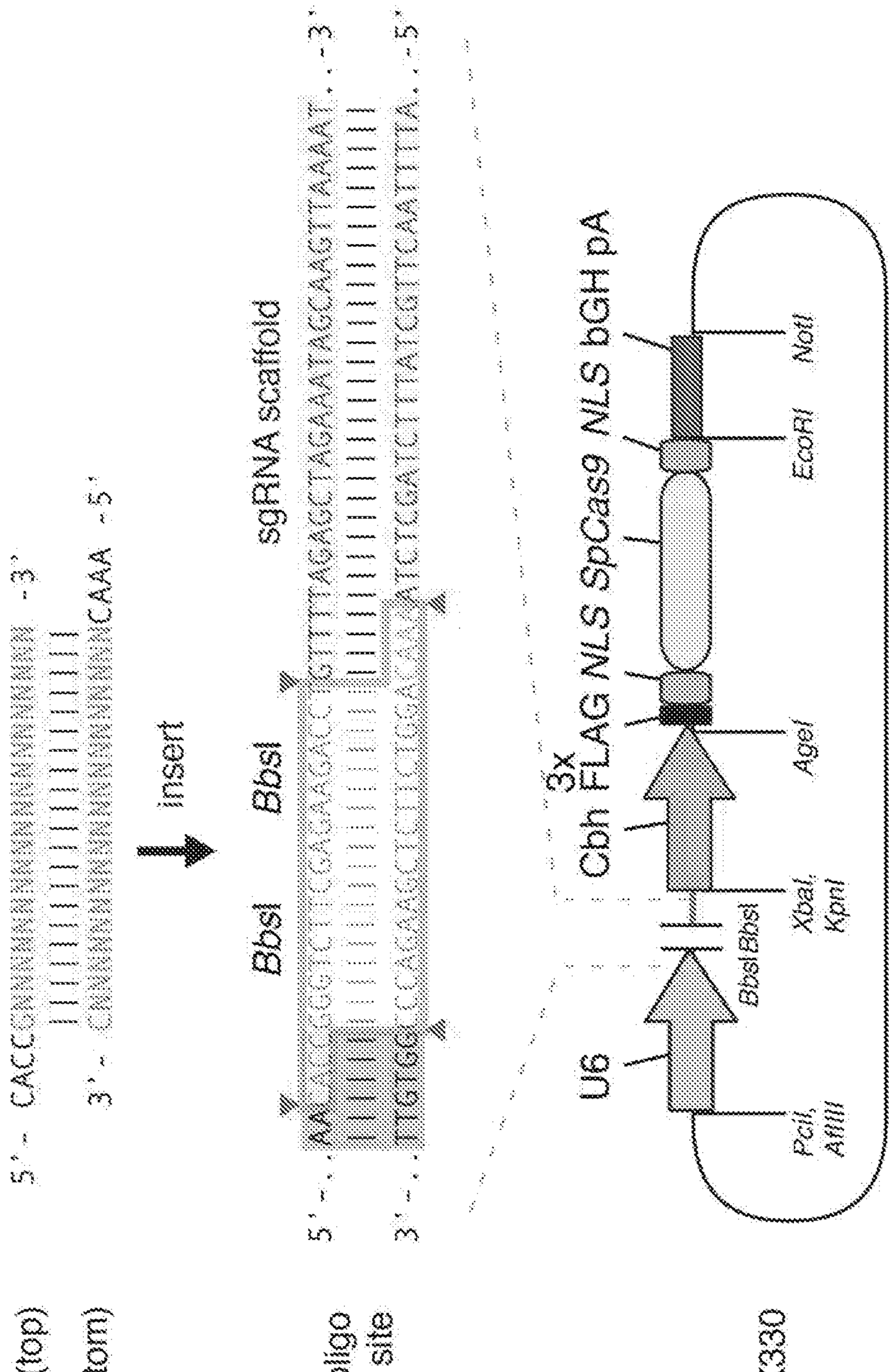


FIG. 22A

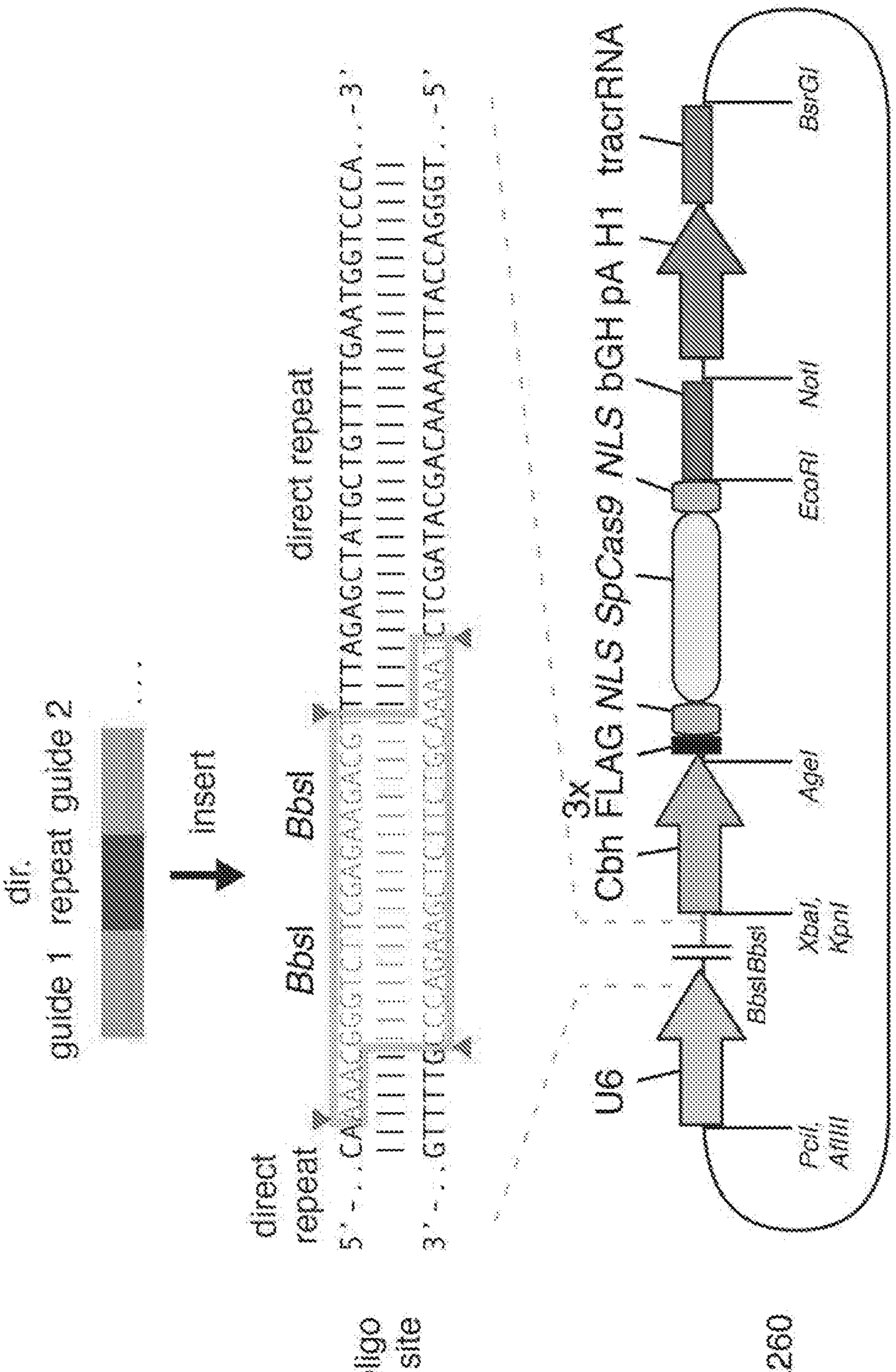

PX260 guide oligo insertion site
5' - . . CACC G N20 GTTTTAGAGCTATGCTGTTTTGAATGGTCCCA . . - 3'
3' - . . CN20 CAAAATCTCGATACGACAAAACTTACCAGGGT . . - 5'
direct repeat
Bbsl
Bbsl
dir. repeat
guide 1
guide 2
insert
U6
Cbh
3x FLAG
NLS SpCas9 NLS
bGH pA
H1
tracrRNA
BsrGI
EcoRI
NotI
BbslBbsl
XbaI, KpnI
AgeI
PciI, AflIII

FIG. 22B

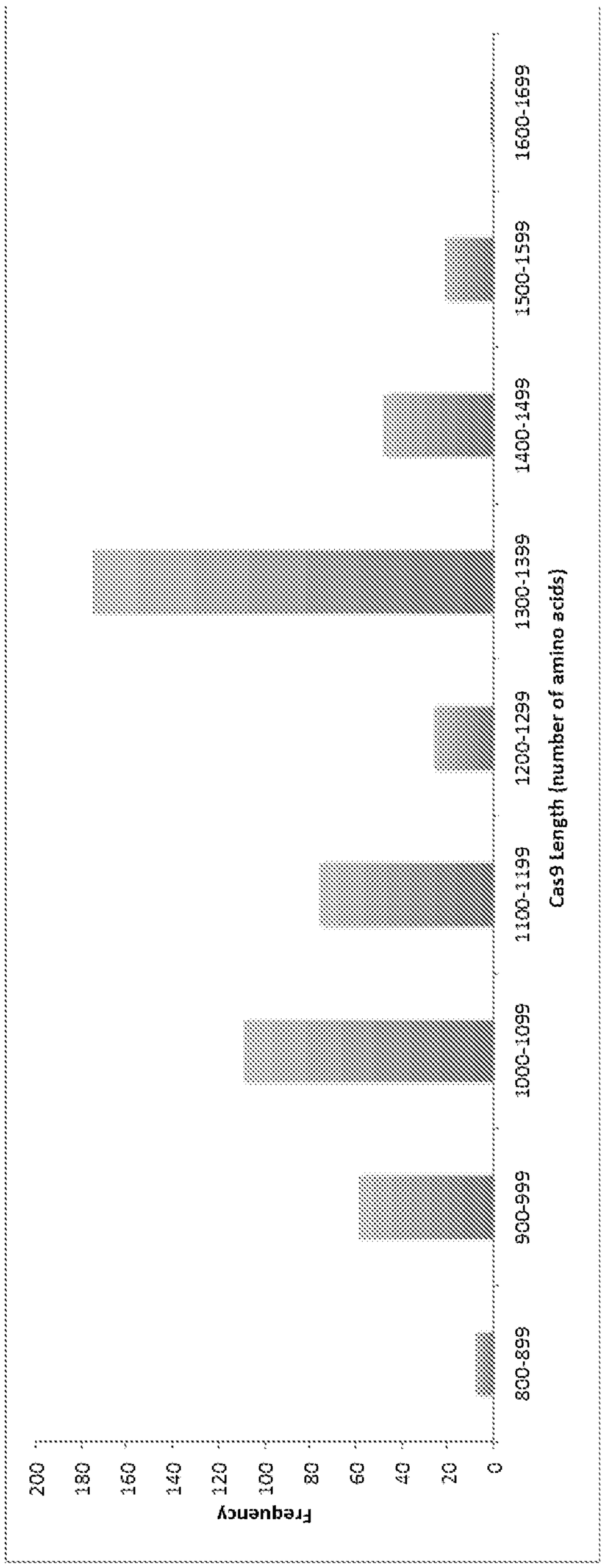

Frequency
200
180
160
140
120
100
80
60
40
20
0
Cas9 Length (number of amino acids)
800-899
900-999
1000-1099
1100-1199
1200-1299
1300-1399
1400-1499
1500-1599
1600-1699

FIG. 23

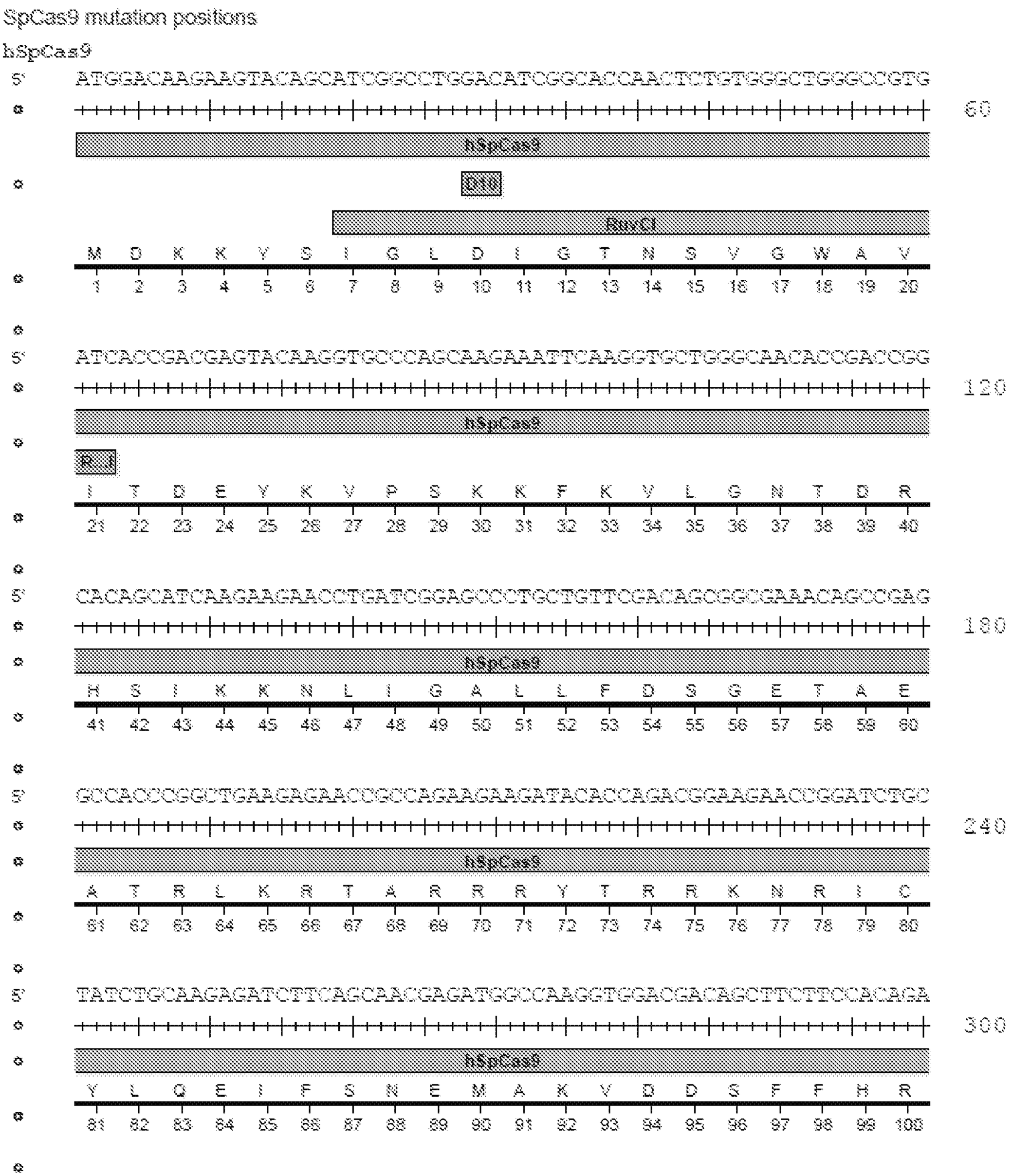

SpCas9 mutation positions
hSpCas9
5'
ATGGACAAGAAGTACAGCATCGGCCTGGACATCGGCACCAACTCTGTGGGCTGGGCCGTG
60
hSpCas9
D10
RuvC
M D K K Y S I G L D I G T N S V G W A V
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
5'
ATCACCGACGAGTACAAGGTGCCCAGCAAGAAATTCAAGGTGCTGGGCAACACCGACCGG
120
hSpCas9
R 1
I T D E Y K V P S K K F K V L G N T D R
21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
5'
CACAGCATCAAGAAGAACCTGATCGGAGCCCTGCTGTTCGACAGCGGCGAAACAGCCGAG
180
hSpCas9
H S I K K N L I G A L L F D S G E T A E
41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60
5'
GCCACCCGGCTGAAGAGAACCGCCAGAAGAAGATACACCAGACGGAAGAACCGGATCTGC
240
hSpCas9
A T R L K R T A R R R Y T R R K N R I C
61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80
5'
TATCTGCAAGAGATCTTCAGCAACGAGATGGCCAAGGTGGACGACAGCTTCTTCCACAGA
300
hSpCas9
Y L Q E I F S N E M A K V D D S F F H R
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

FIG. 24A

hSpCas9
5'
CTGGAAGAGTCCTTCCTGGTGGAAGAGGATAAGAAGCACGAGCGGCACCCCATCTTCGGC
360
hSpCas9
L E E S F L V E E D K K H E R H P I F G
101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120
5'
AACATCGTGGACGAGGTGGCCTACCACGAGAAGTACCCCACCATCTACCACCTGAGAAAG
420
hSpCas9
N I V D E V A Y H E K Y P T I Y H L R K
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140
5'
AAACTGGTGGACAGCACCGACAAGGCCGACCTGCGGCTGATCTATCTGGCCCTGGCCCAC
480
hSpCas9
K L V D S T D K A D L R L I Y L A L A H
141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160
5'
ATGATCAAGTTCCGGGGCCACTTCCTGATCGAGGGCGACCTGAACCCCGACAACAGCGAC
540
hSpCas9
M I K F R G H F L I E G D L N P D N S D
161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
5'
GTGGACAAGCTGTTCATCCAGCTGGTGCAGACCTACAACCAGCTGTTCGAGGAAAACCCC
600
hSpCas9
V D K L F I Q L V Q T Y N Q L F E E N P
181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200
5'
ATCAACGCCAGCGGCGTGGACGCCAAGGCCATCCTGTCTGCCAGACTGAGCAAGAGCAGA
660
hSpCas9
I N A S G V D A K A I L S A R L S K S R
201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220

FIG. 24B

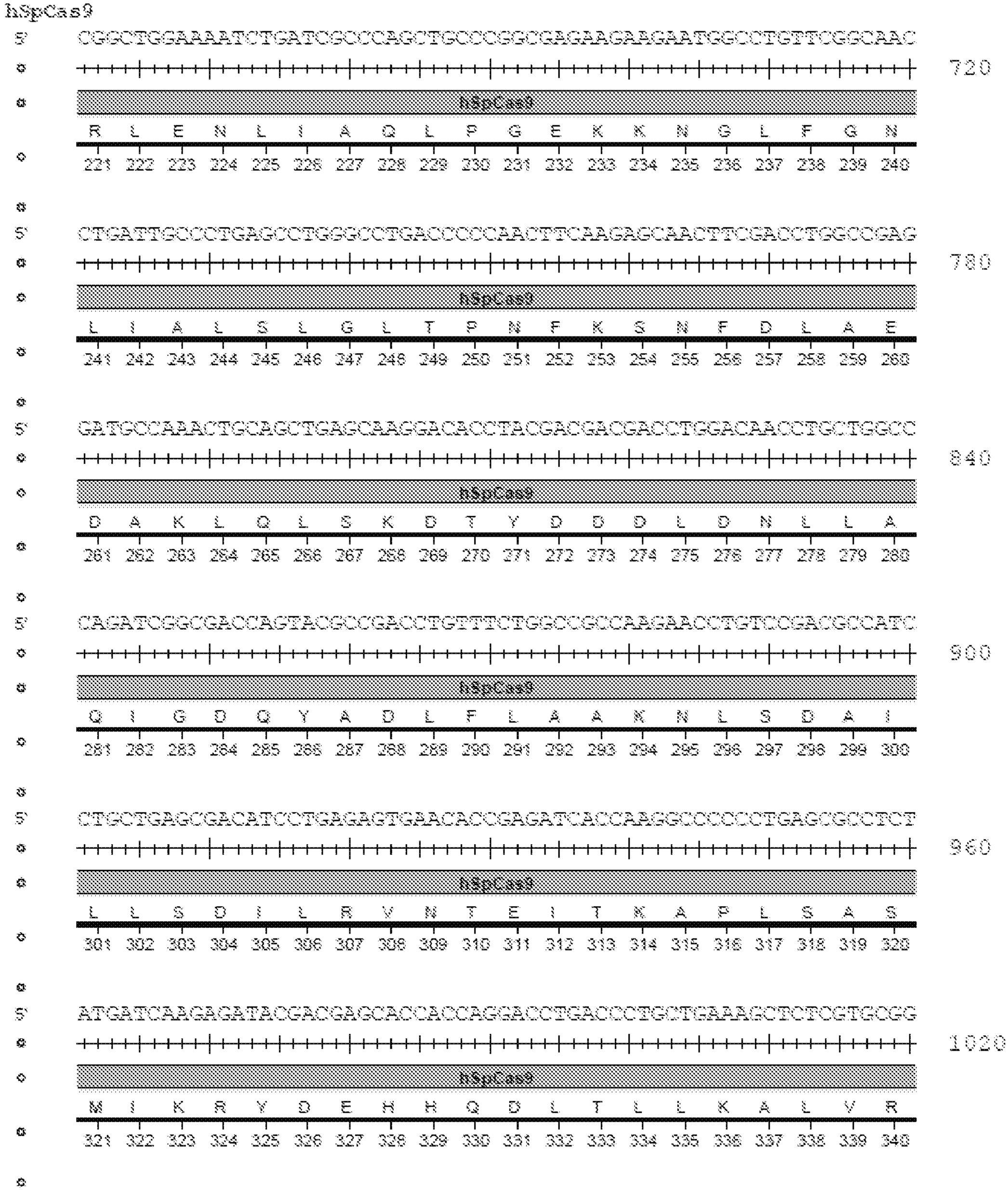

hSpCas9
5'
CGGCTGGAAAATCTGATCGCCCAGCTGCCCGGCGAGAAGAAGAATGGCCTGTTCGGCAAC
720
hSpCas9
R L E N L I A Q L P G E K K N G L F G N
221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240
5'
CTGATTGCCCTGAGCCTGGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAG
780
hSpCas9
L I A L S L G L T P N F K S N F D L A E
241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260
5'
GATGCCAAACTGCAGCTGAGCAAGGACACCTACGACGACGACCTGGACAACCTGCTGGCC
840
hSpCas9
D A K L Q L S K D T Y D D D L D N L L A
261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280
5'
CAGATCGGCGACCAGTACGCCGACCTGTTTCTGGCCGCCAAGAACCTGTCCGACGCCATC
900
hSpCas9
Q I G D Q Y A D L F L A A K N L S D A I
281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300
5'
CTGCTGAGCGACATCCTGAGAGTGAACACCGAGATCACCAAGGCCCCCCTGAGCGCCTCT
960
hSpCas9
L L S D I L R V N T E I T K A P L S A S
301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320
5'
ATGATCAAGAGATACGACGAGCACCACCAGGACCTGACCCTGCTGAAAGCTCTCGTGCGG
1020
hSpCas9
M I K R Y D E H H Q D L T L L K A L V R
321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340

FIG. 24C

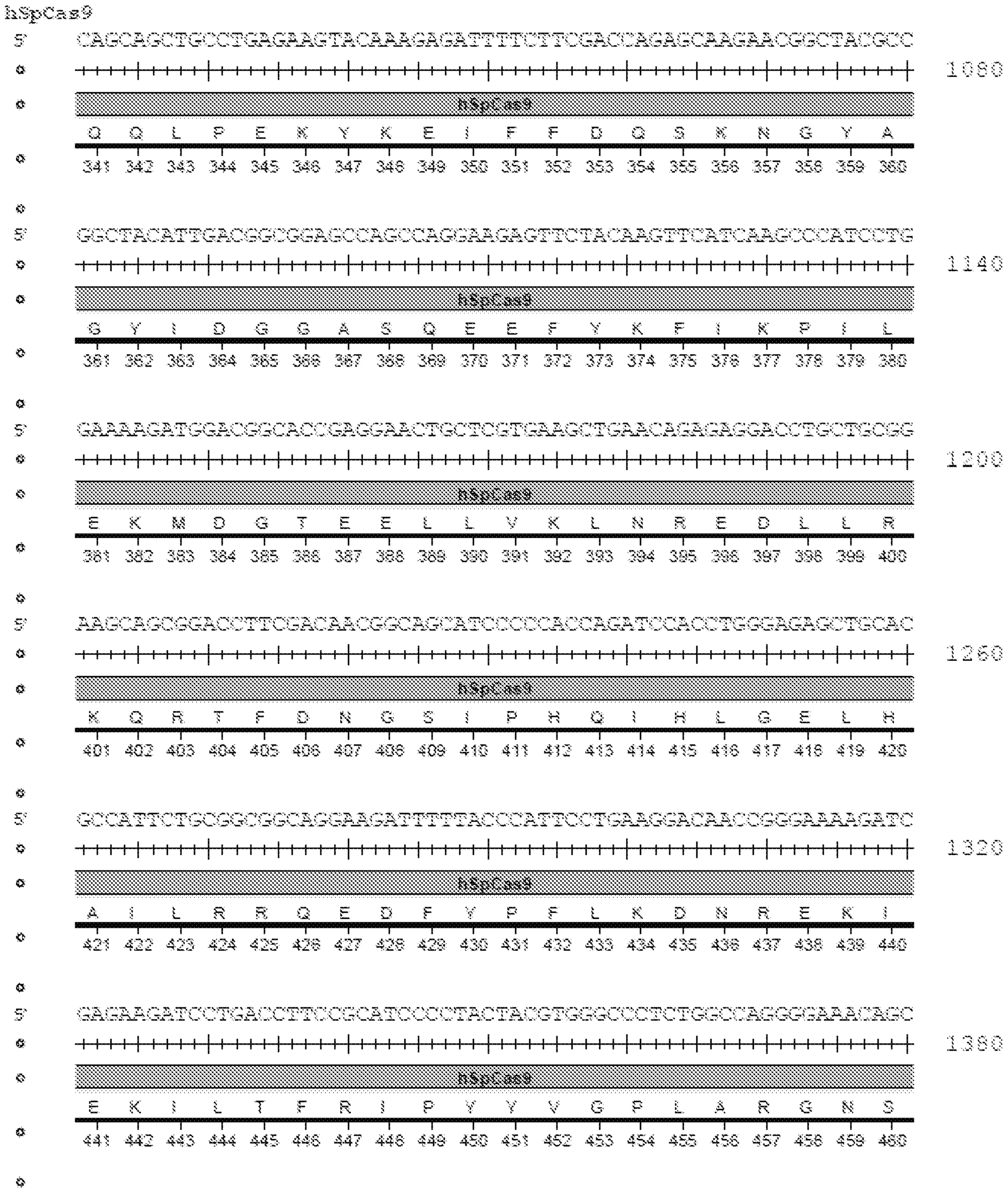

hSpCas9
5'
CAGCAGCTGCCTGAGAAGTACAAAGAGATTTTCTTCGACCAGAGCAAGAACGGCTACGCC
1080
hSpCas9
Q Q L P E K Y K E I F F D Q S K N G Y A
341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360
5'
GGCTACATTGACGGCGGAGCCAGCCAGGAAGAGTTCTACAAGTTCATCAAGCCCATCCTG
1140
hSpCas9
G Y I D G G A S Q E E F Y K F I K P I L
361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380
5'
GAAAAGATGGACGGCACCGAGGAACTGCTCGTGAAGCTGAACAGAGAGGACCTGCTGCGG
1200
hSpCas9
E K M D G T E E L L V K L N R E D L L R
381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400
5'
AAGCAGCGGACCTTCGACAACGGCAGCATCCCCCACCAGATCCACCTGGGAGAGCTGCAC
1260
hSpCas9
K Q R T F D N G S I P H Q I H L G E L H
401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420
5'
GCCATTCTGCGGCGGCAGGAAGATTTTTACCCATTCCTGAAGGACAACCGGGAAAAGATC
1320
hSpCas9
A I L R R Q E D F Y P F L K D N R E K I
421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440
5'
GAGAAGATCCTGACCTTCCGCATCCCCTACTACGTGGGCCCTCTGGCCAGGGGAAACAGC
1380
hSpCas9
E K I L T F R I P Y Y V G P L A R G N S
441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460

FIG. 24D

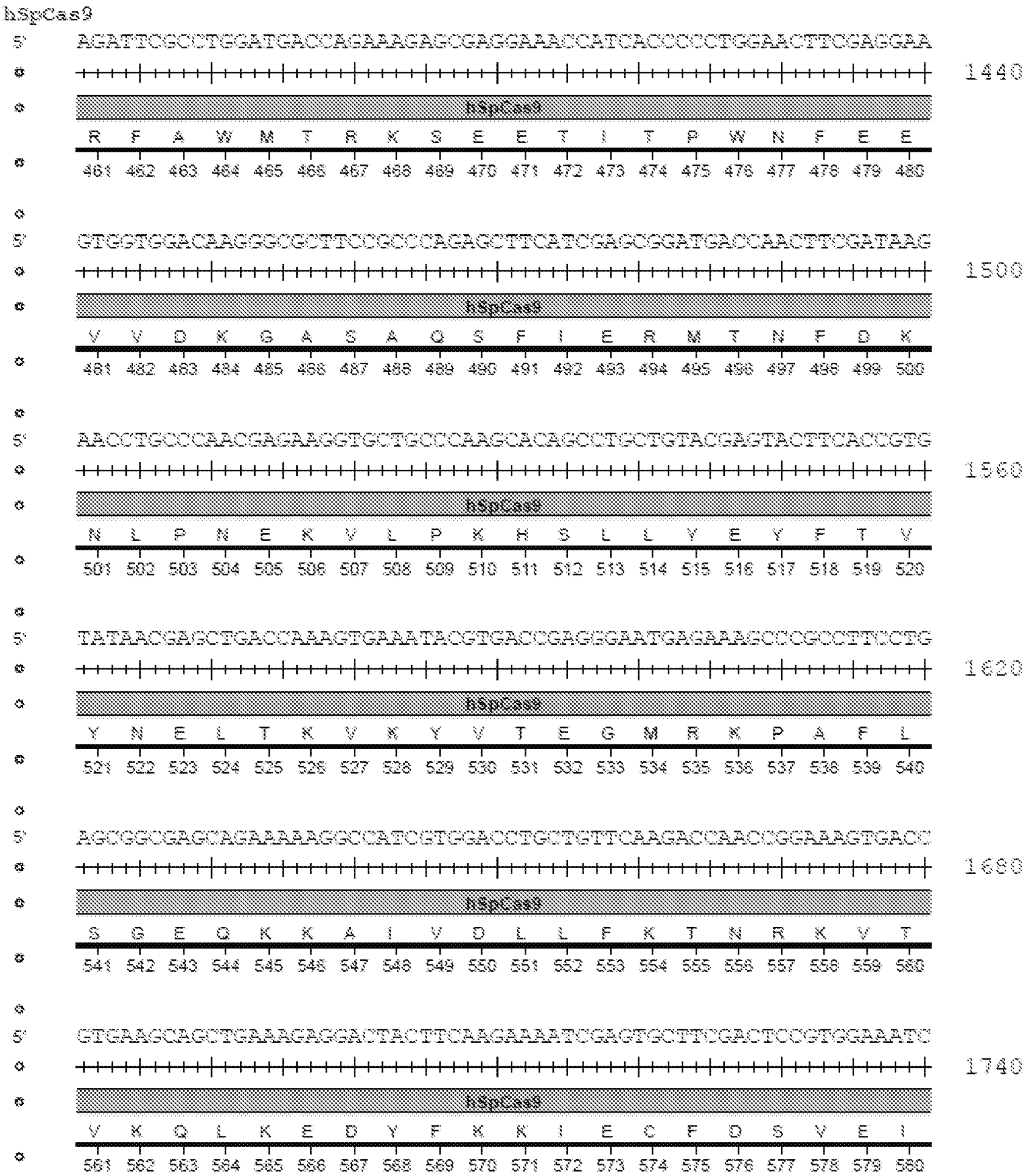

hSpCas9
5'
AGATTCGCCTGGATGACCAGAAAGAGCGAGGAAACCATCACCCCCTGGAACTTCGAGGAA
1440
hSpCas9
R F A W M T R K S E E T I T P W N F E E
461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480
5'
GTGGTGGACAAGGGCGCTTCCGCCCAGAGCTTCATCGAGCGGATGACCAACTTCGATAAG
1500
hSpCas9
V V D K G A S A Q S F I E R M T N F D K
481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500
5'
AACCTGCCCAACGAGAAGGTGCTGCCCAAGCACAGCCTGCTGTACGAGTACTTCACCGTG
1560
hSpCas9
N L P N E K V L P K H S L L Y E Y F T V
501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520
5'
TATAACGAGCTGACCAAAGTGAAATACGTGACCGAGGGAATGAGAAAGCCCGCCTTTCTG
1620
hSpCas9
Y N E L T K V K Y V T E G M R K P A F L
521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540
5'
AGCGGCGAGCAGAAAAAGGCCATCGTGGACCTGCTGTTCAAGACCAACCGGAAAGTGACC
1680
hSpCas9
S G E Q K K A I V D L L F K T N R K V T
541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560
5'
GTGAAGCAGCTGAAAGAGGACTACTTCAAGAAAATCGAGTGCTTCGACTCCGTGGAAATC
1740
hSpCas9
V K Q L K E D Y F K K I E C F D S V E I
561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580

FIG. 24E

hSpCas9
5'
TCCGGCGTGGAAGATCGGTTCAACGCCTCCCTGGGCACATACCACGATCTGCTGAAAATT
1800
hSpCas9
S G V E D R F N A S L G T Y H D L L K I
581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600
5'
ATCAAGGACAAGGACTTCCTGGACAATGAGGAAAACGAGGACATTCTGGAAGATATCGTG
1860
hSpCas9
I K D K D F L D N E E N E D I L E D I V
601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620
5'
CTGACCCTGACACTGTTTGAGGACAGAGAGATGATCGAGGAACGGCTGAAAACCTATGCC
1920
hSpCas9
L T L T L F E D R E M I E E R L K T Y A
621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640
5'
CACCTGTTCGACGACAAAGTGATGAAGCAGCTGAAGCGGCGGAGATACACCGGCTGGGGC
1980
hSpCas9
H L F D D K V M K Q L K R R R Y T G W G
641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660
5'
AGGCTGAGCCGGAAGCTGATCAACGGCATCCGGGACAAGCAGTCCGGCAAGACAATCCTG
2040
hSpCas9
R L S R K L I N G I R D K Q S G K T I L
661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680
5'
GATTTCCTGAAGTCCGACGGCTTCGCCAACAGAAACTTCATGCAGCTGATCCACGACGAC
2100
hSpCas9
D F L K S D G F A N R N F M Q L I H D D
681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700

FIG. 24F

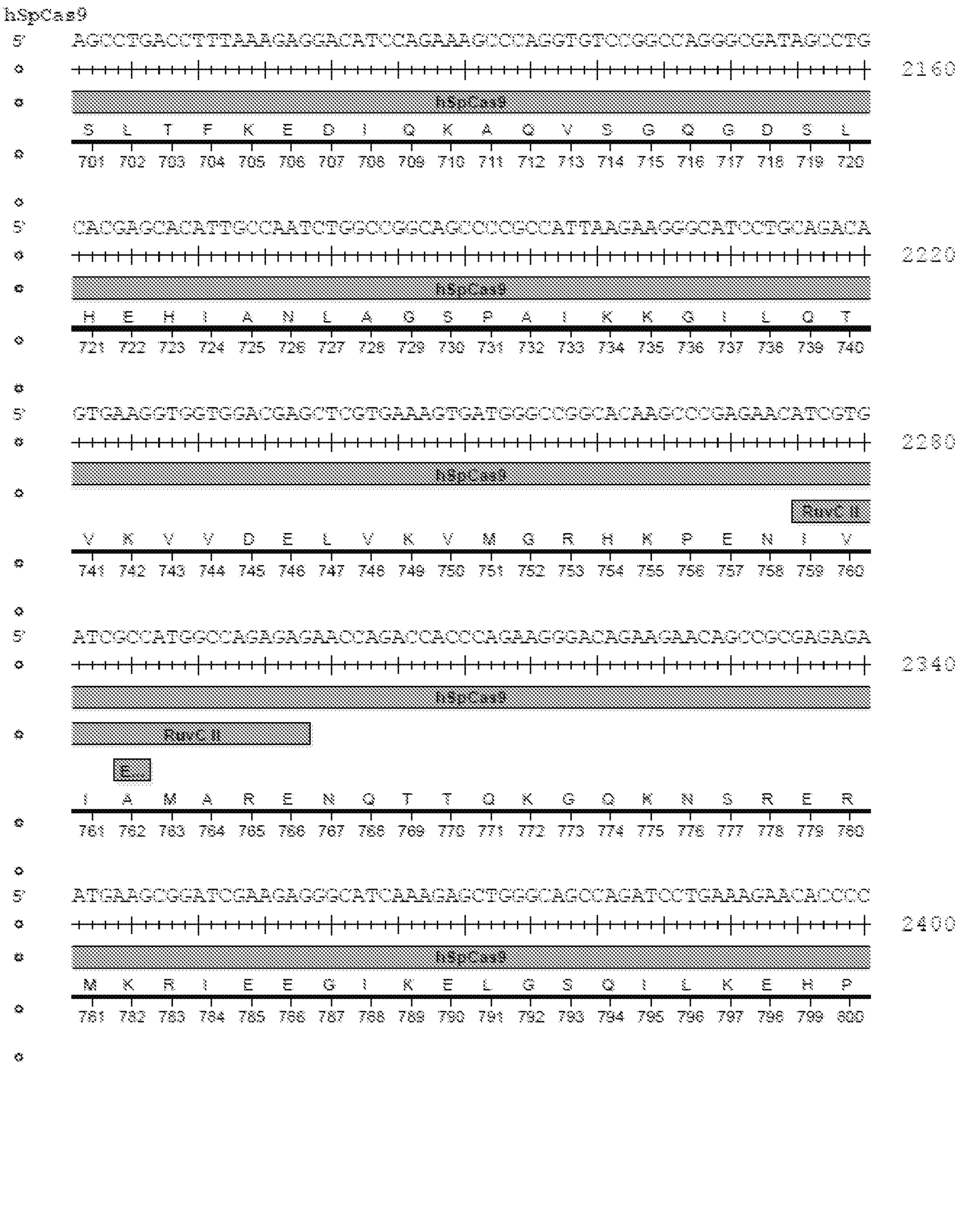

hSpCas9
5'
2160
hSpCas9
S L T F K E D I Q K A Q V S G Q G D S L
701 702 703 704 705 706 707 706 709 710 711 712 713 714 715 716 717 718 719 720
2220
hSpCas9
H E H I A N L A G S P A I K K G I L Q T
721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740
GTGAAGGTGGTGGACGAGCTCGTGAAAGTGATGGGCCGGCACAAGCCCGAGAACATCGTG
2280
hSpCas9
RuvC II
V K V V D E L V K V M G R H K P E N I V
741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760
ATCGCCATGGCCAGAGAGAACCAGACCACCCAGAAGGGACAGAAGAACAGCCGCGAGAGA
2340
hSpCas9
RuvC II
I A M A R E N Q T T Q K G Q K N S R E R
761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780
ATGAAGCGGATCGAAGAGGGCATCAAAGAGCTGGGCAGCCAGATCCTGAAAGAACACCCC
2400
hSpCas9
M K R I E E G I K E L G S Q I L K E H P
781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800

FIG. 24G

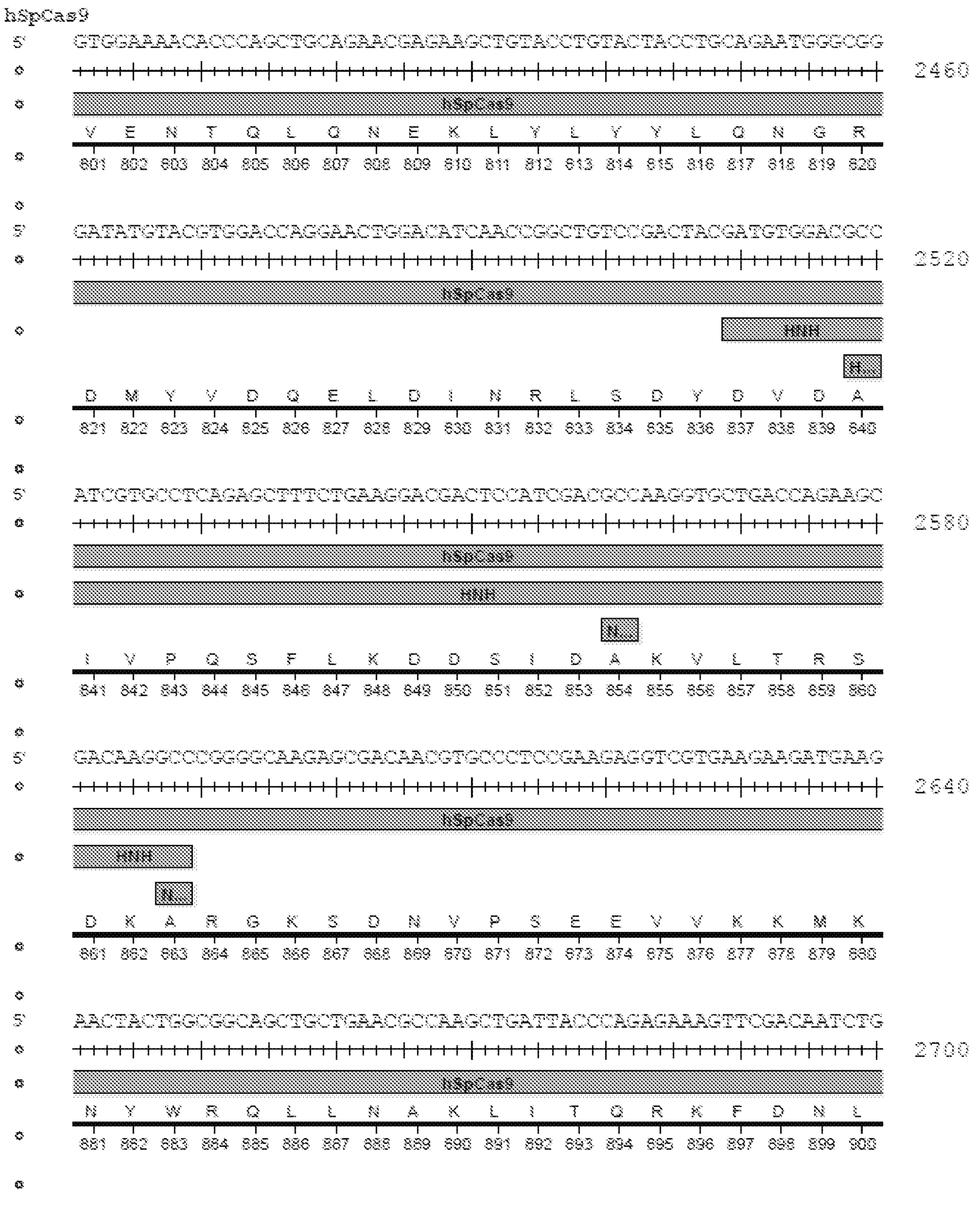

hSpCas9
5'
GTGGAAAACACCCAGCTGCAGAACGAGAAGCTGTACCTGTACTACCTGCAGAATGGGCGG
2460
hSpCas9
V E N T Q L Q N E K L Y L Y Y L Q N G R
801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820
5'
GATATGTACGTGGACCAGGAACTGGACATCAACCGGCTGTCCGACTACGATGTGGACGCC
2520
hSpCas9
HNH
H...
D M Y V D Q E L D I N R L S D Y D V D A
821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840
5'
ATCGTGCCTCAGAGCTTTCTGAAGGACGACTCCATCGACGCCAAGGTGCTGACCAGAAGC
2580
hSpCas9
HNH
N...
I V P Q S F L K D D S I D A K V L T R S
841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860
5'
GACAAGGCCCGGGGCAAGAGCGACAACGTGCCCTCCGAAGAGGTCGTGAAGAAGATGAAG
2640
hSpCas9
HNH
N...
D K A R G K S D N V P S E E V V K K M K
861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880
5'
AACTACTGGCGGCAGCTGCTGAACGCCAAGCTGATTACCCAGAGAAAGTTCGACAATCTG
2700
hSpCas9
N Y W R Q L L N A K L I T Q R K F D N L
881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900

FIG. 24H

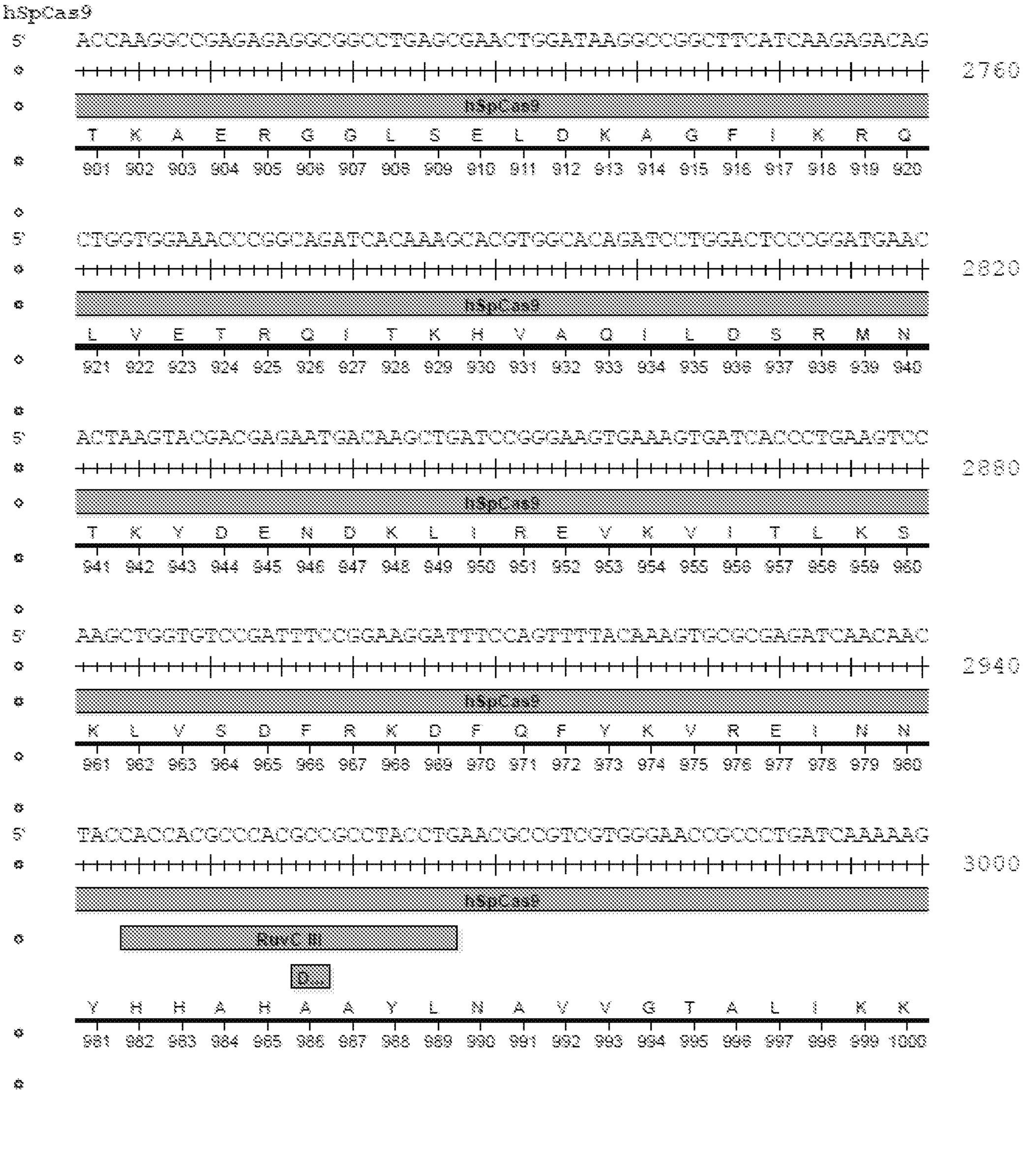

hSpCas9
5'
ACCAAGGCCGAGAGAGGCGGCCTGAGCGAACTGGATAAGGCCGGCTTCATCAAGAGACAG
2760
hSpCas9
T K A E R G G L S E L D K A G F I K R Q
901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920
5'
CTGGTGGAAACCCGGCAGATCACAAAGCACGTGGCACAGATCCTGGACTCCCGGATGAAC
2820
hSpCas9
L V E T R Q I T K H V A Q I L D S R M N
921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940
5'
ACTAAGTACGACGAGAATGACAAGCTGATCCGGGAAGTGAAAGTGATCACCCTGAAGTCC
2880
hSpCas9
T K Y D E N D K L I R E V K V I T L K S
941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960
5'
AAGCTGGTGTCCGATTTCCGGAAGGATTTCCAGTTTTACAAAGTGCGCGAGATCAACAAC
2940
hSpCas9
K L V S D F R K D F Q F Y K V R E I N N
961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980
5'
TACCACCACGCCCACGCCGCCTACCTGAACGCCGTCGTGGGAACCGCCCTGATCAAAAAG
3000
hSpCas9
RuvC NI
D...
Y H H A H A A Y L N A V V G T A L I K K
981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000

FIG. 24I

hSpCas9
5'
TACCCTAAGCTGGAAAGCGAGTTCGTGTACGGCGACTACAAGGTGTACGACGTGCGGAAG
3060
hSpCas9
Y P K L E S E F V Y G D Y K V Y D V R K
1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020
ATGATCGCCAAGAGCGAGCAGGAAATCGGCAAGGCTACCGCCAAGTACTTCTTCTACAGC
3120
hSpCas9
M I A K S E Q E I G K A T A K Y F F Y S
1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040
AACATCATGAACTTTTTCAAGACCGAGATTACCCTGGCCAACGGCGAGATCCGGAAGCGG
3180
hSpCas9
N I M N F F K T E I T L A N G E I R K R
1041 1042 1043 1044 1045 1046 1047 1048 1049 1050 1051 1052 1053 1054 1055 1056 1057 1058 1059 1060
CCTCTGATCGAGACAAACGGCGAAACCGGGGAGATCGTGTGGGATAAGGGCCGGGATTTT
3240
hSpCas9
P L I E T N G E T G E I V W D K G R D F
1061 1062 1063 1064 1065 1066 1067 1068 1069 1070 1071 1072 1073 1074 1075 1076 1077 1078 1079 1080
GCCACCGTGCGGAAAGTGCTGAGCATGCCCCAAGTGAATATCGTGAAAAAGACCGAGGTG
3300
hSpCas9
A T V R K V L S M P Q V N I V K K T E V
1081 1082 1083 1084 1085 1086 1087 1088 1089 1090 1091 1092 1093 1094 1095 1096 1097 1098 1099 1100
CAGACAGGCGGCTTCAGCAAAGAGTCTATCCTGCCCAAGAGGAACAGCGATAAGCTGATC
3360
hSpCas9
Q T G G F S K E S I L P K R N S D K L I
1101 1102 1103 1104 1105 1106 1107 1108 1109 1110 1111 1112 1113 1114 1115 1116 1117 1118 1119 1120

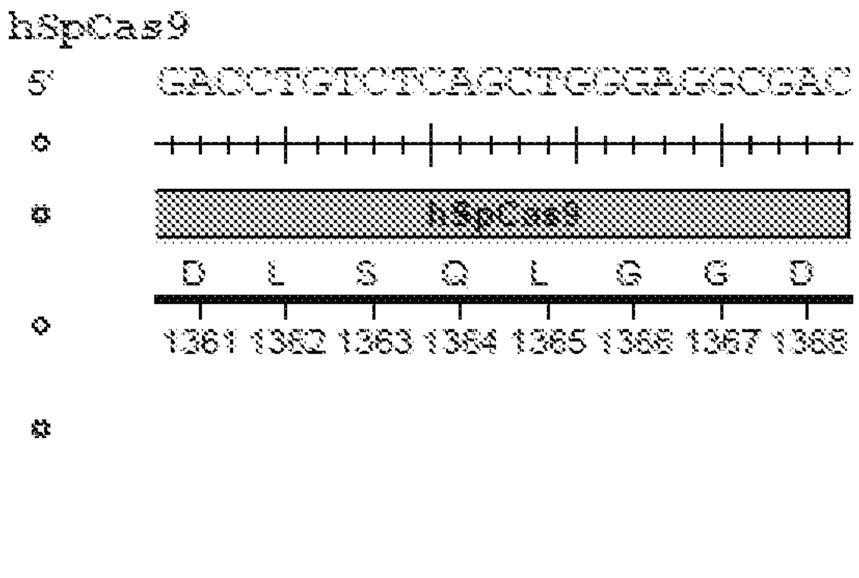
FIG. 24J

hSpCas9
5'
GCCAGAAAGAAGGACTGGGACCCTAAGAAGTACGGCGGCTTCGACAGCCCCACCGTGGCC
3420
hSpCas9
A R K K D W D P K K Y G G F D S P T V A
1121 1122 1123 1124 1125 1126 1127 1128 1129 1130 1131 1132 1133 1134 1135 1136 1137 1138 1139 1140
5'
TATTCTGTGCTGGTGGTGGCCAAAGTGGAAAAGGGCAAGTCCAAGAAACTGAAGAGTGTG
3480
hSpCas9
Y S V L V V A K V E K G K S K K L K S V
1141 1142 1143 1144 1145 1146 1147 1148 1149 1150 1151 1152 1153 1154 1155 1156 1157 1158 1159 1160
5'
AAAGAGCTGCTGGGGATCACCATCATGGAAAGAAGCAGCTTCGAGAAGAATCCCATCGAC
3540
hSpCas9
K E L L G I T I M E R S S F E K N P I D
1161 1162 1163 1164 1165 1166 1167 1168 1169 1170 1171 1172 1173 1174 1175 1176 1177 1178 1179 1180
5'
TTTCTGGAAGCCAAGGGCTACAAAGAAGTGAAAAAGGACCTGATCATCAAGCTGCCTAAG
3600
hSpCas9
F L E A K G Y K E V K K D L I I K L P K
1181 1182 1183 1184 1185 1186 1187 1188 1189 1190 1191 1192 1193 1194 1195 1196 1197 1198 1199 1200
5'
TACTCCCTGTTCGAGCTGGAAAACGGCCGGAAGAGAATGCTGGCCTCTGCCGGCGAACTG
3660
hSpCas9
Y S L F E L E N G R K R M L A S A G E L
1201 1202 1203 1204 1205 1206 1207 1208 1209 1210 1211 1212 1213 1214 1215 1216 1217 1218 1219 1220
5'
CAGAAGGGAAACGAACTGGCCCTGCCCTCCAAATATGTGAACTTCCTGTACCTGGCCAGC
3720
hSpCas9
Q K G N E L A L P S K Y V N F L Y L A S
1221 1222 1223 1224 1225 1226 1227 1228 1229 1230 1231 1232 1233 1234 1235 1236 1237 1238 1239 1240

FIG. 24K

hSpCas9
5'
CACTATGAGAAGCTGAAGGGCTCCCCGAGGATAATGAGCAGAAACAGCTGTTTGTGGAA
3780
hSpCas9
H Y E K L K G S P E D N E Q K Q L F V E
1241 1242 1243 1244 1245 1246 1247 1248 1249 1250 1251 1252 1253 1254 1255 1256 1257 1258 1259 1260
5'
CAGCACAAGCACTACCTGGACGAGATCATCGAGCAGATCAGCGAGTTCTCCAAGAGAGTG
3840
hSpCas9
Q H K H Y L D E I I E Q I S E F S K R V
1261 1262 1263 1264 1265 1266 1267 1268 1269 1270 1271 1272 1273 1274 1275 1276 1277 1278 1279 1280
5'
ATCCTGGCCGACGCTAATCTGGACAAAGTGCTGTCCGCCTACAACAAGCACCGGGATAAG
3900
hSpCas9
I L A D A N L D K V L S A Y N K H R D K
1281 1282 1283 1284 1285 1286 1287 1288 1289 1290 1291 1292 1293 1294 1295 1296 1297 1298 1299 1300
5'
CCCATCAGAGAGCAGGCCGAGAATATCATCCACCTGTTTACCCTGACCAATCTGGGAGCC
3960
hSpCas9
P I R E Q A E N I I H L F T L T N L G A
1301 1302 1303 1304 1305 1306 1307 1308 1309 1310 1311 1312 1313 1314 1315 1316 1317 1318 1319 1320
5'
CCTGCCGCCTTCAAGTACTTTGACACCACCATCGACCGGAAGAGGTACACCAGCACCAAA
4020
hSpCas9
P A A F K Y F D T T I D R K R Y T S T K
1321 1322 1323 1324 1325 1326 1327 1328 1329 1330 1331 1332 1333 1334 1335 1336 1337 1338 1339 1340
5'
GAGGTGCTGGACGCCACCCTGATCCACCAGAGCATCACCGGCCTGTACGAGACACGGATC
4080
hSpCas9
E V L D A T L I H Q S I T G L Y E T R I
1341 1342 1343 1344 1345 1346 1347 1348 1349 1350 1351 1352 1353 1354 1355 1356 1357 1358 1359 1360

FIG. 24L


hSpCas9
5'
GACCTGTCTCAGCTGGGAGGCGAC
hSpCas9
D L S Q L G G D
1361 1362 1363 1364 1365 1366 1367 1368

4104

FIG. 24M

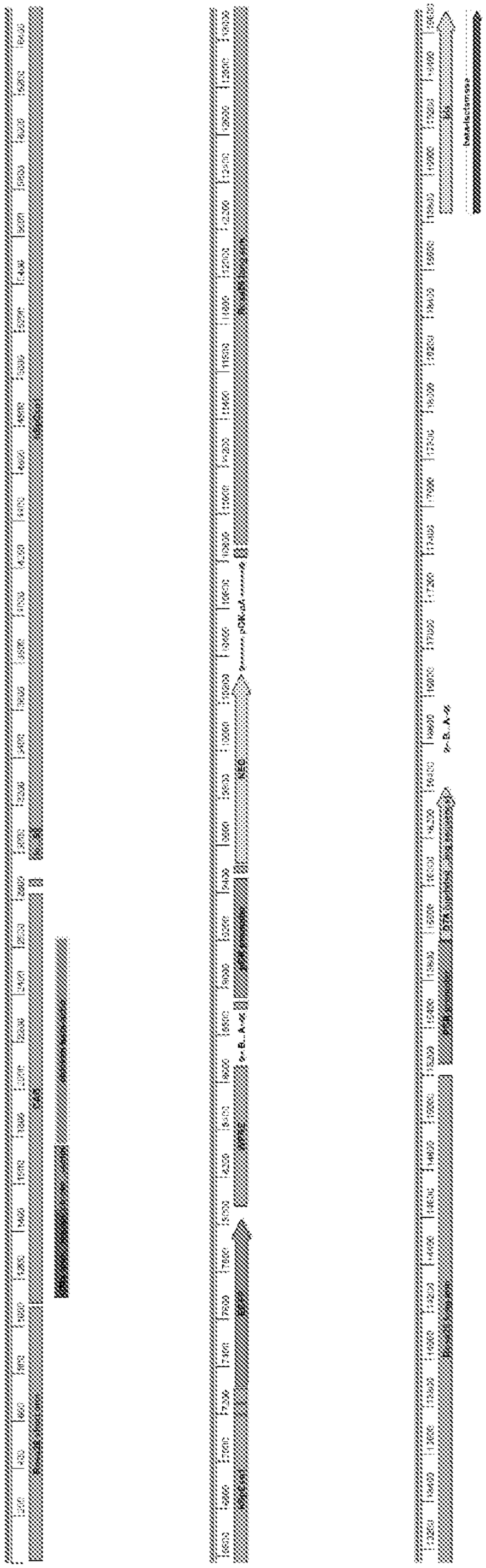
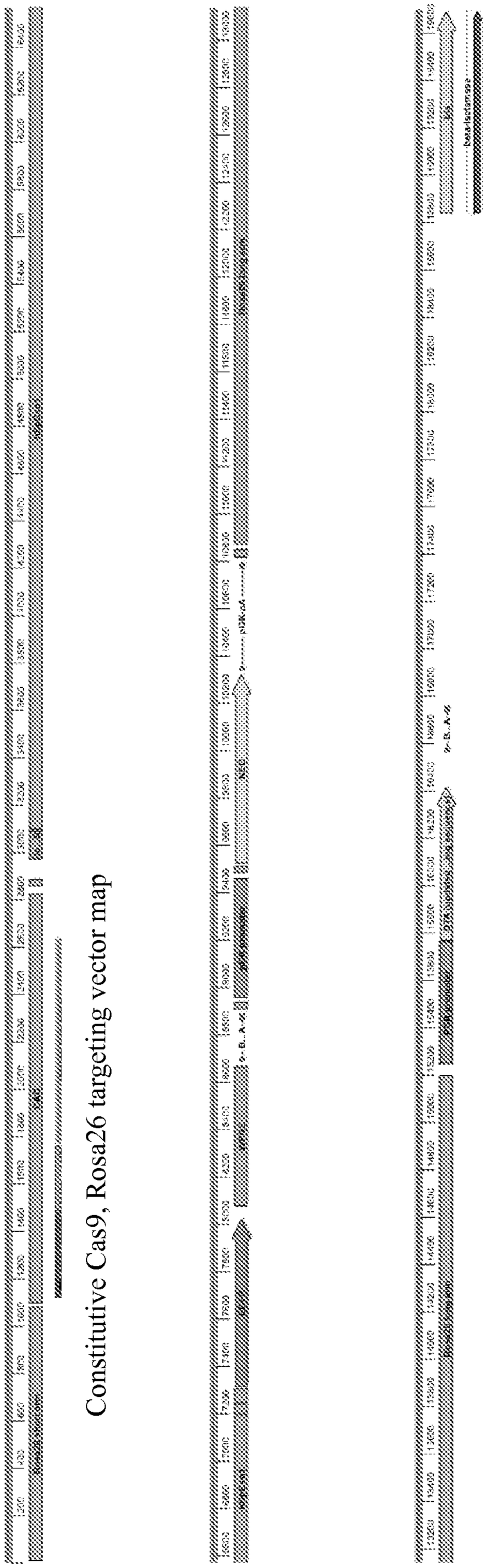

Conditional Cas9, Rosa26 targeting vector map
pGKneoA
NEO
Rosa26 homology arm

FIG. 25A



Constitutive Cas9, Rosa26 targeting vector map

FIG. 25B

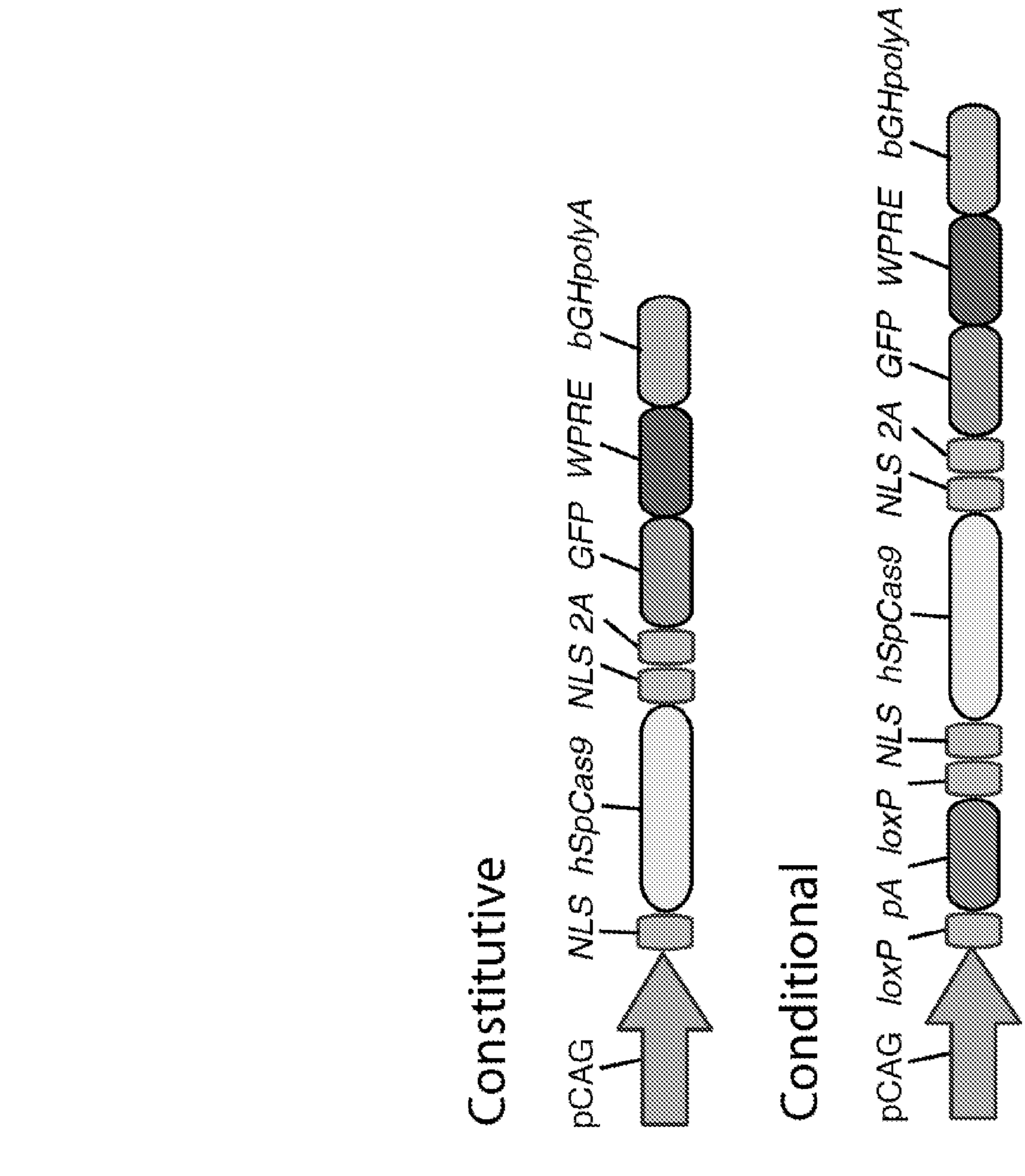
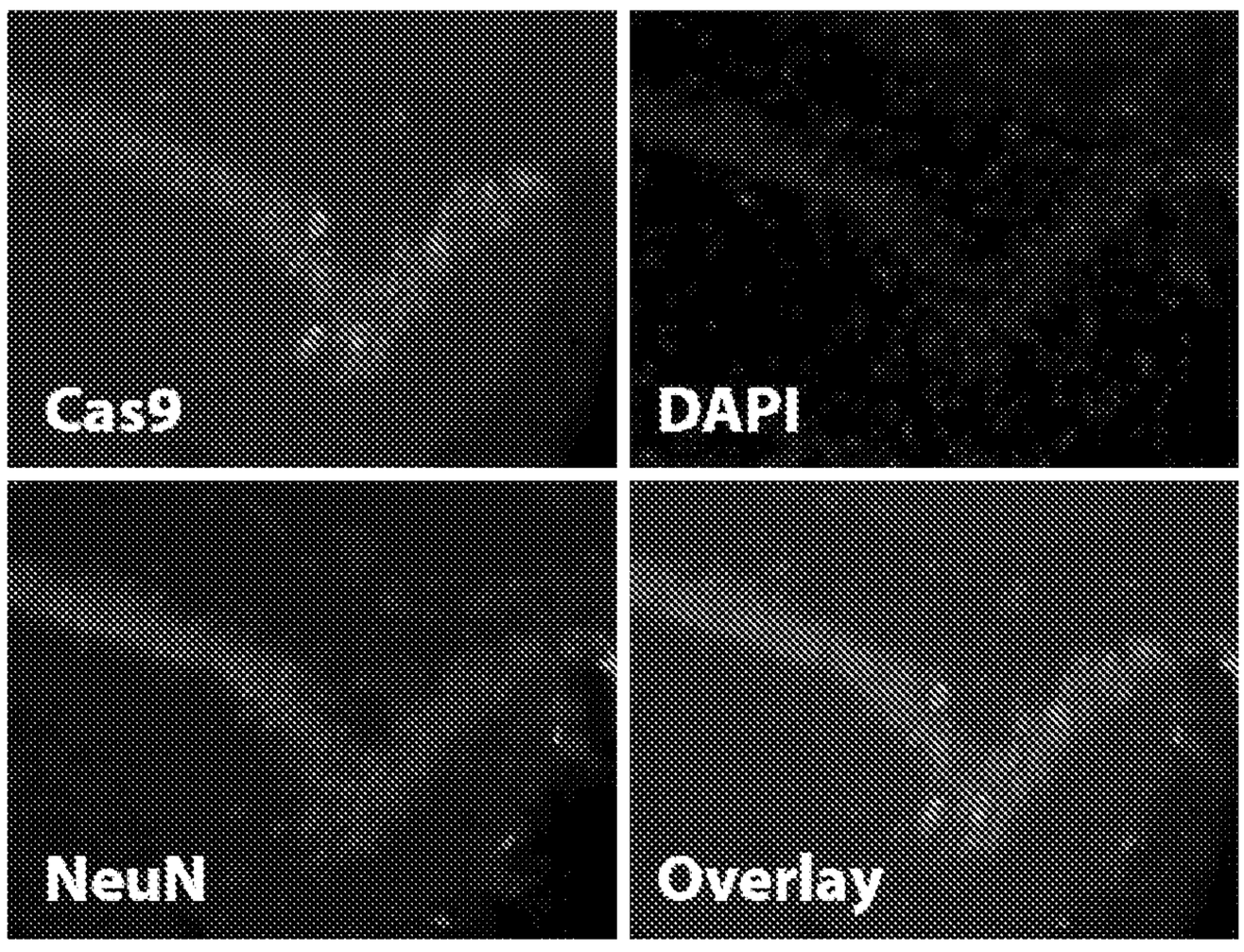

Constitutive
pCAG
NLS hSpCas9 NLS 2A GFP WPRE bGHpolyA
Conditional
pCAG loxP pA loxP NLS hSpCas9 NLS 2A GFP WPRE bGHpolyA

FIG. 26


Cas9 Expression in Mouse Hippocampus (AAV)
Cas9
DAPI
NeuN
Overlay

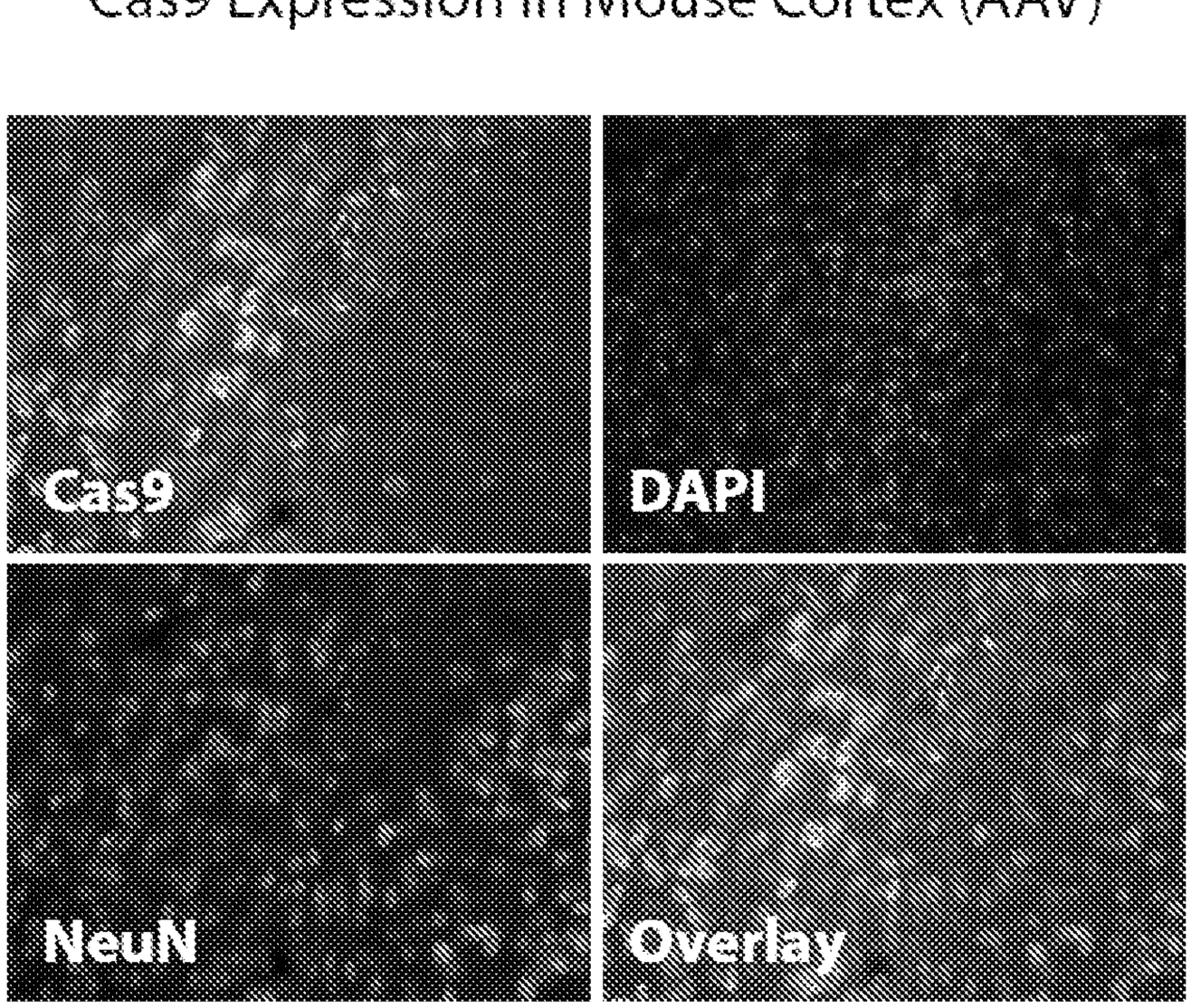

Cas9 Expression in Mouse Cortex (AAV)
Cas9
DAPI
NeuN
Overlay

FIG. 27

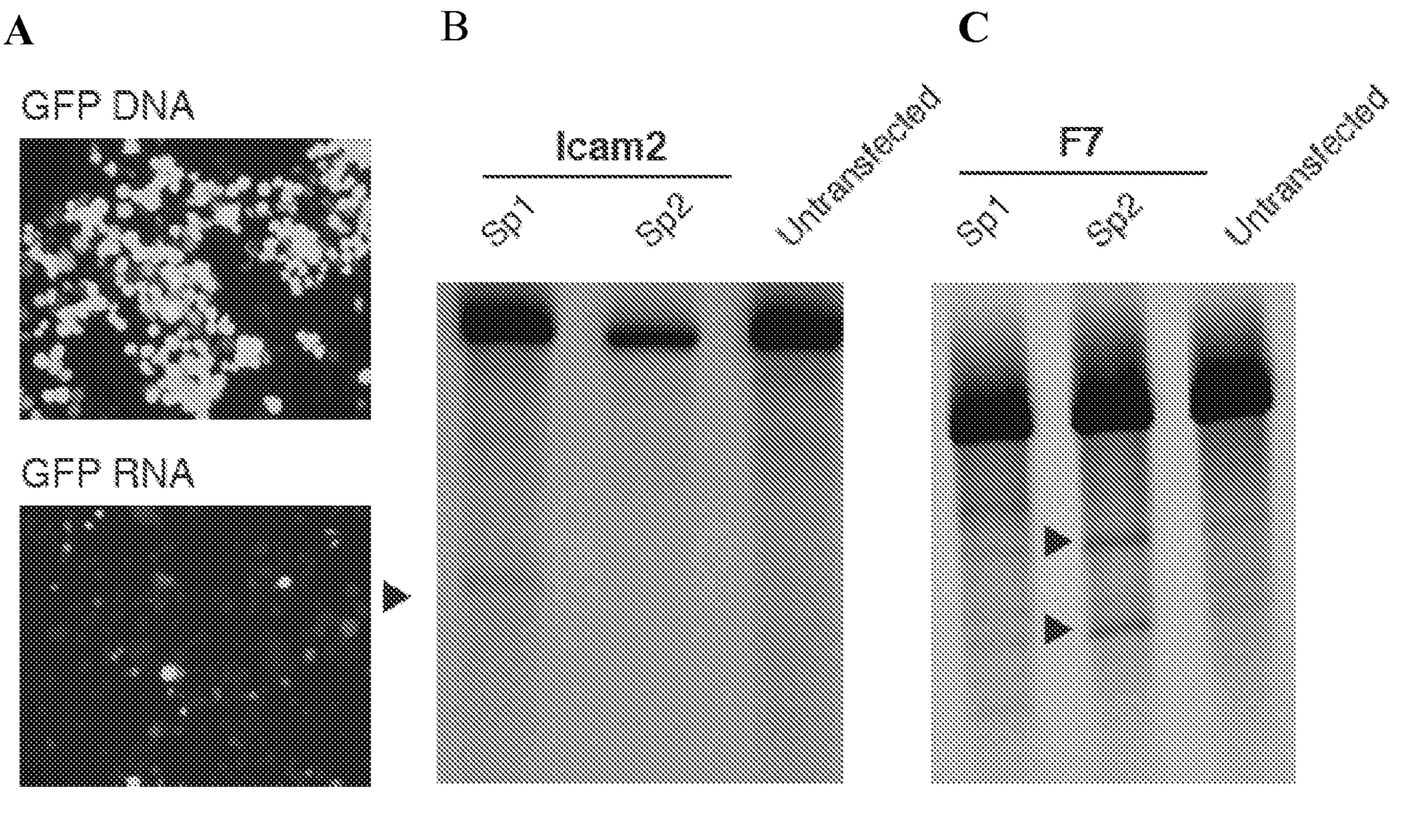

A
B
C
GFP DNA
GFP RNA
Icam2
F7
Sp1
Sp2
Untransfected
Sp1
Sp2
Untransfected

FIG. 28

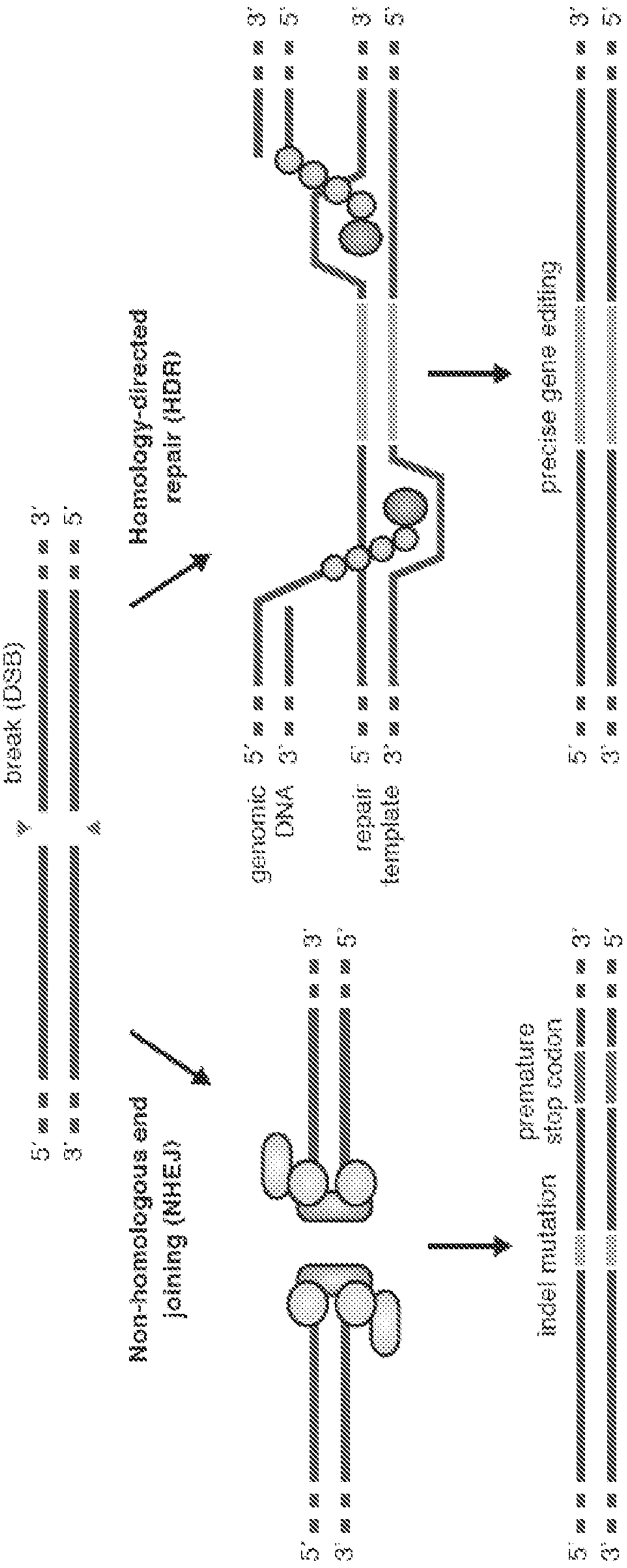

double stranded break (DSB)
5'
3'
Homology-directed repair (HDR)
genomic DNA
repair template
precise gene editing
Non-homologous end joining (NHEJ)
premature stop codon
indel mutation

FIG. 29

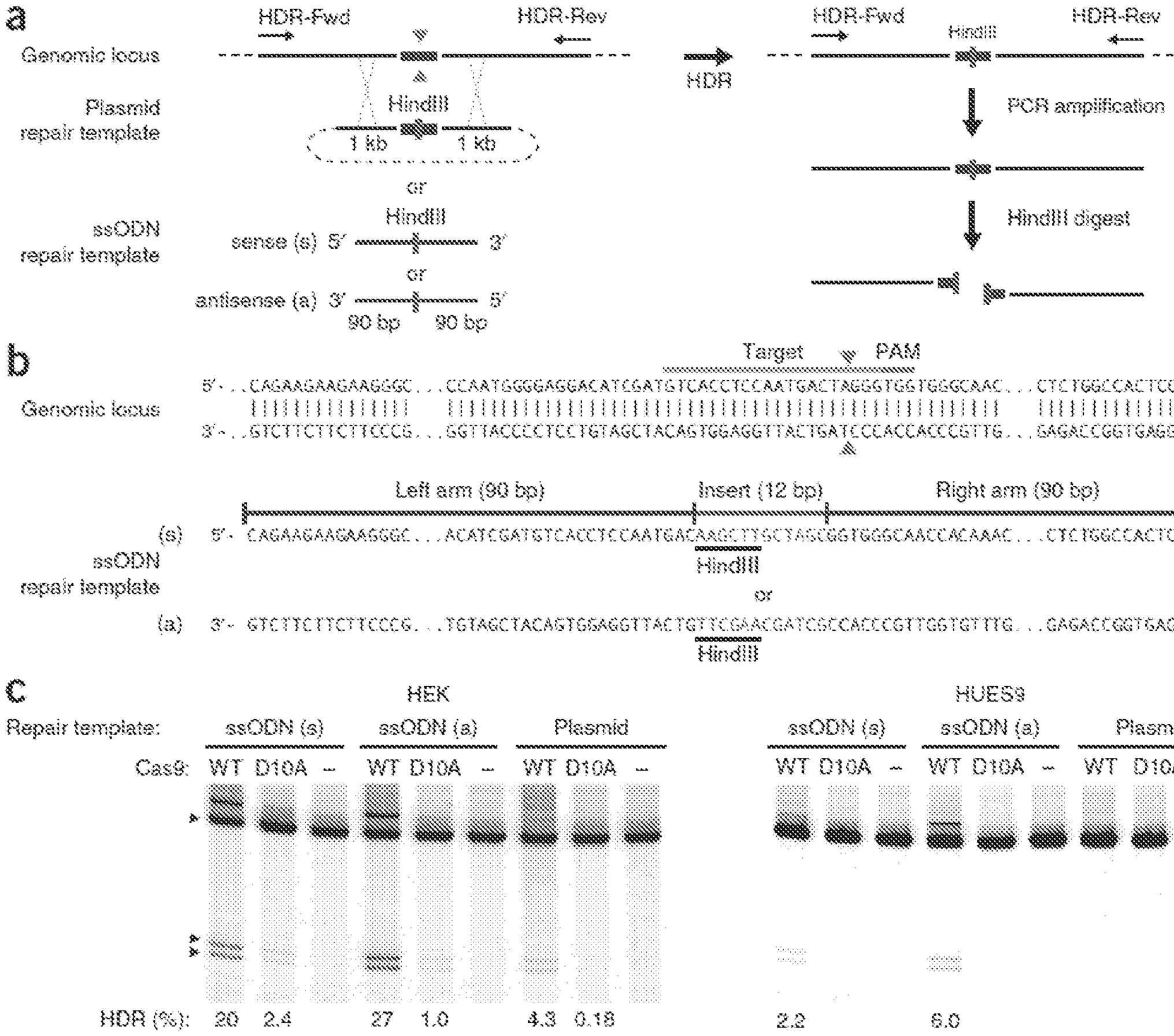

a
Genomic locus
Plasmid repair template
ssODN repair template
HDR-Fwd
HDR-Rev
HindIII
1 kb
1 kb
or
sense (s) 5′ 3′
antisense (a) 3′ 5′
90 bp
90 bp
HDR
PCR amplification
HindIII digest
b
Genomic locus
Target
PAM
5′- . . CAGAAGAAGAAGGGC . . . CCAATGGGGAGGACATCGATGTCACCTCCAATGACTAGGGTGGTGGGCAAC . . . CTCTGGCCACTCCCT . . -3′
3′- . . GTCTTCTTCTTCCCG . . . GGTTACCCCTCCTGTAGCTACAGTGGAGGTTACTGATCCCACCACCCGTTG . . . GAGACCGGTGAGGGA . . -5′
ssODN repair template
Left arm (90 bp)
Insert (12 bp)
Right arm (90 bp)
(s) 5′- CAGAAGAAGAAGGGC . . . ACATCGATGTCACCTCCAATGACAAGCTTGCTAGCGGTGGGCAACCACAAAC . . . CTCTGGCCACTCCCT -3′
HindIII
or
(a) 3′- GTCTTCTTCTTCCCG . . . TGTAGCTACAGTGGAGGTTACTGTTCGAACGATCGCCACCCGTTGGTGTTTG . . . GAGACCGGTGAGGGA -5′
HindIII
c
Repair template:
ssODN (s)
ssODN (a)
Plasmid
Cas9:
WT D10A –
HEK
HUES9
HDR (%):
20 2.4 27 1.0 4.3 0.18
2.2 6.0

FIG. 30A-C

Repair Strategy for Cystic Fibrosis deltaF508 Mutation

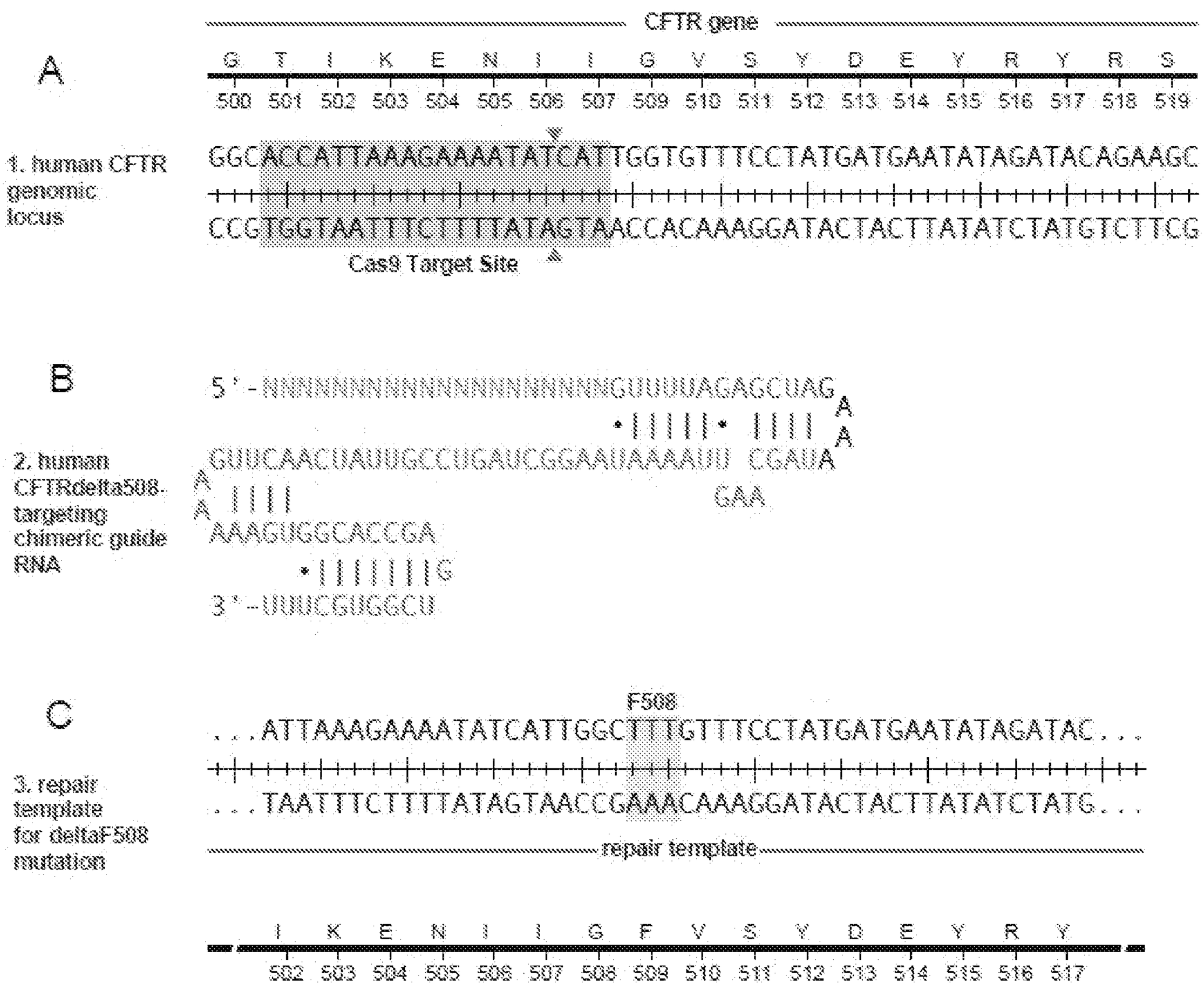
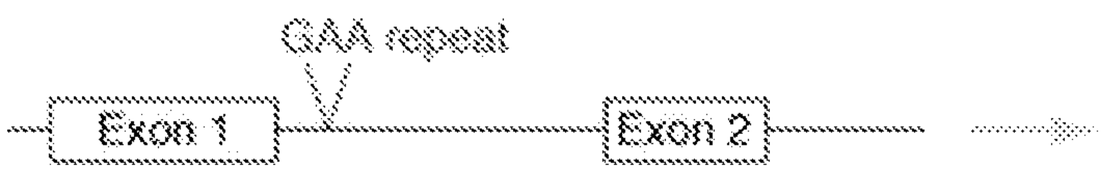


FIG. 31

A

GAA repeat expansion in *FXN* intron 1



B

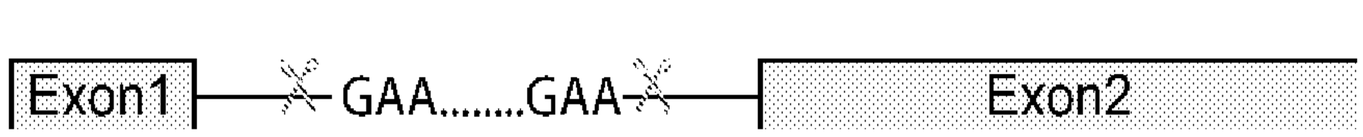


FIG. 32

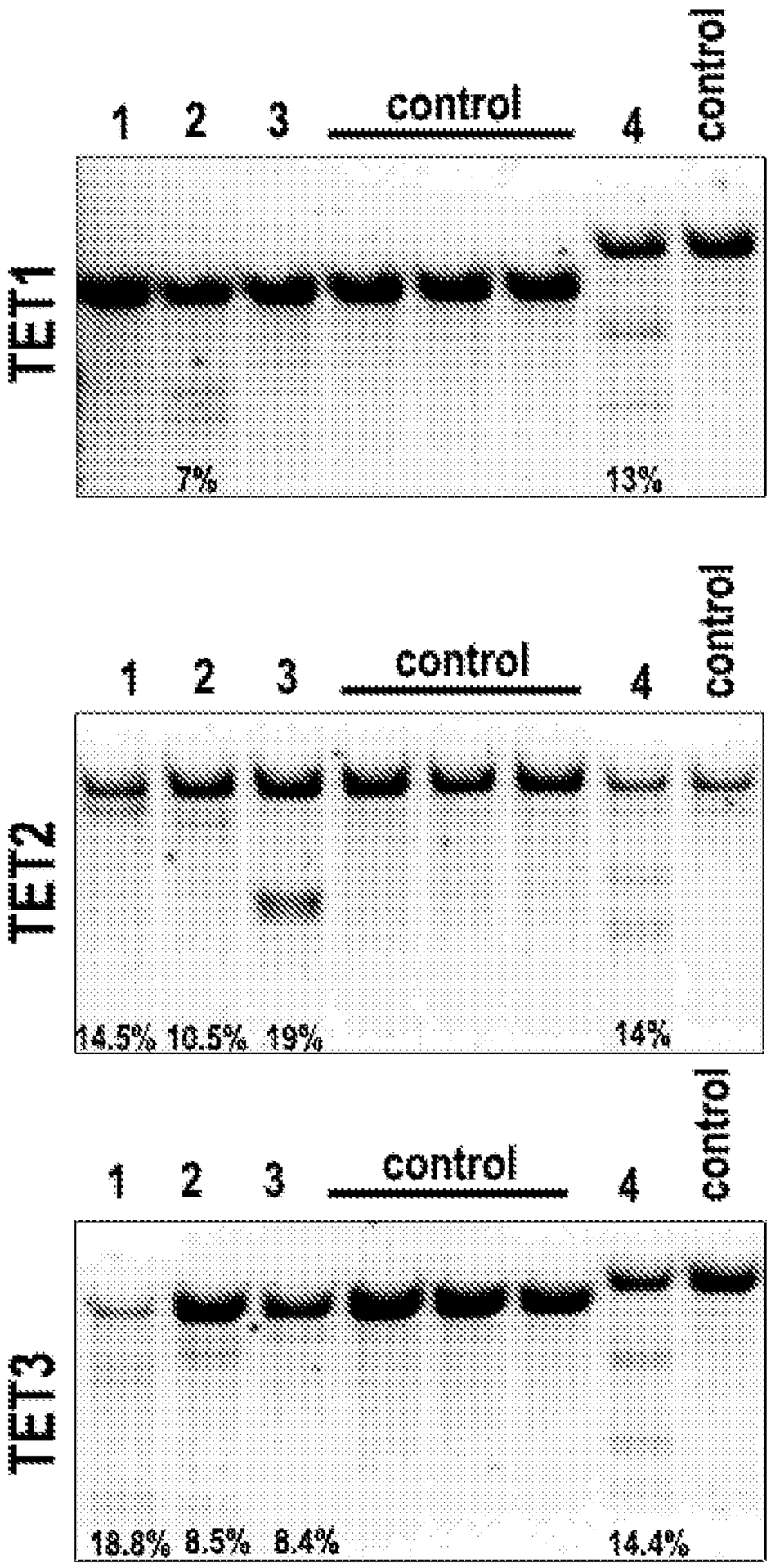

1
2
3
control
4
control
TET1
7%
13%
TET2
14.5%
10.5%
19%
14%
TET3
18.8%
8.5%
8.4%
14.4%
DNMT1
DNMT3a
18.5%
20.4%
11.5%
DNMT3b
16.3%
21%
14%

FIG. 33

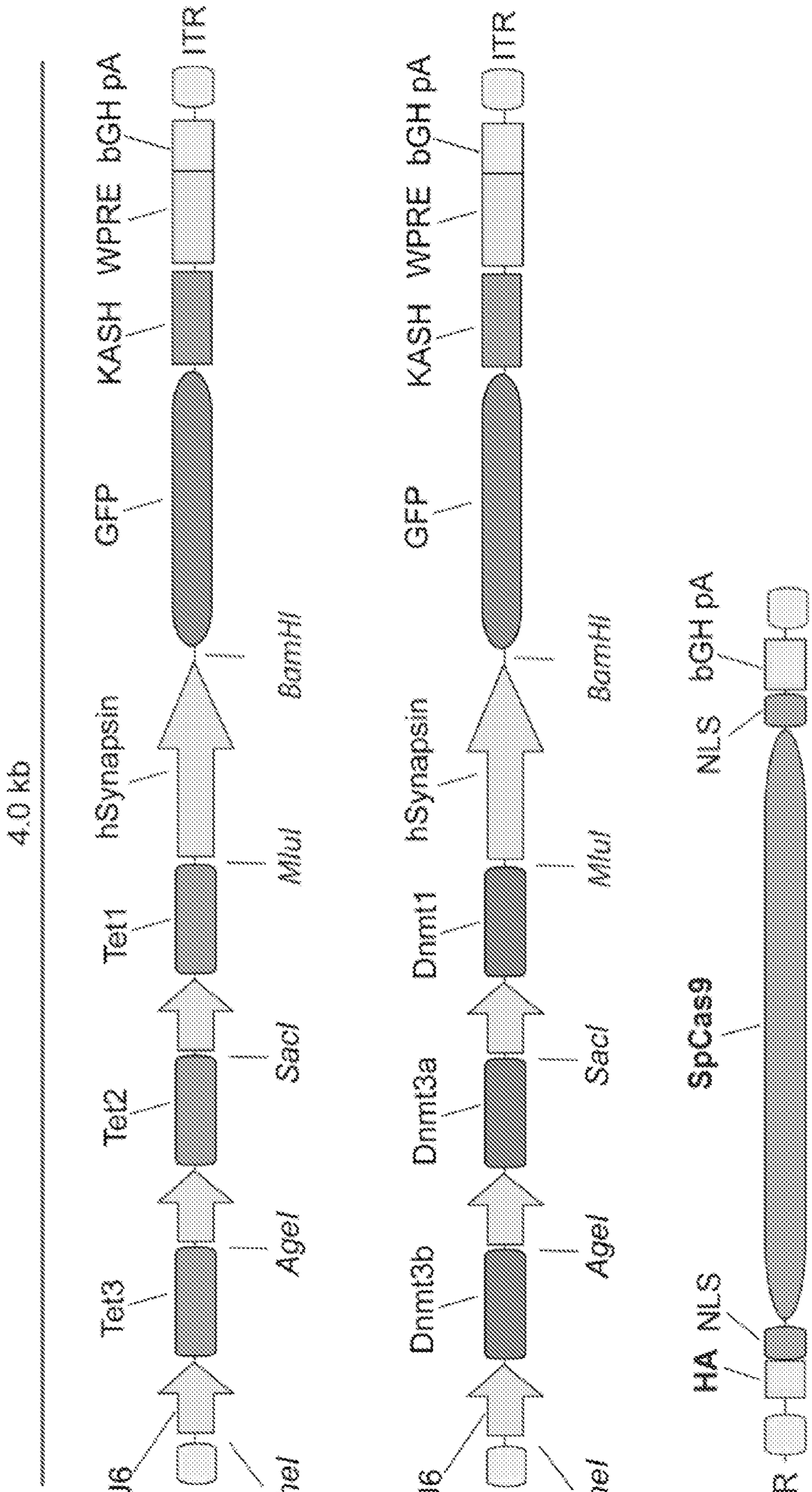

a
4.0 kb
ITR
bGH pA
WPRE
KASH
GFP
BamHI
hSynapsin
Tet1
MluI
Tet2
SacI
Tet3
AgeI
U6
ITR
NotI/NheI
Dnmt1
Dnmt3a
Dnmt3b
SpCas9
NLS
HA
bGH pA
ITR

FIG. 34

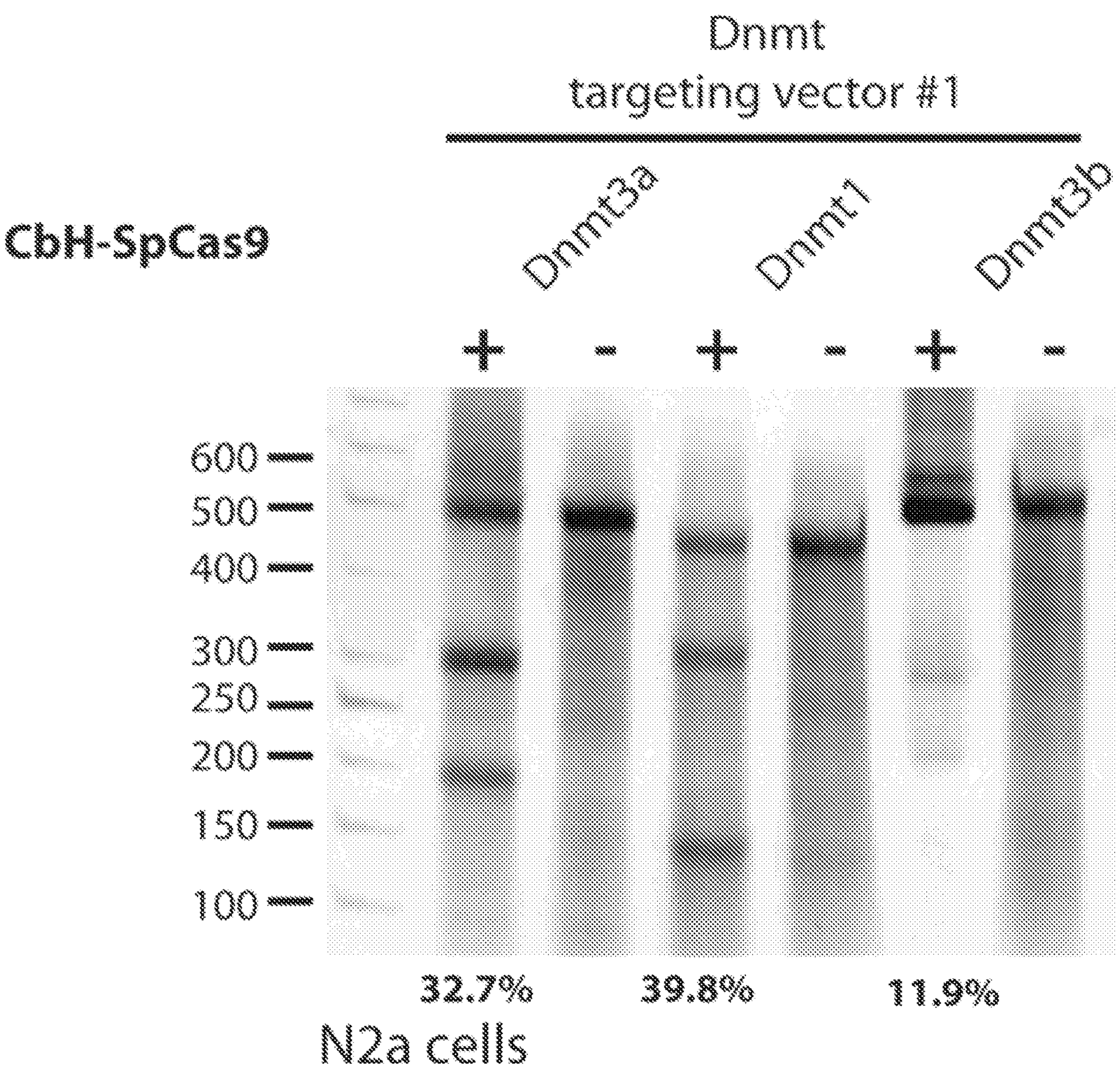

Dnmt
targeting vector #1
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+
-
+
-
+
-
600
500
400
300
250
200
150
100
32.7%
39.8%
11.9%
N2a cells

FIG. 35

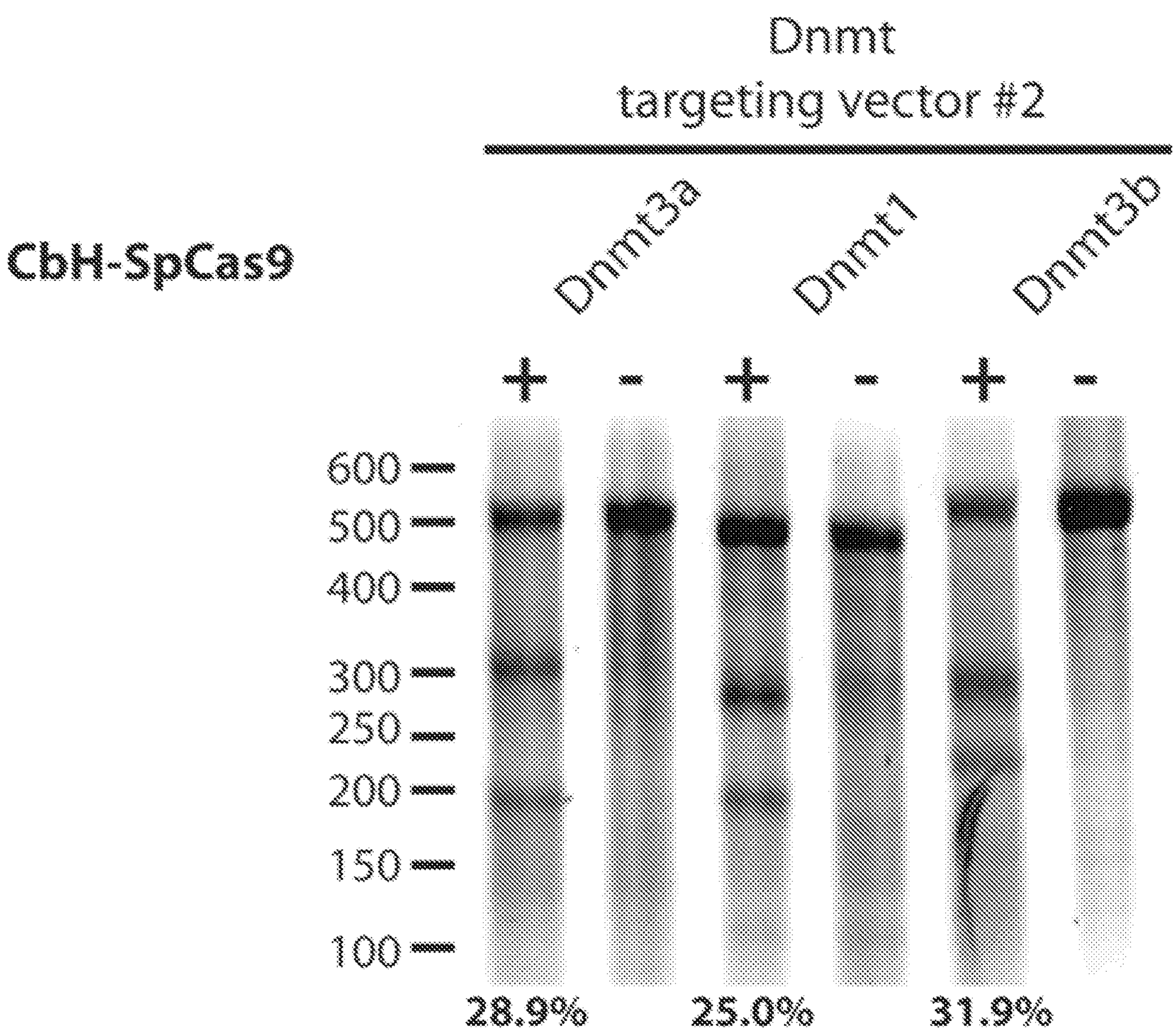

Dnmt
targeting vector #2
CbH-SpCas9
Dnmt3a
Dnmt1
Dnmt3b
+
-
+
-
+
-
600
500
400
300
250
200
150
100
28.9%
25.0%
31.9%
N2a cells

FIG. 36

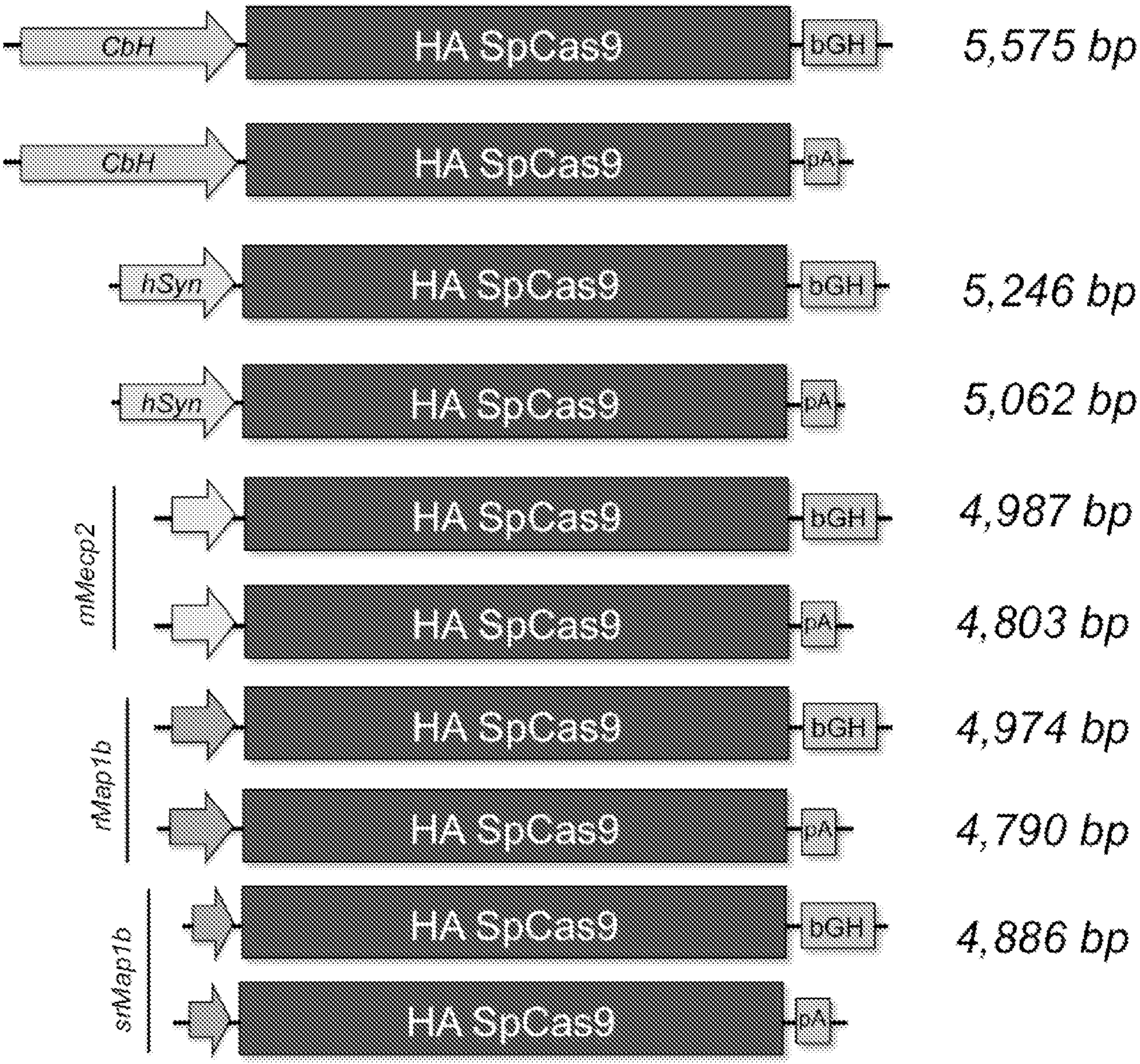

CbH
HA SpCas9
bGH
5,575 bp
CbH
HA SpCas9
pA
hSyn
HA SpCas9
bGH
5,246 bp
hSyn
HA SpCas9
pA
5,062 bp
mMecp2
HA SpCas9
bGH
4,987 bp
HA SpCas9
pA
4,803 bp
rMap1b
HA SpCas9
bGH
4,974 bp
HA SpCas9
pA
4,790 bp
srMap1b
HA SpCas9
bGH
4,886 bp
HA SpCas9
pA

FIG. 37

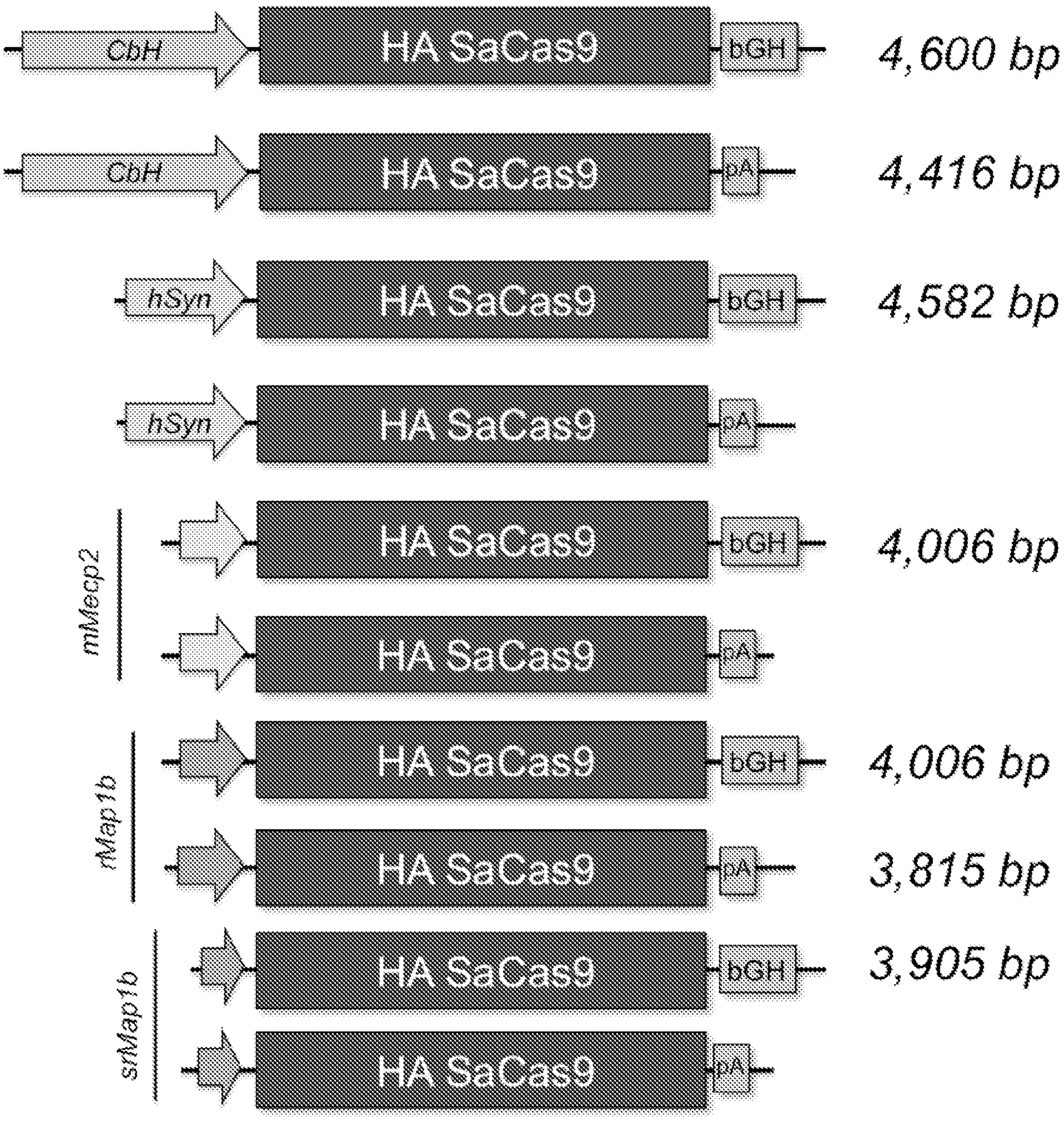

CbH
HA SaCas9
bGH
4,600 bp
CbH
HA SaCas9
pA
4,416 bp
hSyn
HA SaCas9
bGH
4,582 bp
hSyn
HA SaCas9
pA
mMecp2
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
mMap1b
HA SaCas9
bGH
4,006 bp
HA SaCas9
pA
3,815 bp
smMap1b
HA SaCas9
bGH
3,905 bp
HA SaCas9
pA

FIG. 38

Expression of SpCas9 & SaCas9 in N2a cells

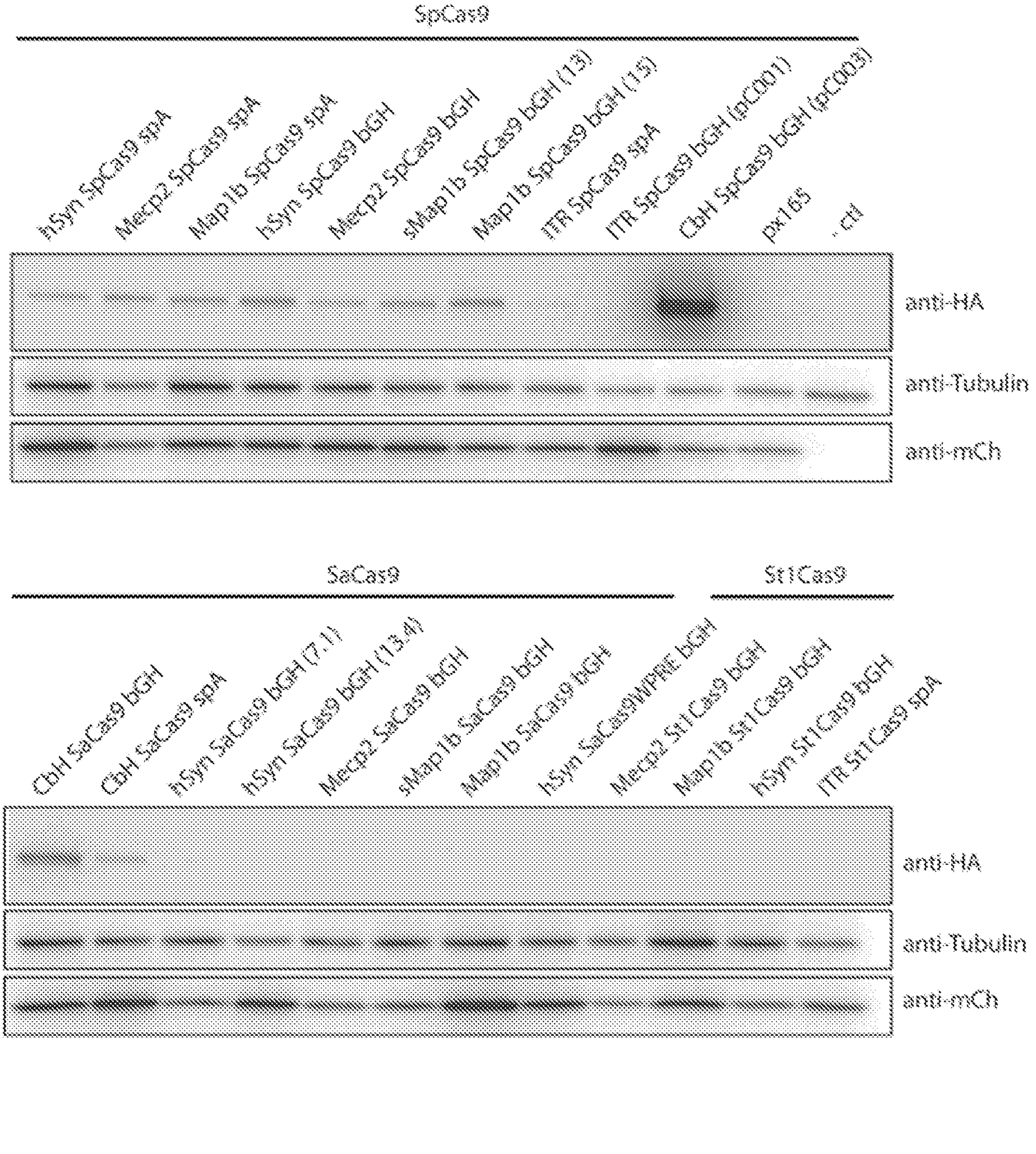


FIG. 39

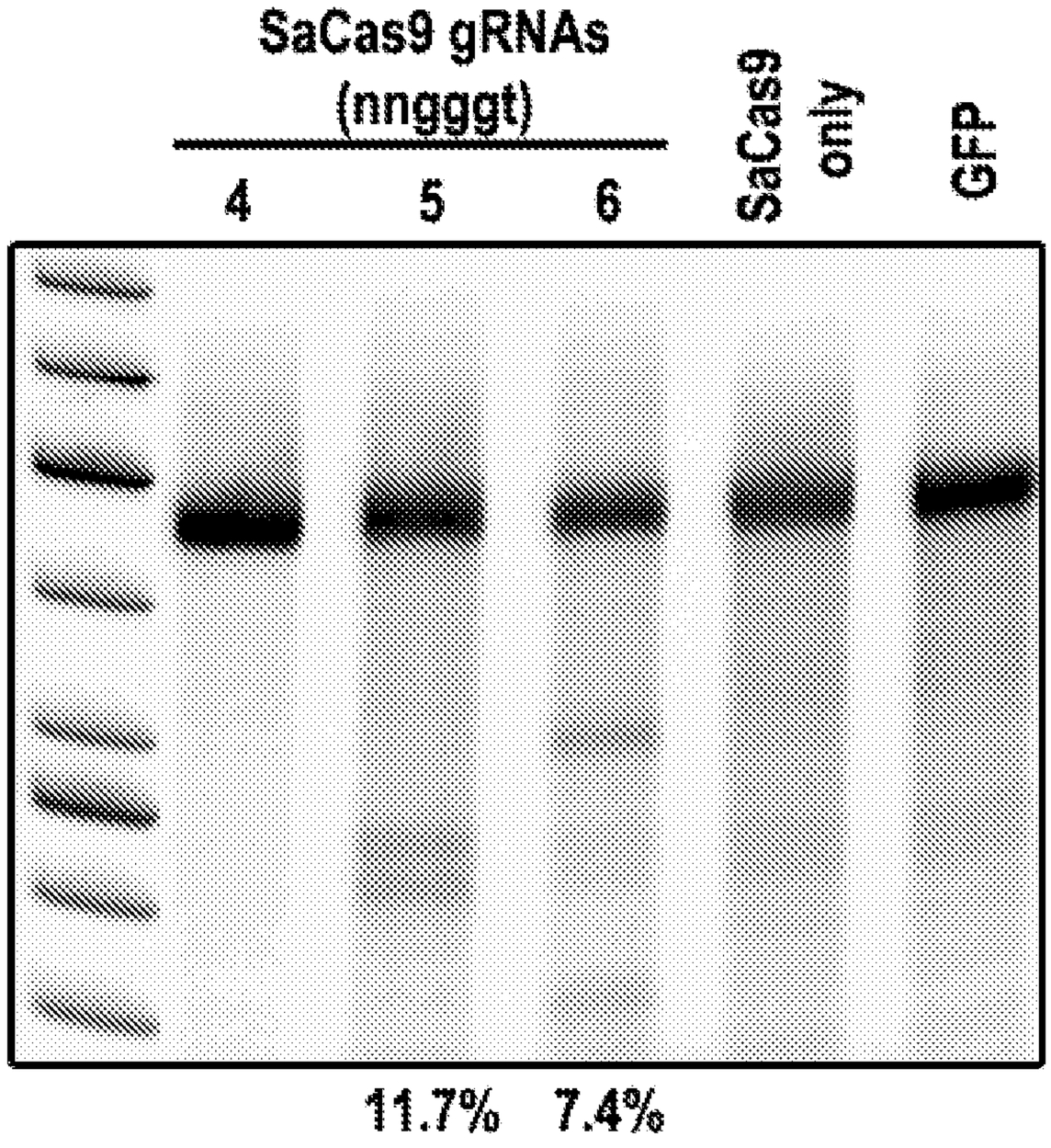


FIG. 40

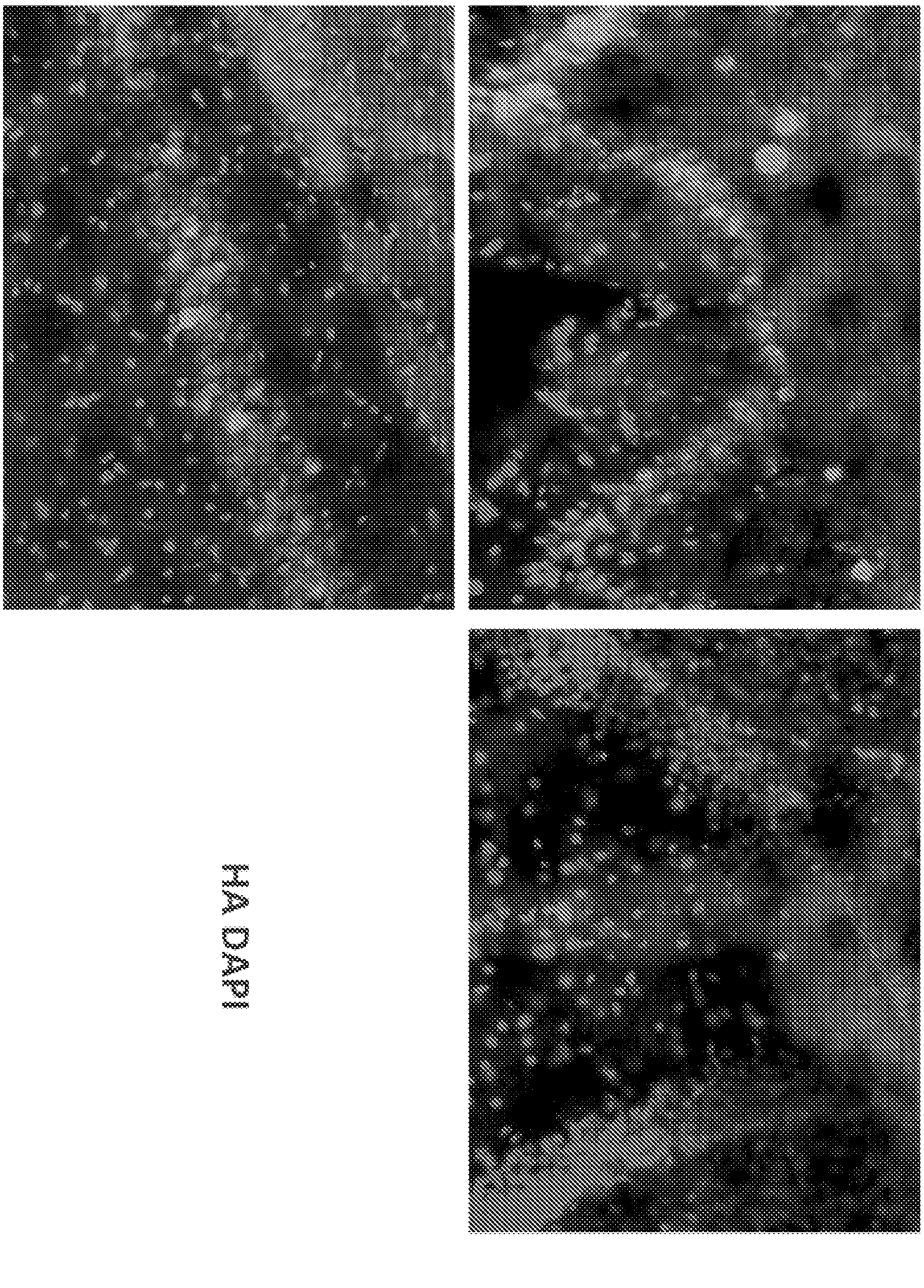


FIG. 41

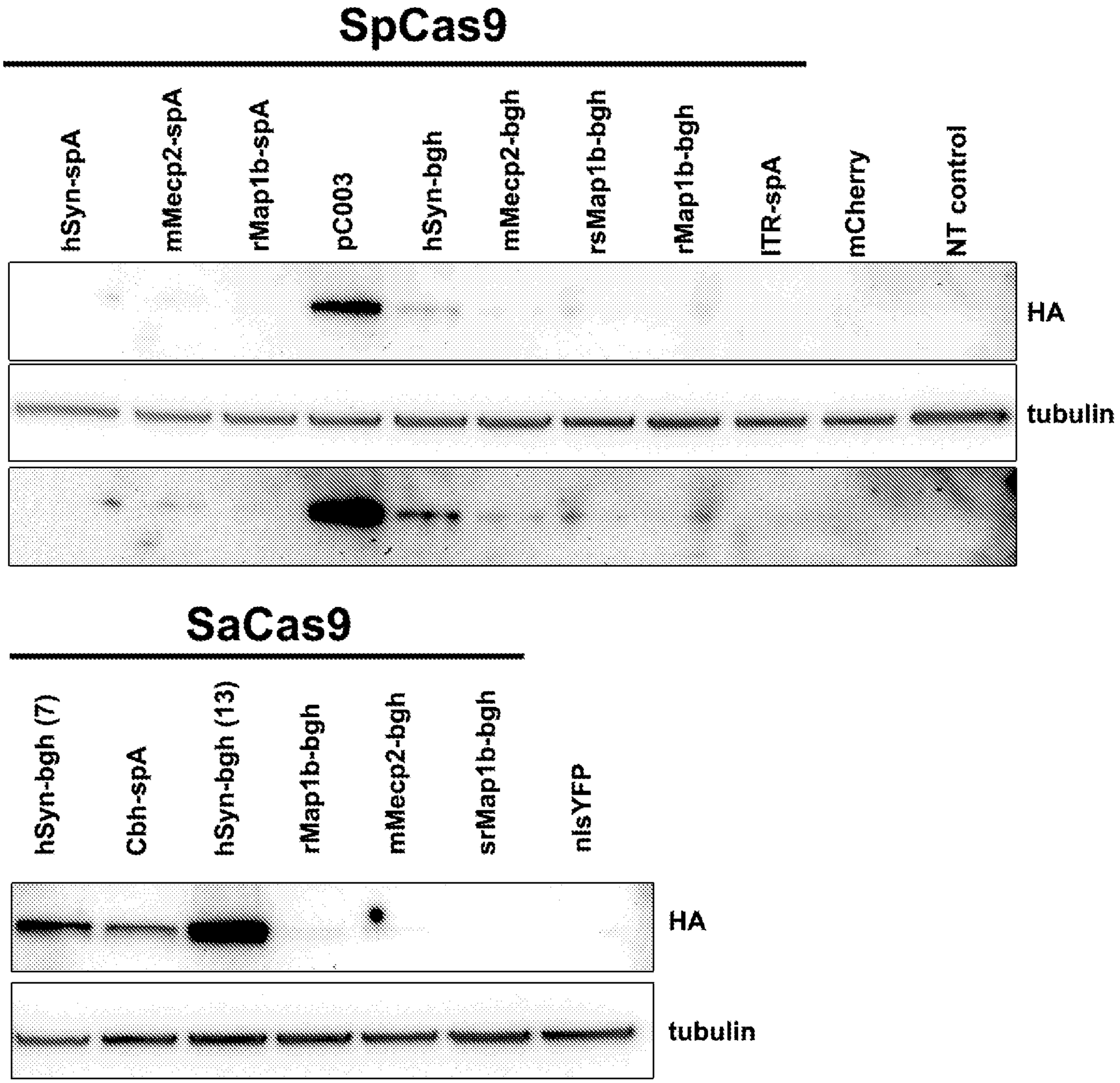

SpCas9
hSyn-spA
mMecp2-spA
rMap1b-spA
pC003
hSyn-bgh
mMecp2-bgh
srMap1b-bgh
rMap1b-bgh
ITR-spA
mCherry
NT control
HA
tubulin
SaCas9
hSyn-bgh (7)
Cbh-spA
hSyn-bgh (13
rMap1b-bgh
mMecp2-bgh
srMap1b-bgh
nlsYFP
HA
tubulin

FIG. 42

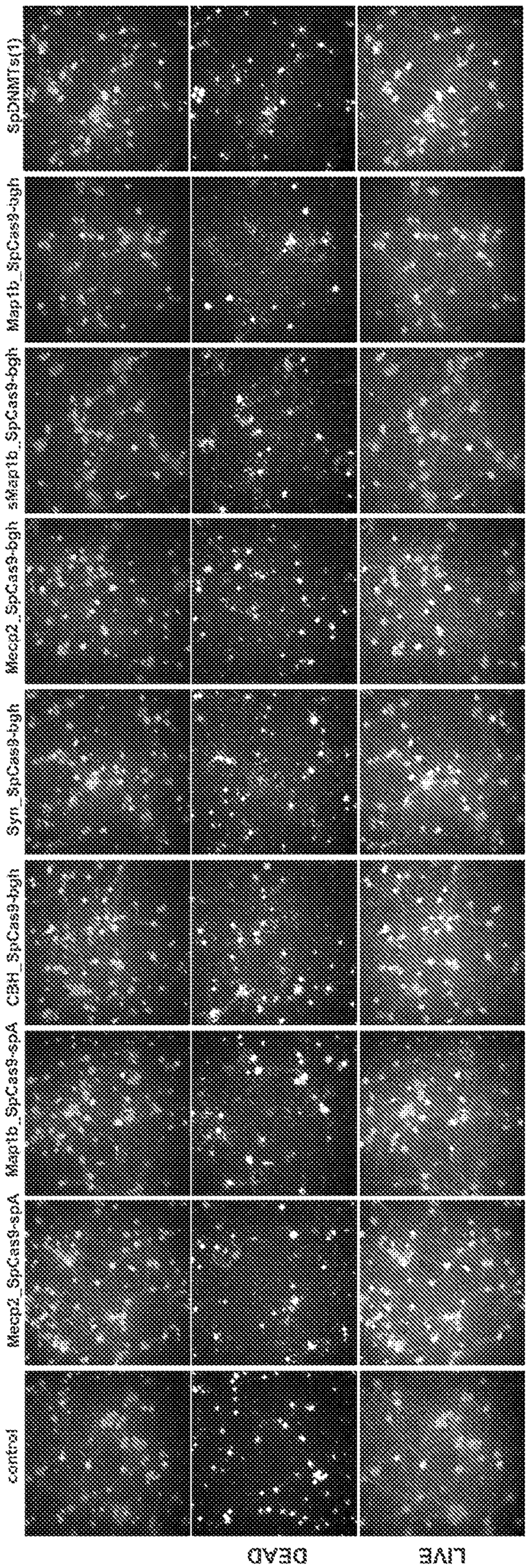

SpCMVTs(1)
Map1b_SpCas9-bgh
Mecp2_SpCas9-bgh
sMap1b_SpCas9-bgh
Syn_SpCas9-bgh
CBH_SpCas9-bgh
Map1b_SpCas9-spA
Mecp2_SpCas9-spA
control
DEAD
LIVE

FIG. 43

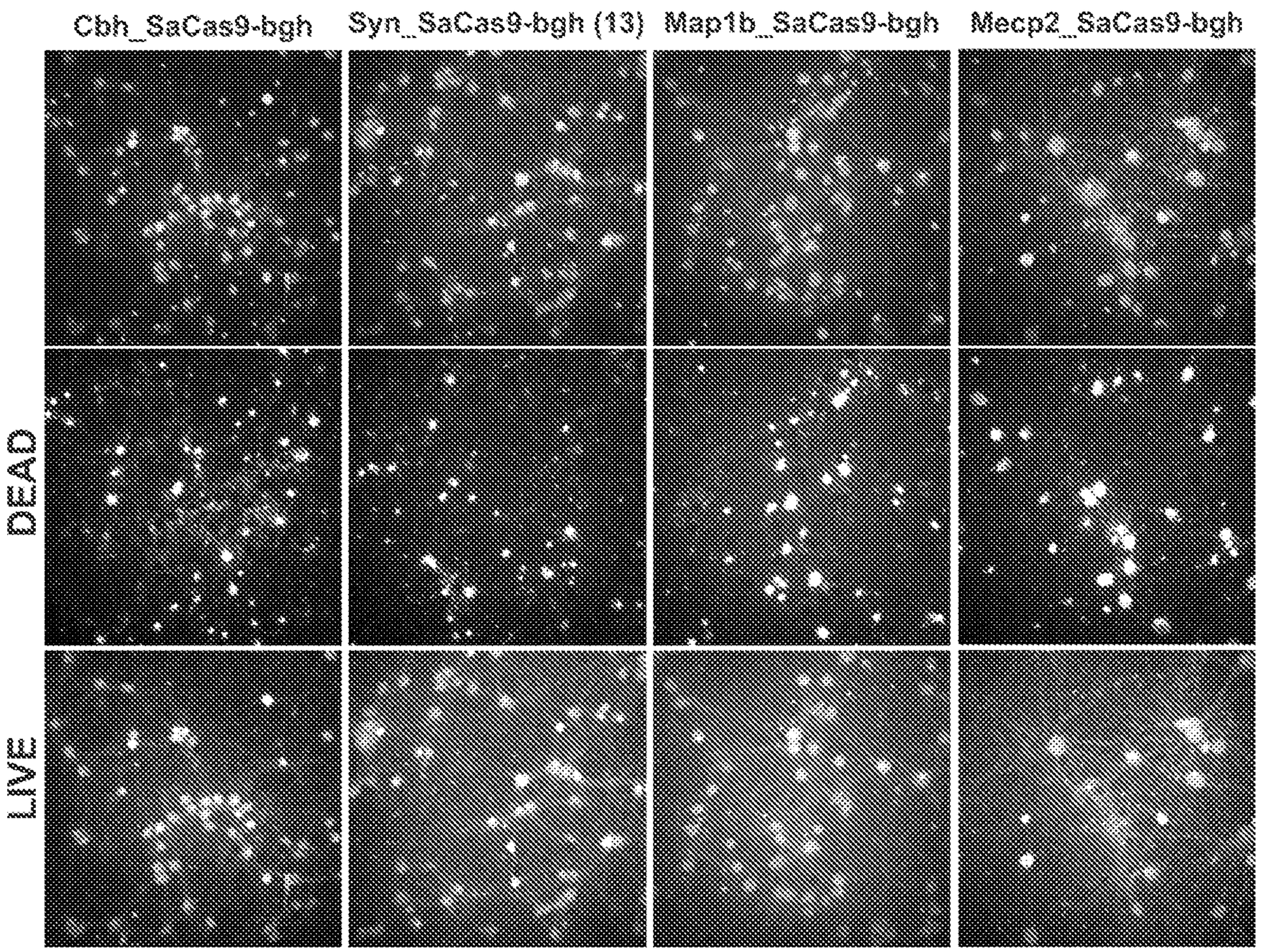

Cbh_SaCas9-bgh
Syn_SaCas9-bgh (13)
Map1b_SaCas9-bgh
Mecp2_SaCas9-bgh
LIVE
DEAD

FIG. 44

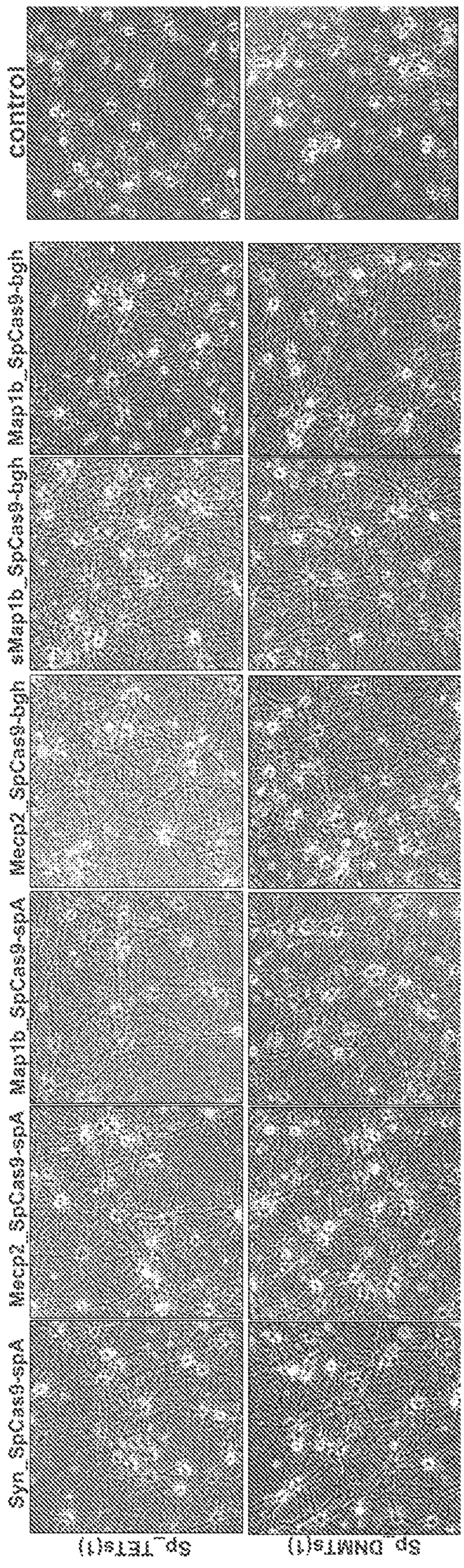

control
sMap1b_SpCas9-bgh
Map1b_SpCas9-bgh
Mecp2_SpCas9-spA
Map1b_SpCas9-spA
Mecp2_SpCas9-bgh
Syn_SpCas9-spA
Sp_TETs(1)
Sp_DNMTs(1)

FIG. 45

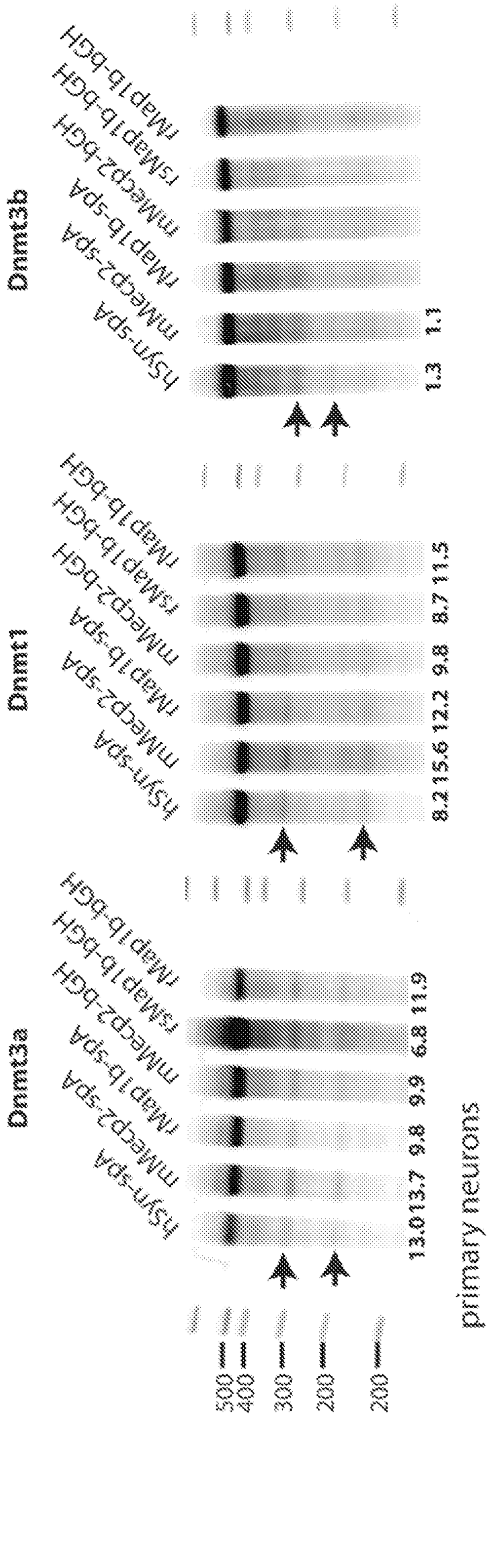

0mm3b
hSyn-sPA
mMeCP2-sPA
mMap1b-sPA
mMap1b-bGH
hSyn-sPA
mMeCP2-sPA
mMap1b-sPA
mMap1b-bGH
1.3
1.1
0mm1
hSyn-sPA
mMeCP2-sPA
mMap1b-sPA
mMap1b-bGH
8.2
15.6
12.2
9.8
8.7
11.5
0mm3a
hSyn-sPA
mMeCP2-sPA
mMap1b-sPA
mMap1b-bGH
13.7
8.8
9.9
6.8
11.9
200
200
300
300
primary neurons

FIG. 46

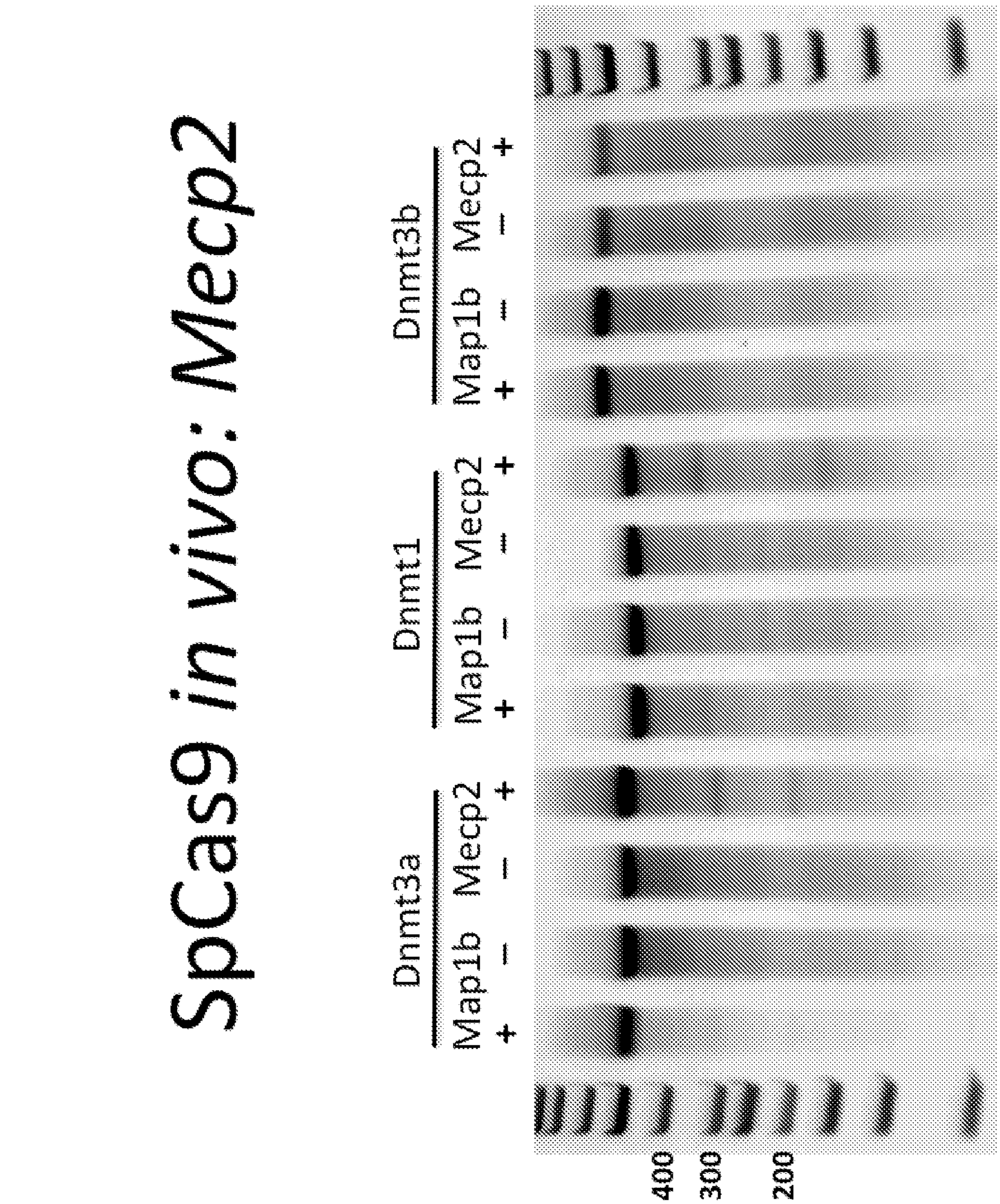

SpCas9 in vivo: Mecp2
Dnmt1
Map1b + Mecp2 +
Dnmt3b
Map1b + Mecp2 +
Dnmt3a
Map1b + Mecp2 +
17.0%
12.0%
400
300
200

FIG. 47

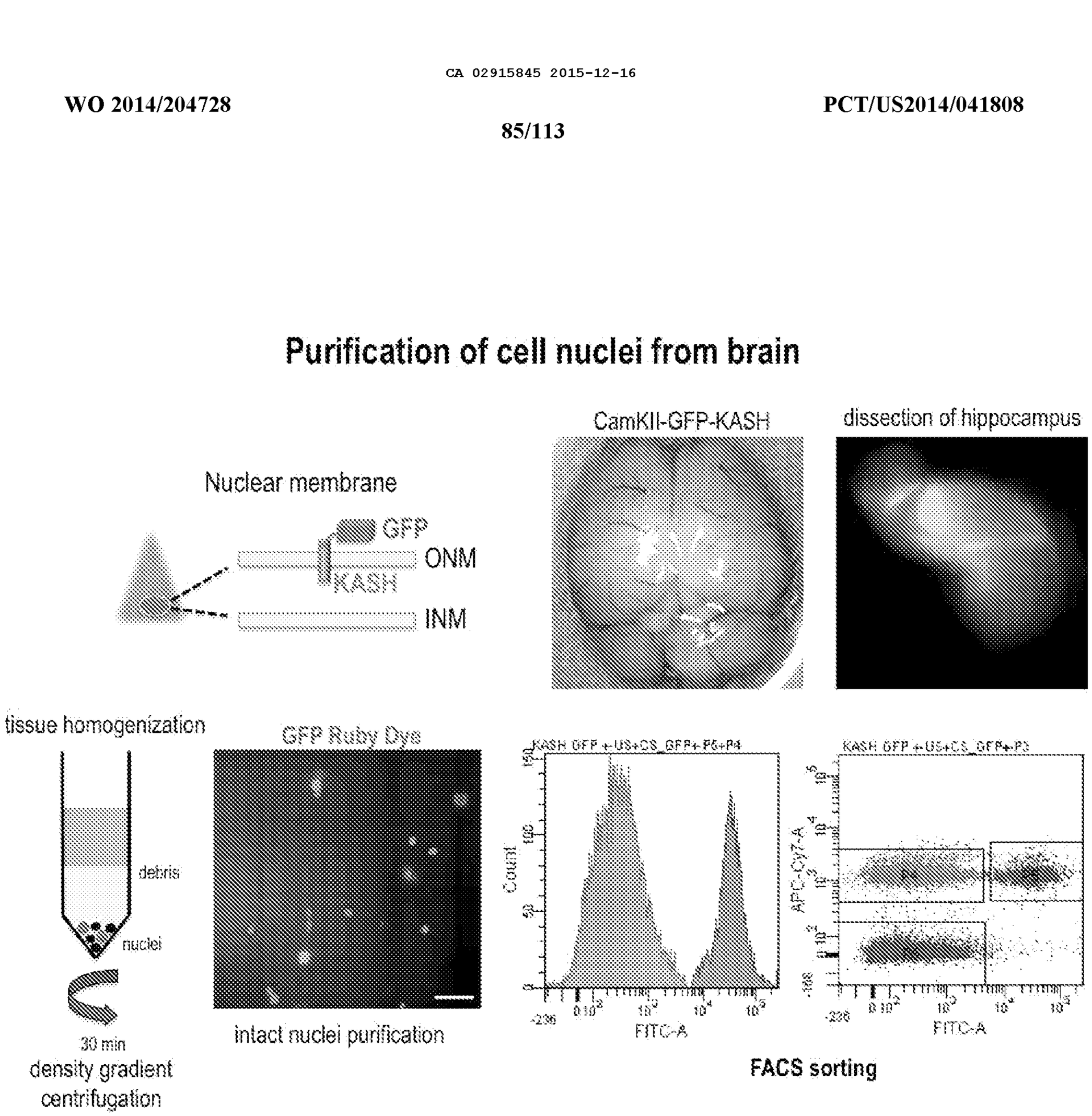

Purification of cell nuclei from brain
CamKII-GFP-KASH
dissection of hippocampus
Nuclear membrane
GFP
ONM
KASH
INM
tissue homogenization
GFP Ruby Dye
KASH GFP +US+CS_GFP+P5+P4
KASH GFP +US+CS_GFP+P3
debris
nuclei
30 min
density gradient
centrifugation
intact nuclei purification
Count
APC-Cy7-A
FITC-A
FACS sorting

FIG. 48

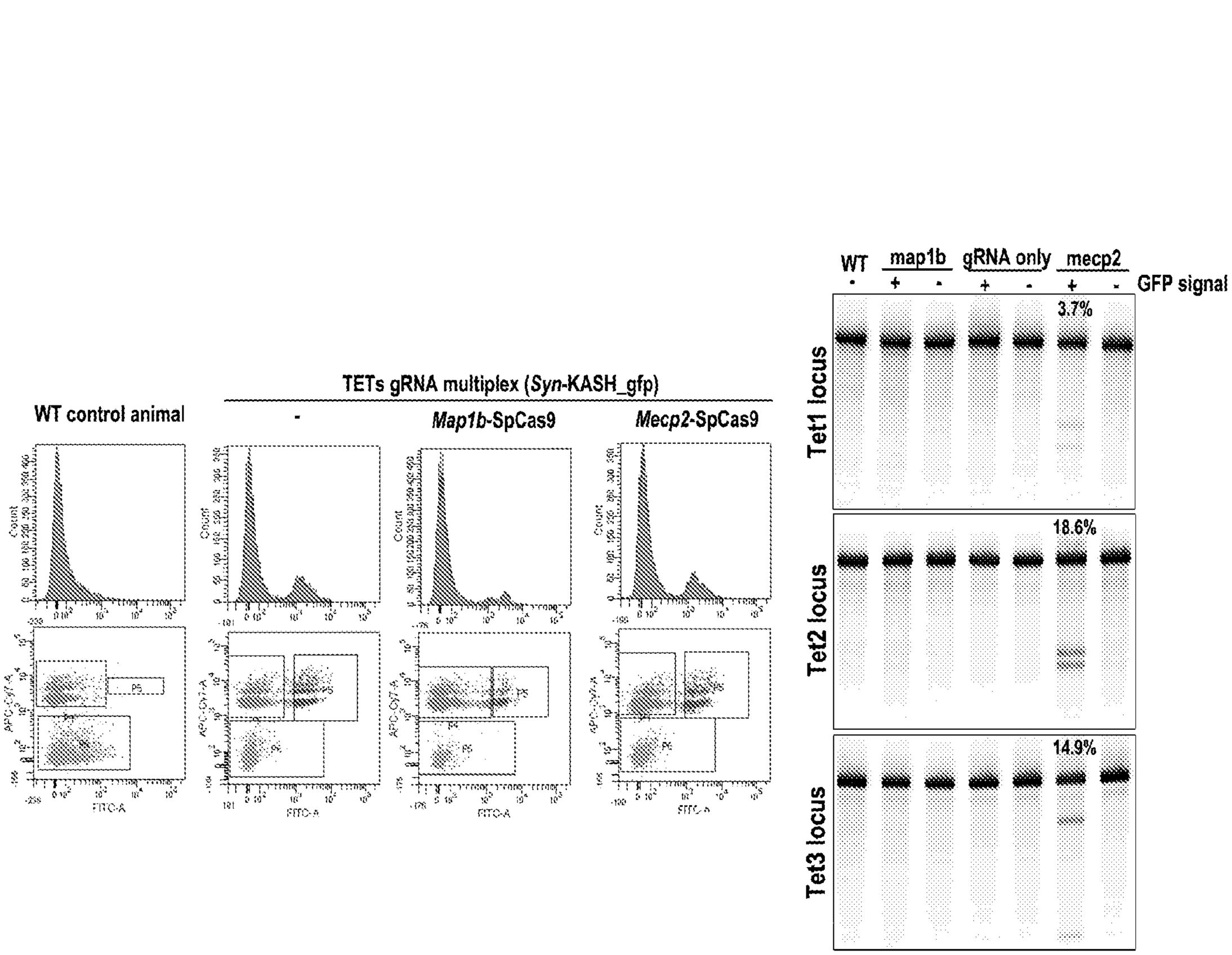

TETs gRNA multiplex (Syn-KASH_gfp)
WT control animal
-
Map1b-SpCas9
Mecp2-SpCas9
Count
APC-Cy7-A
FITC-A
P5
WT
map1b
gRNA only
mecp2
GFP signal
Tet1 locus
Tet2 locus
Tet3 locus
3.7%
18.6%
14.9%

FIG. 49

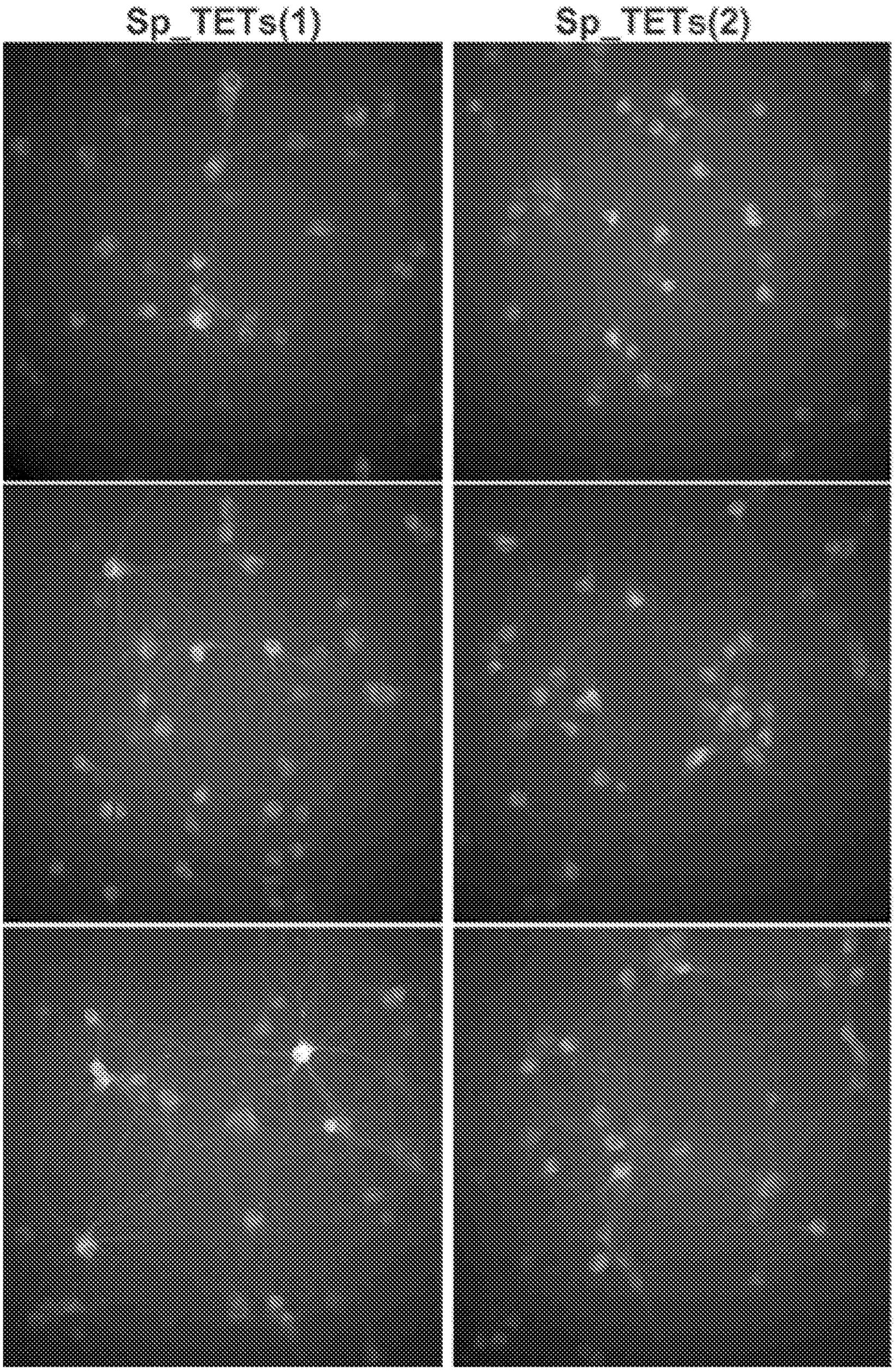
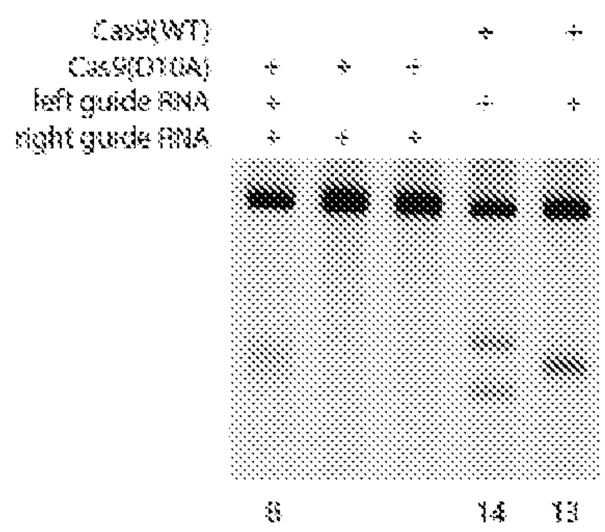
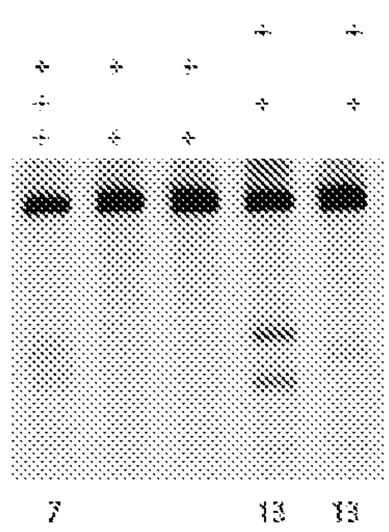


FIG. 50

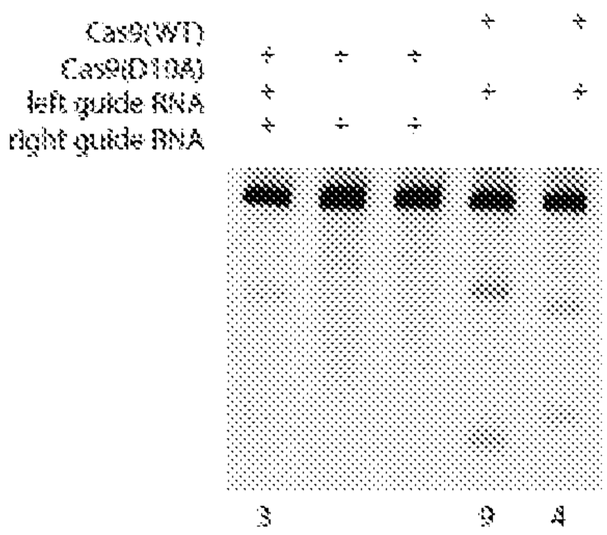
	pattern	overhang
1	CCnNNNNNNNNNNNNNNNNnnnnnnnnnnnnnnNNNNNNNNNNNNNNNNnGG	38
2	CCnNNNNNNNNNNNNNNNNnnnnnnnnnnnnNNNNNNNNNNNNNNNNnGG	34
3	CCnNNNNNNNNNNNNNNNNnnnnnnnnnnNNNNNNNNNNNNNNNNnGG	30
4	CCnNNNNNNNNNNNNNNNNnnnnnnnnnNNNNNNNNNNNNNNNNnGG	26
5	CCnNNNNNNNNNNNNNNNNnnnnnnnnNNNNNNNNNNNNNNNNnGG	22
6	CCnNNNNNNNNNNNNNNNNnnnnnnnNNNNNNNNNNNNNNNNnGG	18
7	CCnNNNNNNNNNNNNNNNNnnnnnnNNNNNNNNNNNNNNNNnGG	14
8	CCnNNNNNNNNNNNNNNNNnnnnnNNNNNNNNNNNNNNNNnGG	10
9	CCnNNNNNNNNNNNNNNNNnnnnNNNNNNNNNNNNNNNNnGG	6
10	CCnNNNNNNNNNNNNNNNNnnnNNNNNNNNNNNNNNNNnGG	4
11	CCnNNNNNNNNNNNNNNNNnnNNNNNNNNNNNNNNNNnGG	3
12	CCnNNNNNNNNNNNNNNNNnNNNNNNNNNNNNNNNNnGG	2
13	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	1
14	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	0
15	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	1
16	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	2
17	CCnNNNNNNNNNNNNNNNNNNNNNNNNNNNNnGG	3
18	CCnnnGG	4
19	CCGG	8
20	GGCC	10
21	GGNNCC	12
22	GGNNNCC	14
23	GGNNNNCC	16
24	GGNNNNNNNNCC	20



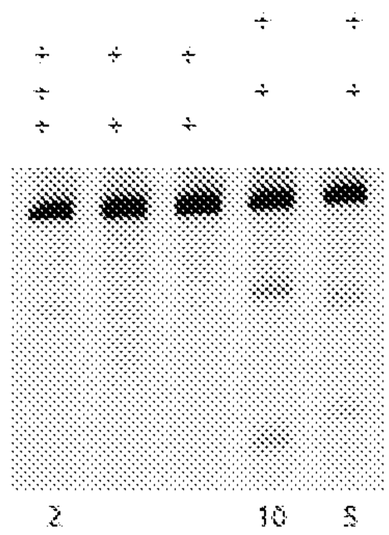
Pattern 1



Pattern 2



Pattern 3



Pattern 4

FIG. 51

Reverse PCR primers for generating U6-guide RNA expression Cassette (pair with U6 forward primer)

FE0260	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTGCCGCATTGCCGCGAAGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 1
FE0261	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCACATCGACCTGGTGGCCACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 2
FE0262	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAAAGTGCGGTTTCGTCCTTTCCACAAG	Left guide RNA 3
FE0263	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAAGACAAAGTACAAAAGTGCGGTTTCGTCCTTTCCACAAG	Left guide RNA 4
FE0264	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTGCCGCATTGCCGCGAAGCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 5
FE0265	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAAGGCAGATGCTGCTGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 6
FE0266	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGAGTCCAGACAGAAGAAGAAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 7
FE0267	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAAAGTGCGGTTTCGTCCTTTCCACAAG	Left guide RNA 8
FE0268	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGCGAAGAAAGAAAGGTCCCGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 9
FE0269	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTCCACATCGACCTGGTGGCCACAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 10
FE0270	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGCCCCAGGGGTAAGGTGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 11
FE0271	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCCTCCTGGCCCAGGGTAAGGTGGTTTCGTCCTTTCCACAAG	Left guide RNA 12
FE0272	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGAGGGACAAAGTACAAAGTGCGGTTTCGTCCTTTCCACAAG	Left guide RNA 13
FE0273	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGGTTCCTAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 14
FE0274	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGTGAAGGTGTGGTTCCAAAGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 15
FE0275	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCCCAAGGACAAAGTACAAAGTGGTTTCGTCCTTTCCACAAG	Left guide RNA 16
FE0276	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTGGCCCCAGGGTAAGGTGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 17
FE0277	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGTGAAGGTGTGGGTCCTAGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 18
FE0278	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGAGGACAAAGTACAAAACGGCGGTTTCGTCCTTTCCACAAG	Left guide RNA 19
FE0279	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGTAGTCGAAGGGGCCACAGATTCGTGGTTTCGTCCTTTCCACAAG	Left guide RNA 20
FE0280	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACTTCACCCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 21
FE0281	AAAAAAAGCACCGACTCGGTGCCACTGTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCCCTAGTCACTGGGAGGTGAGTGGTTCGTCCTTTCCACAAG	Left guide RNA 22
FE0282	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCAGAGGAGCCACTGGGGCCTGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 23
FE0283	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCACTTTCACCCTGGGCCAGGGGGTGTTTCGTCCTTTCCACAAG	Left guide RNA 24
FE0284	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCCTATTGGCCTGGGTTCCTGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 1
FE0285	AAAAAAAGCACCGACTCGGTGCCACTGTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGACTGGCCTGCTGGTGGCAAGGTGGTTCGTCCTTTCCACAAG	Right guide RNA 2
FE0286	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCTGCCTCTGCTCCAGGCTTCCTGGCCGGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 3
FE0287	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCTCCAGCTTCTGGGCCGTGTTGGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 4
FE0288	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATTGGCCTGCTTCCTGGTGGCAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 5
FE0289	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGCCAGGCAGGCCACTGGGGCTGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 6
FE0290	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCCTTATTCTGGCTCCGGACTCCGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 7
FE0291	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACACCGGGGGGACAAAGTACAAAGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 8
FE0292	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCTTCCTTTGCTCCGGACTCAGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 9
FE0293	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTGAGGTGACTGGGAGCCCTGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 10
FE0294	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGGCCAGGGAGGGAAGGGGAGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 11
FE0295	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGCAGCGAAGGGGCTCCAGAATGGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 12
FE0296	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCGGTTCCTGGAACCAGCCCTTGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 13
FE0297	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCAGAGGAGGGAGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 14
FE0298	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCACCTGGGCCCAGGGAGGGAGGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 15
FE0299	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTCGAAACCAACACCTTTCAGGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 16
FE0300	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGAAGGGAAGGGGGCCACAGATTCGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 17
FE0301	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCAGTCAGGGAGGAAGGGGTCAGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 18
FE0302	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGTTCTCGAAACCAACACCTTTCAGGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 19
FE0303	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACAGGTGAAGGTGTGGTTTCAGGGTGGTTTCGTCCTTTCCACAAG	Right guide RNA 20
FE0304	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAACCGGAGGGACAAAGTACAAAGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 21
FE0305	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCAACCCACGAGGAGCCAAAGTGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 22
FE0306	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGTTTCTCTATCTGTGCCCCGGGTGTTTCGTCCTTTCCACAAG	Right guide RNA 23
FE0307	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGAGGACAAAGTACAAAAGTGCGGTTTCGTCCTTTCCACAAG	Right guide RNA 24

FIG. 52

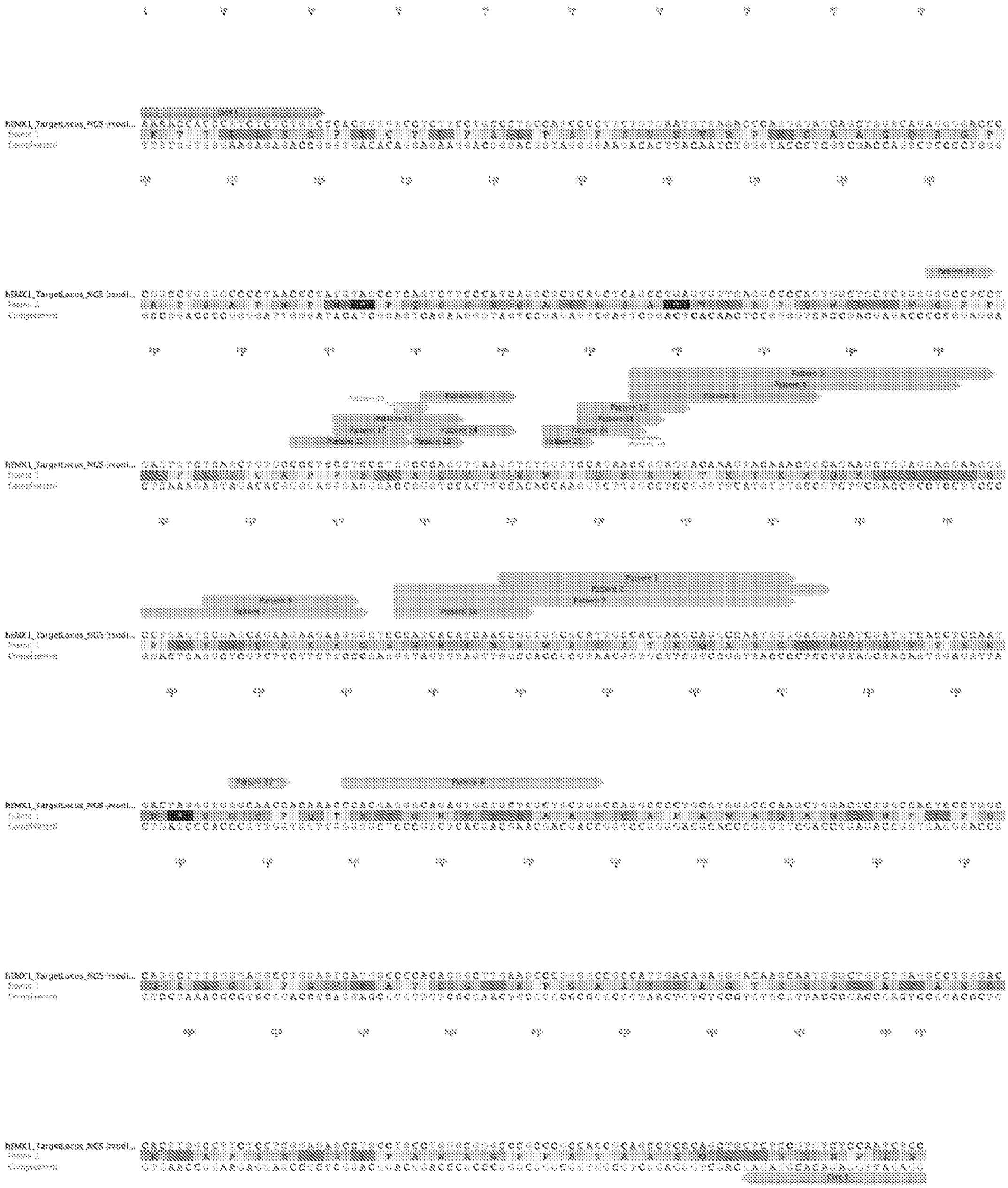


FIG. 53

lane #	Cas9 to use	Left gRNA	Right gRNA	Overhang (D10A) - number of bases protruding from 5'
1	D10A	left 23	left 12	-36
2	D10A	right 4	left 9	-25
3	D10A	left 23	right 23	-16
4	D10A	right 7	left 10	-15
5	D10A	right 16	left 3	-8
6	D10A	right 22	right 6	26
7	D10A	left 12	right 16	31
8	D10A	left 12	right 13	34
9	D10A	left 10	right 1	38
10	D10A	right 23	right 16	51
11	D10A	right 23	right 13	54
12	D10A	left 3	right 7	57
13	D10A	left 12	right 4	65
14	D10A	left 12	right 3	69
15	D10A	left 3	right 10	76
16	D10A	right 23	right 4	85
17	D10A	left 12	right 9	95
18	D10A	left 12	right 10	115
19	D10A	right 23	right 10	135
20	D10A	left 12	right 2	145
21	D10A	left 12	left 22	181
22	D10A	right 23	left 22	201
23	D10A	left 12	right 6	222
24	D10A	right 23	right 6	242

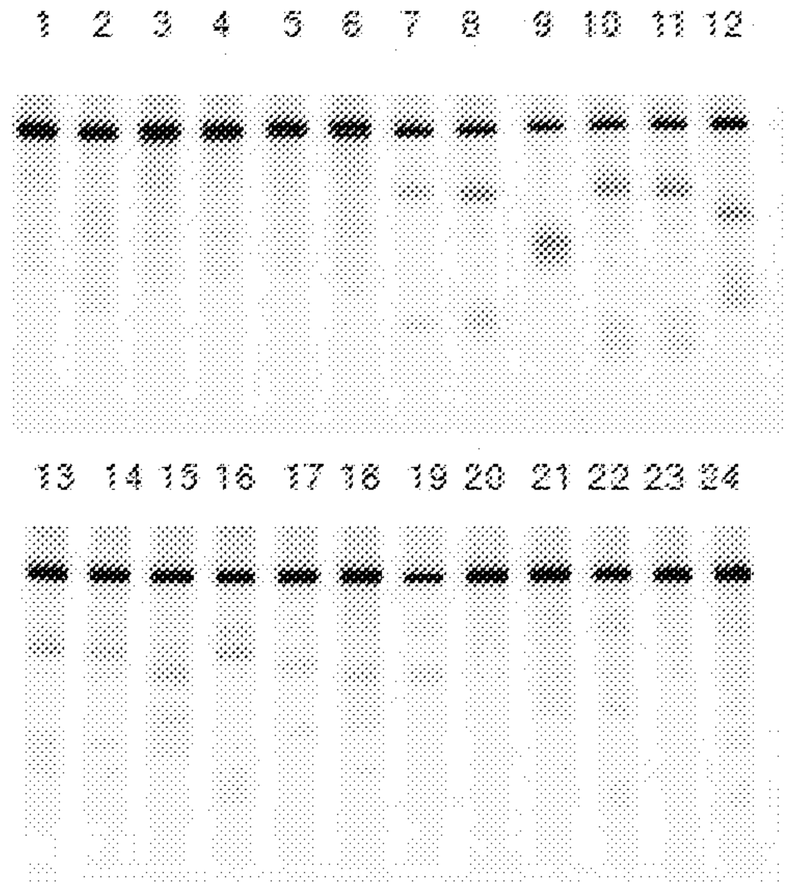


FIG. 54

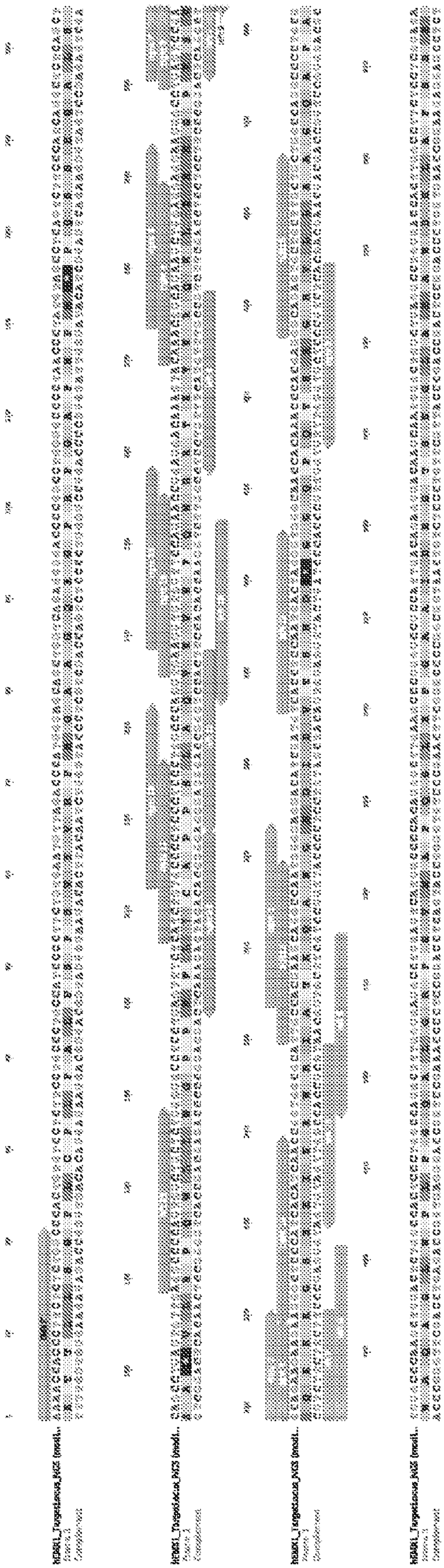
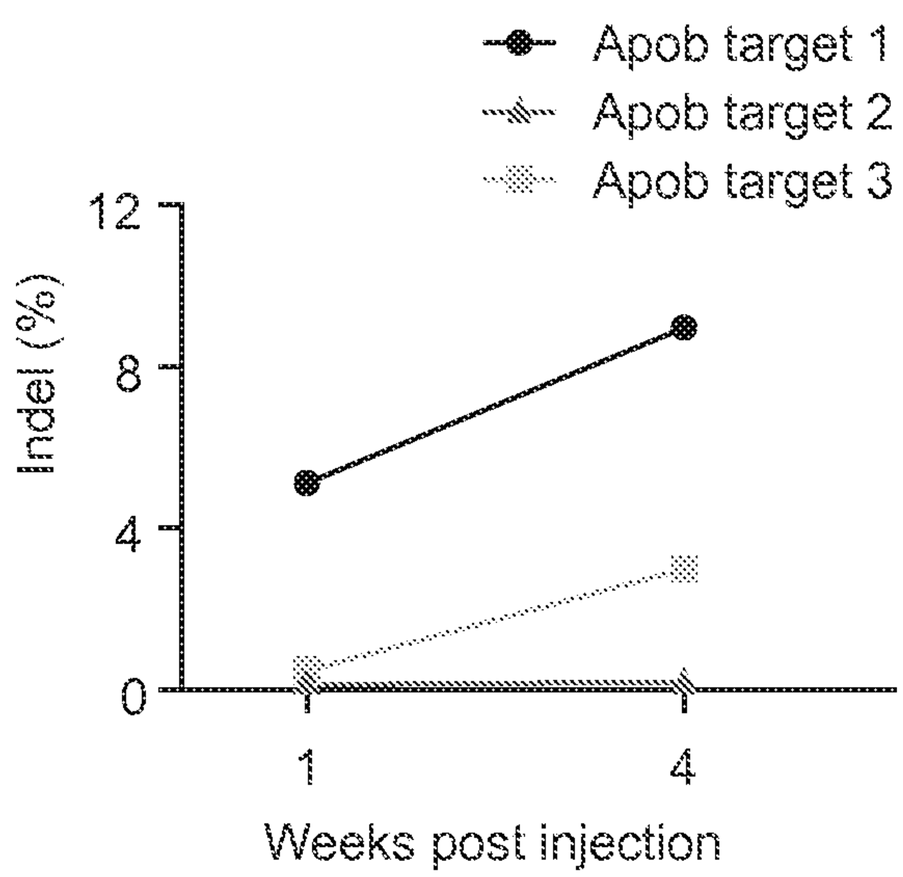
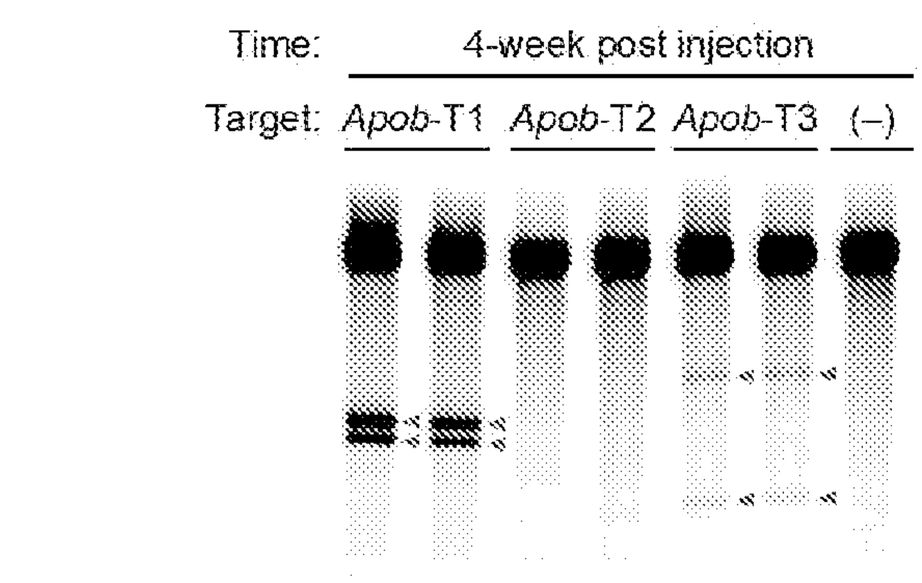


FIG. 55

a

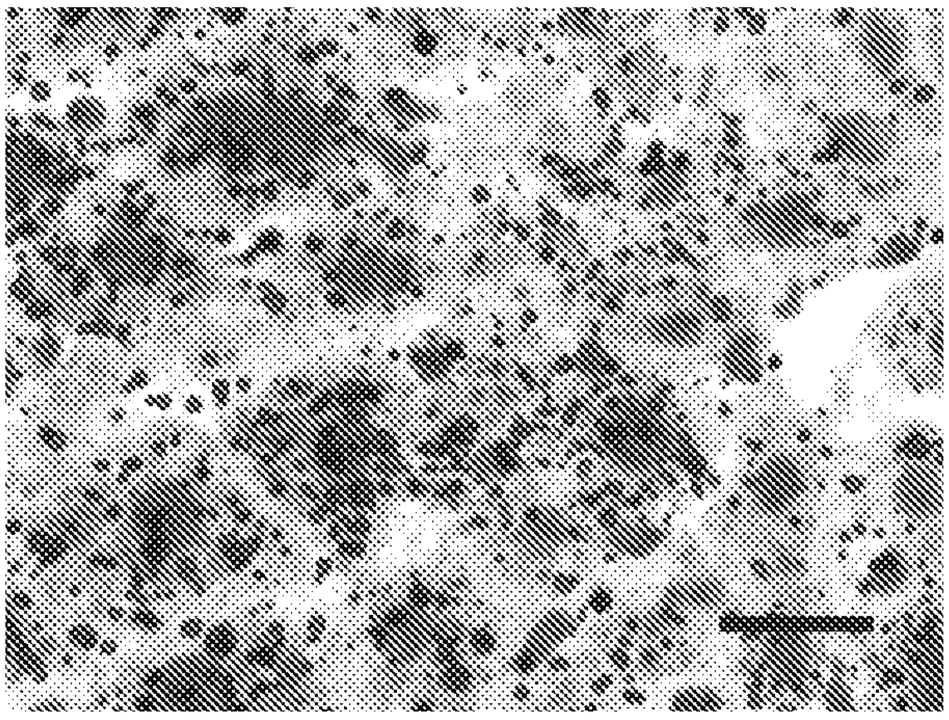


b

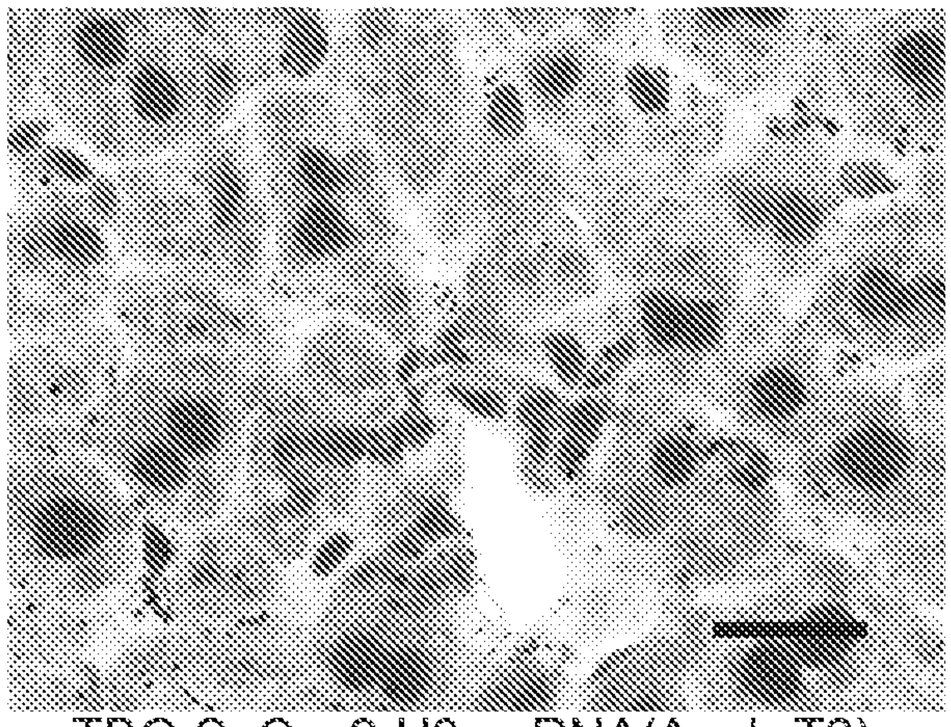


ApoB-T1 = Target 1; ApoB-T2 = Target 2, etc.

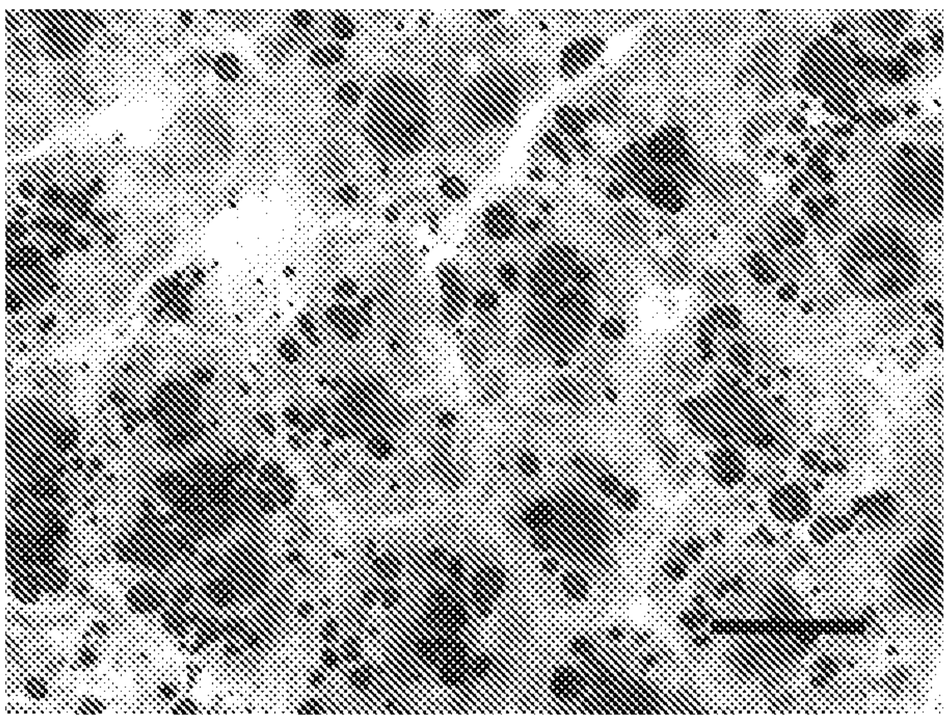
FIG. 56



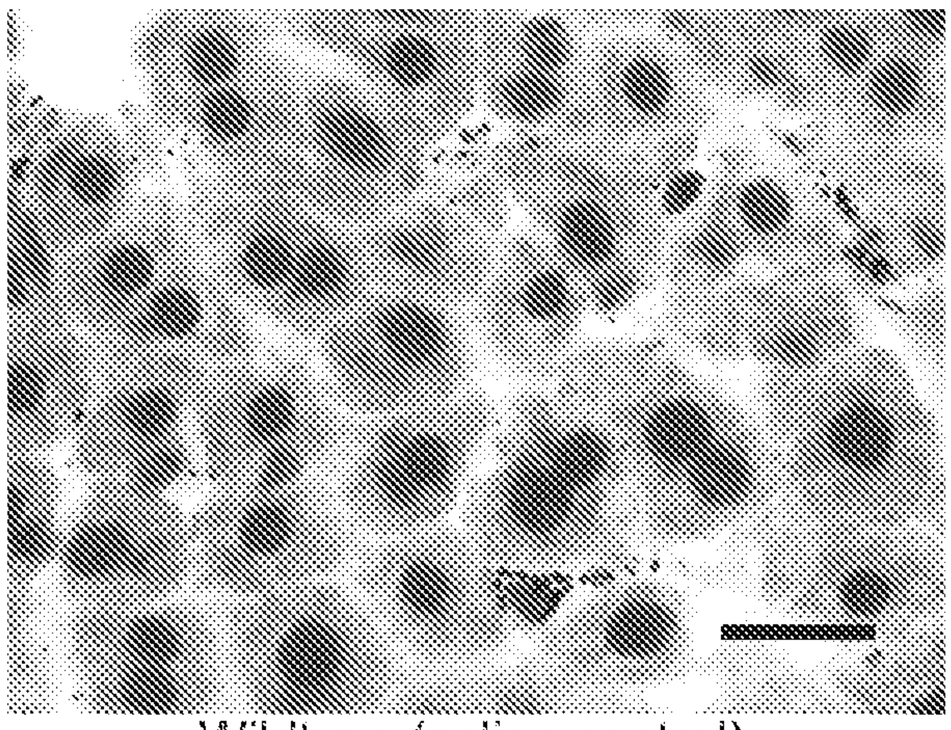
TBG-SaCas9-U6-sgRNA(Apob-T1)
4wk post injection



TBG-SaCas9-U6-sgRNA(Apob-T2)
4wk post injection



TBG-SaCas9-U6-sgRNA(Apob-T3)
4wk post injection



Wildtype (saline control)
4wk post injection

FIG. 57

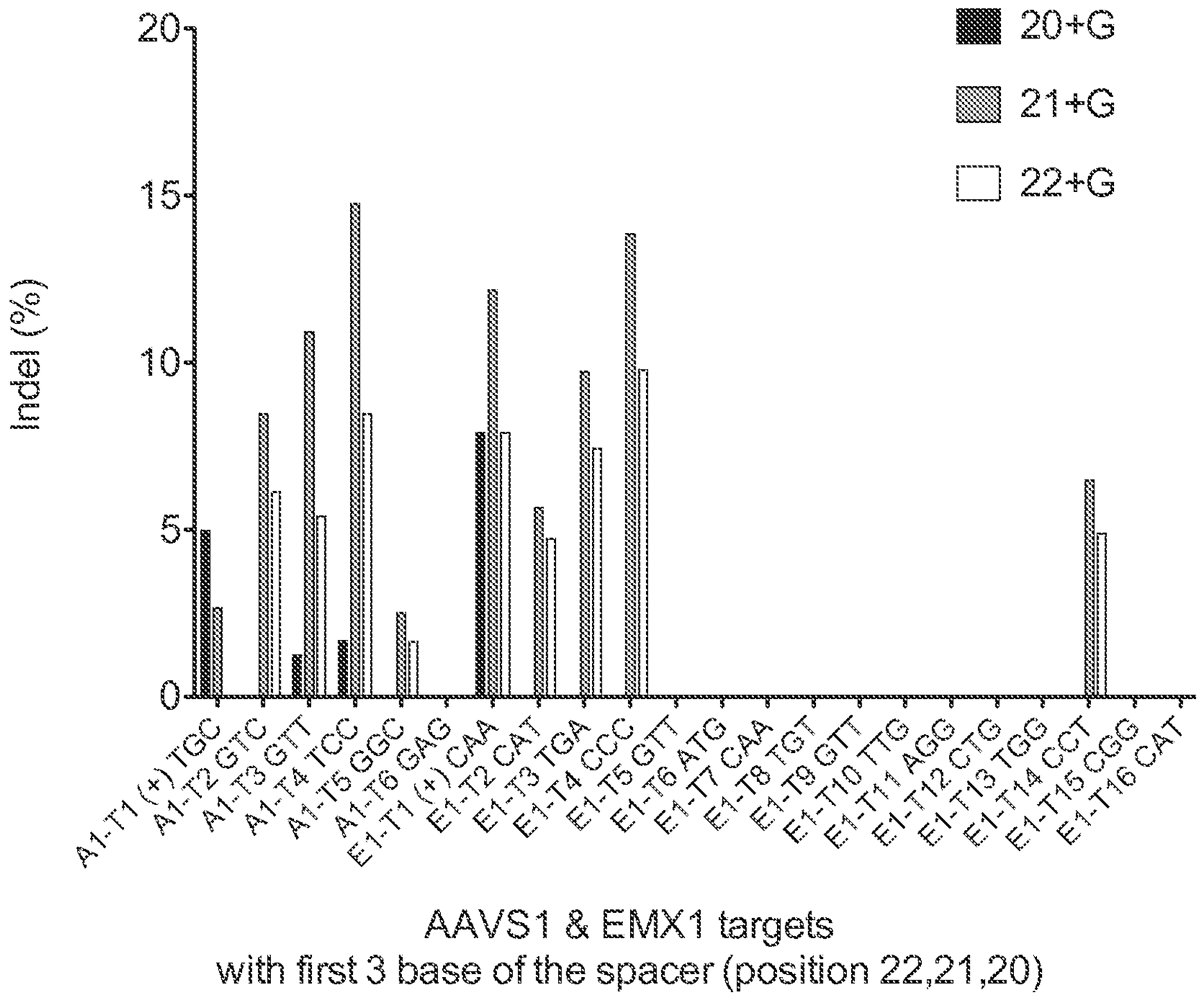

20+G
21+G
22+G
Indel (%)
0
5
10
15
20
A1-T1 (+) TGC
A1-T2 GTC
A1-T3 GTT
A1-T4 TCC
A1-T5 GGC
A1-T6 GAG
E1-T1 (+) CAA
E1-T2 CAT
E1-T3 TGA
E1-T4 CCC
E1-T5 GTT
E1-T6 ATG
E1-T7 CAA
E1-T8 TGT
E1-T9 GTT
E1-T10 TTG
E1-T11 AGG
E1-T12 CTG
E1-T13 TGG
E1-T14 CCT
E1-T15 CGG
E1-T16 CAT
AAVS1 & EMX1 targets
with first 3 base of the spacer (position 22,21,20)

FIG. 58

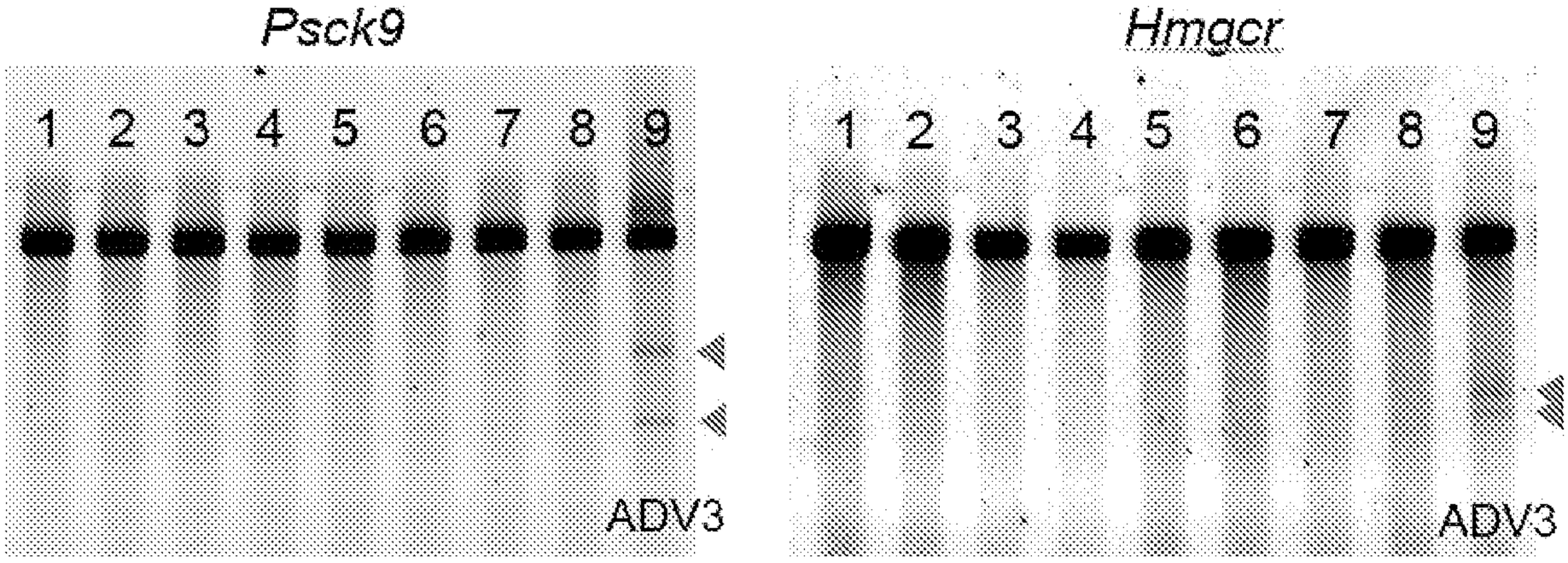


FIG. 59

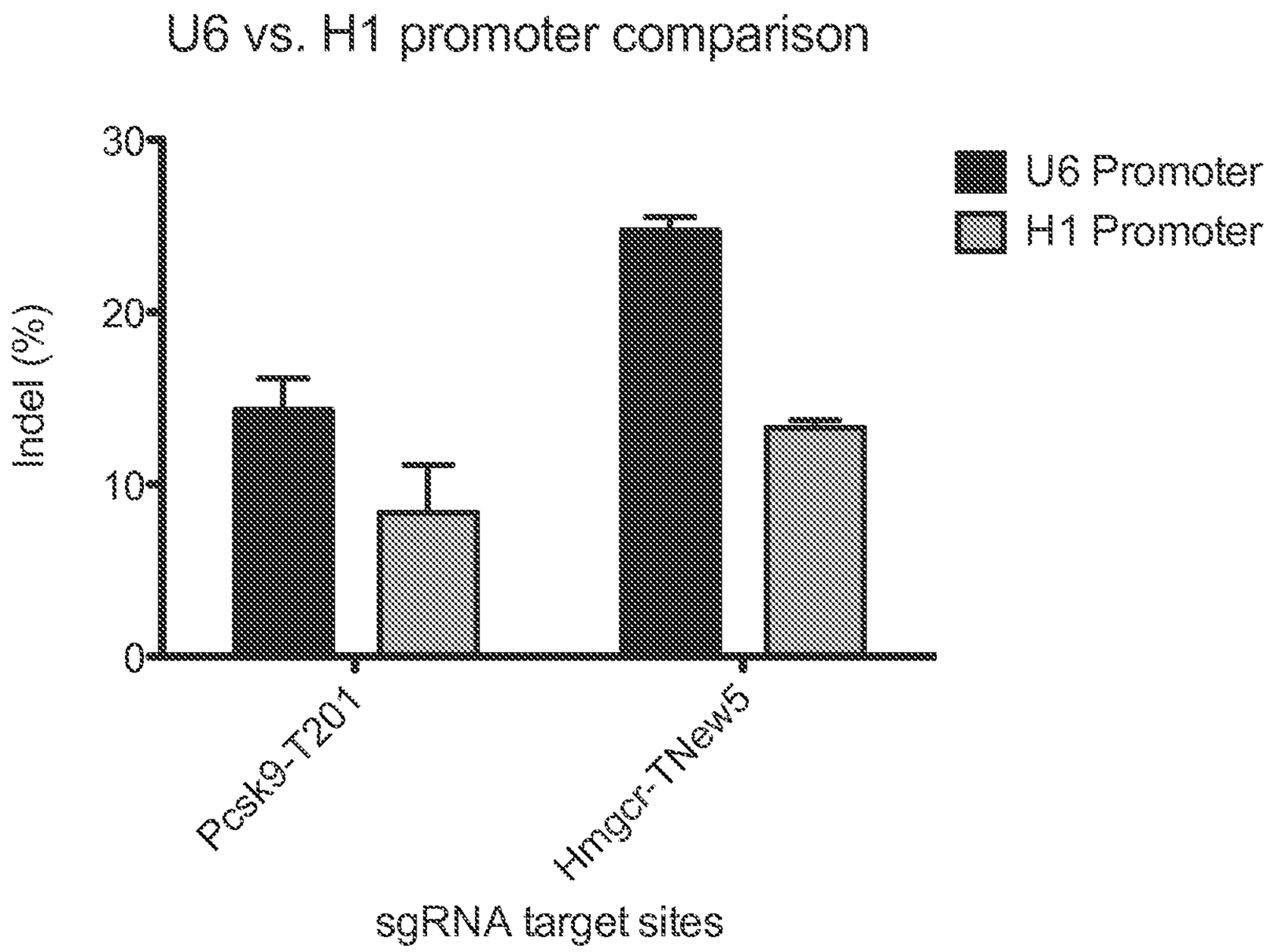

U6 vs. H1 promoter comparison
U6 Promoter
H1 Promoter
Indel (%)
30
20
10
0
Pcsk9-T201
Hmgcr-TNew5
sgRNA target sites

FIG. 60

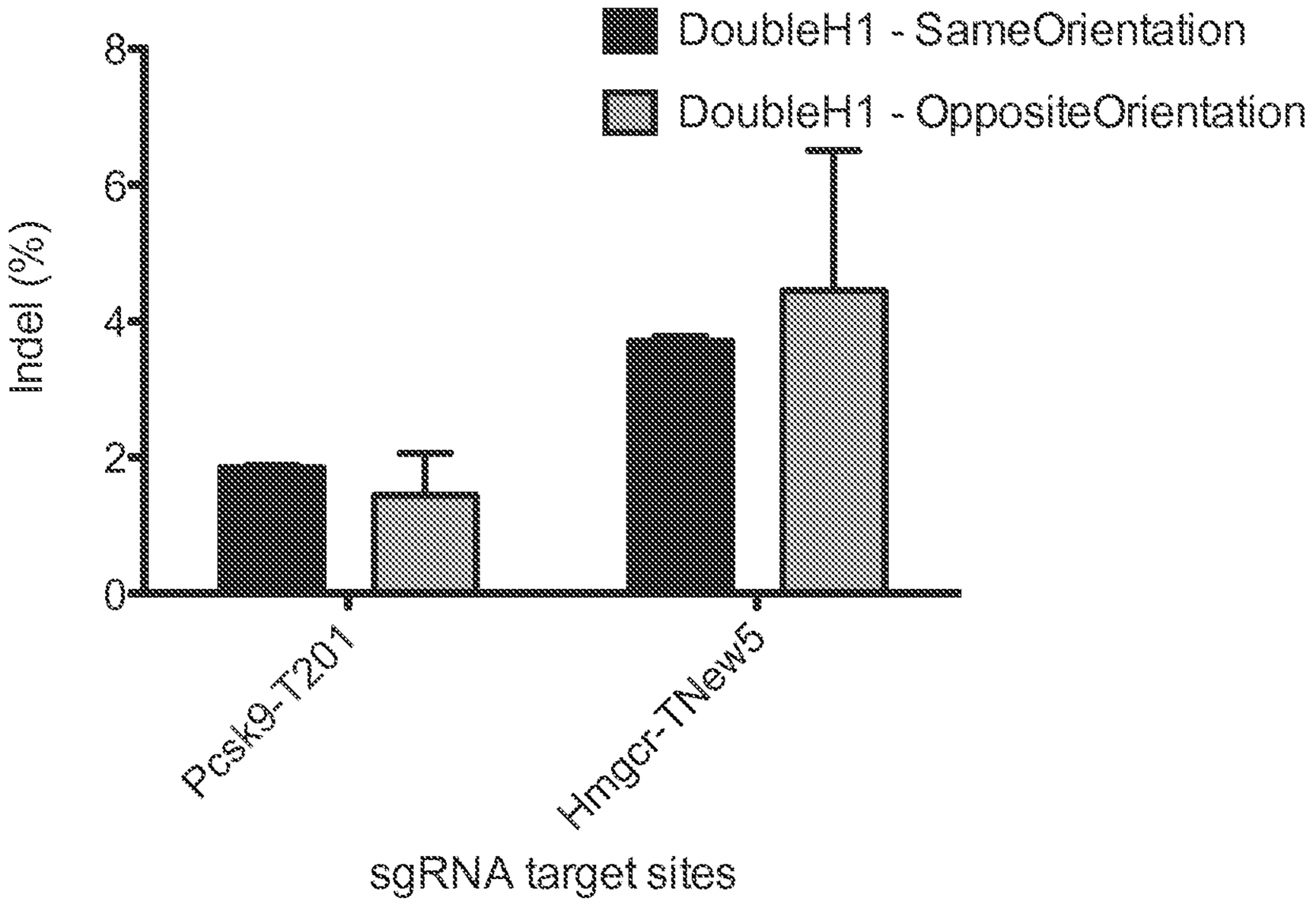

Double short H1 promoter test
DoubleH1 - SameOrientation
DoubleH1 - OppositeOrientation
Indel (%)
0
2
4
6
8
Pcsk9-T201
Hmgcr-TNew5
sgRNA target sites

FIG. 61

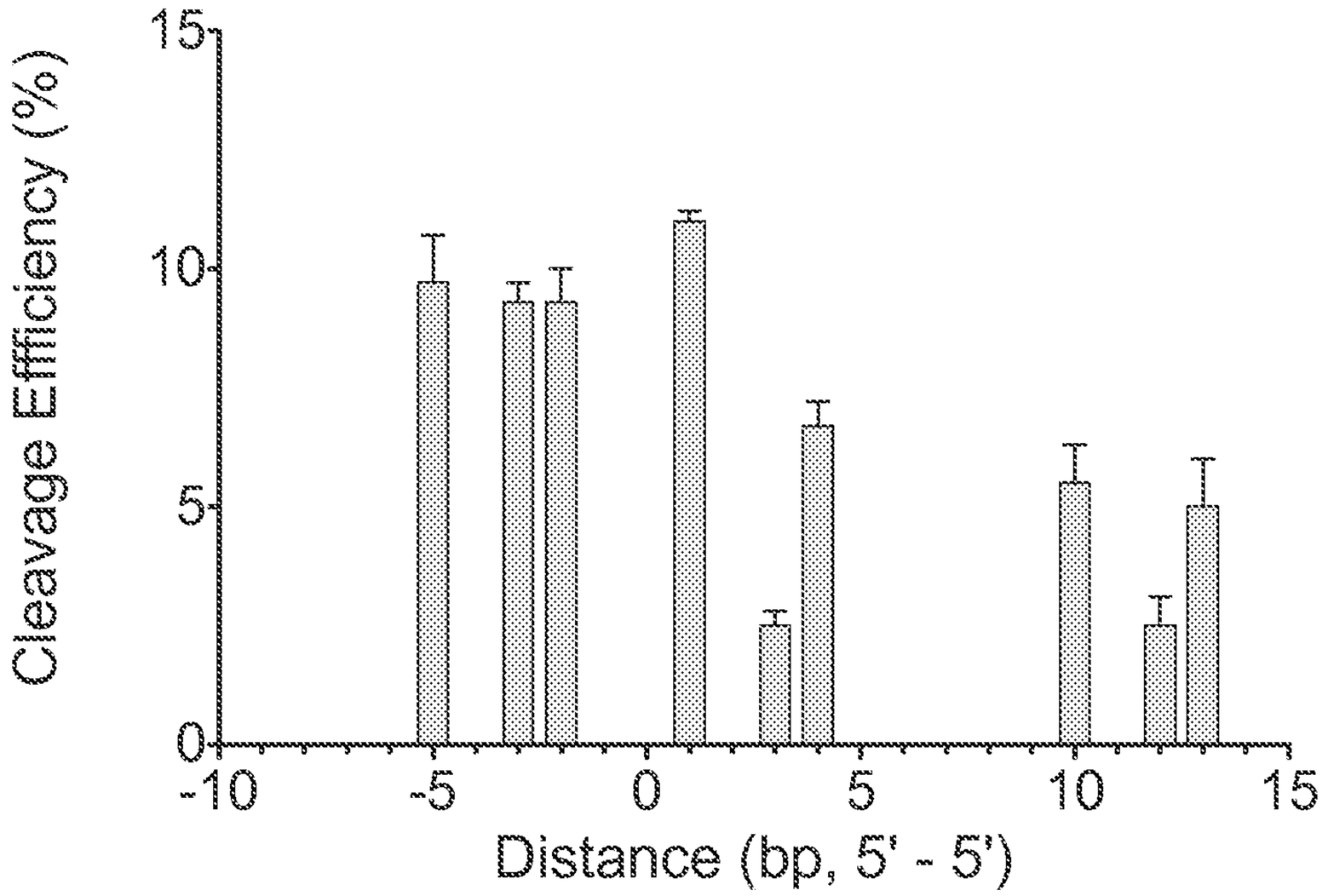


FIG. 62

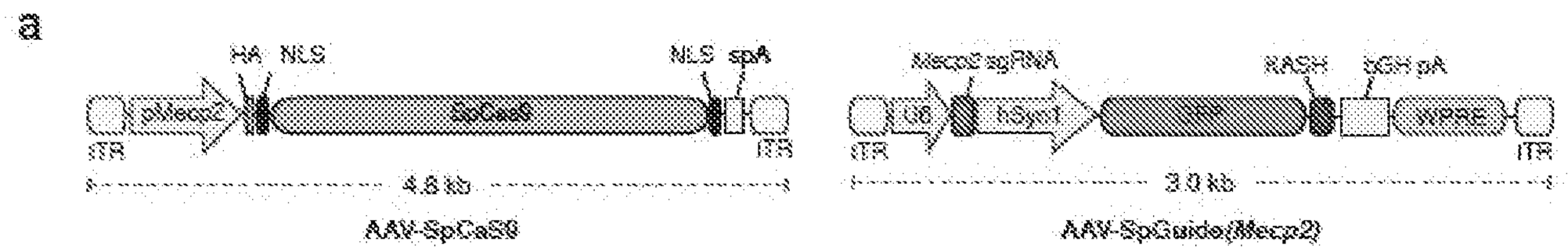

a
HA NLS
NLS spA
pMecp2
SpCas9
ITR
ITR
4.8 kb
AAV-SpCas9
MecpS sgRNA
U6
hSyn1
KASH
SGH pA
WPRE
ITR
ITR
3.0 kb
AAV-SpGuide(Mecp2)
b
HA-SpCas9
GFP-KASH
merge
c
% of labeled (1) neurons expressing labeled (2)
100
50
0
(1) GFP+
(2) SpCas9+
(1) SpCas9+
(2) GFP+
d
target 5
PAM
DNA
target
Mecp2 sgRNA
Mecp2 locus
ex
CCATTCTGCAGAGCCAG-------CAGAGG
CCATTCTGCAGAGCCAGAGGC------AGG
CCATTCTGCAGAGCC---------CAGAGG
CCATTCTGCAGAGCCAG--------AGAGG
CCATTCTGCAGAGC----------AGAGG
CCATTCTGCAGAGCCCC--------CAGAGG
CCATTCTGCAGAGCCAGAGAGC-----AGG
CCATTCTGCAGAGCCAG-8bp-CAGAGG
CCATTCTGCAGAGCCAG-33bp----AGG
CCATTCTGCAGAGCCAG-------AAGAGG
CCATTCTGCAGAGCCAG----CACAGAGG
CCATTCTGCAGAGCCA--------CAGAGG
CCATTCTGCAGAGCCA-----GTCAGAGG
e
SpCas9 + +
Mecp2 sgRNA — +
indel (%) N.D. 13
f
SpCas9 + +
Mecp2 sgRNA — +
MeCP2
GAPDH
MeCP2 fold change
1.0
0.5
0
**
g
SpCas9 only
SpCas9 + MecP2 sgRNA
MeCP2
DAPI
h
% of MeCP2+ nuclei in total number of nuclei
100
75
50
25
0
****
SpCas9 + +
Mecp2 sgRNA — +

FIG. 63

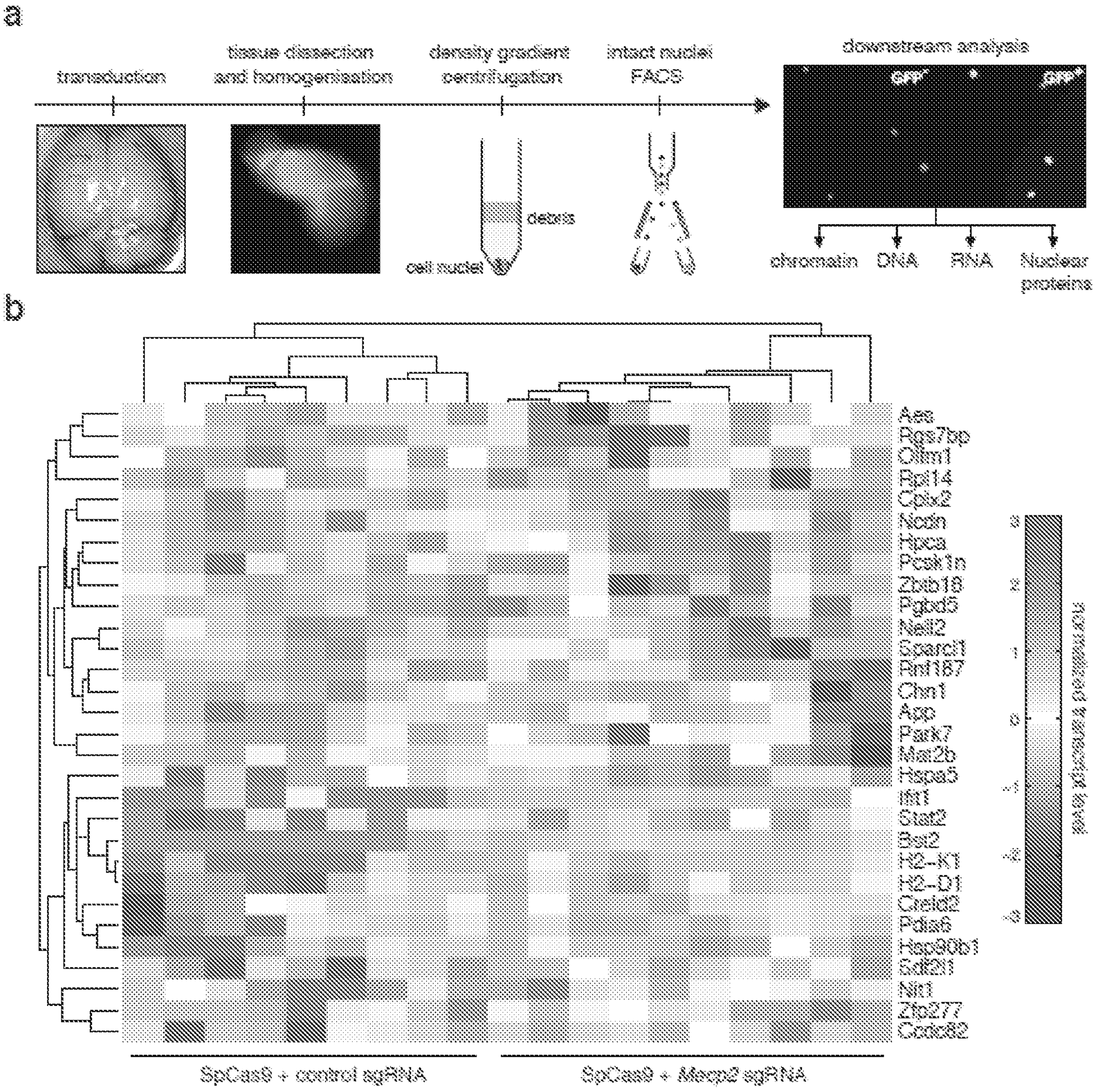

a
transduction
tissue dissection and homogenization
density gradient centrifugation
intact nuclei FACS
downstream analysis
GFP
GFP
debris
cell nuclei
chromatin
DNA
RNA
Nuclear proteins
b
Aes
Rgs7bp
Olfm1
Rpl14
Cplx2
Nostrin
Hpca
Pcsk1n
Zbtb18
Pgbd5
Nell2
Stard1
Rnf167
Chn1
App
Park7
Mar2b
Hspa5
Ifit1
Stat2
Bst2
H2-K1
H2-D1
Creld2
Pdia6
Hsp90b1
Sdf2l1
Nit1
Zfp277
Cdc42
SpCas9 + control sgRNA
SpCas9 + Mecp2 sgRNA

FIG. 64

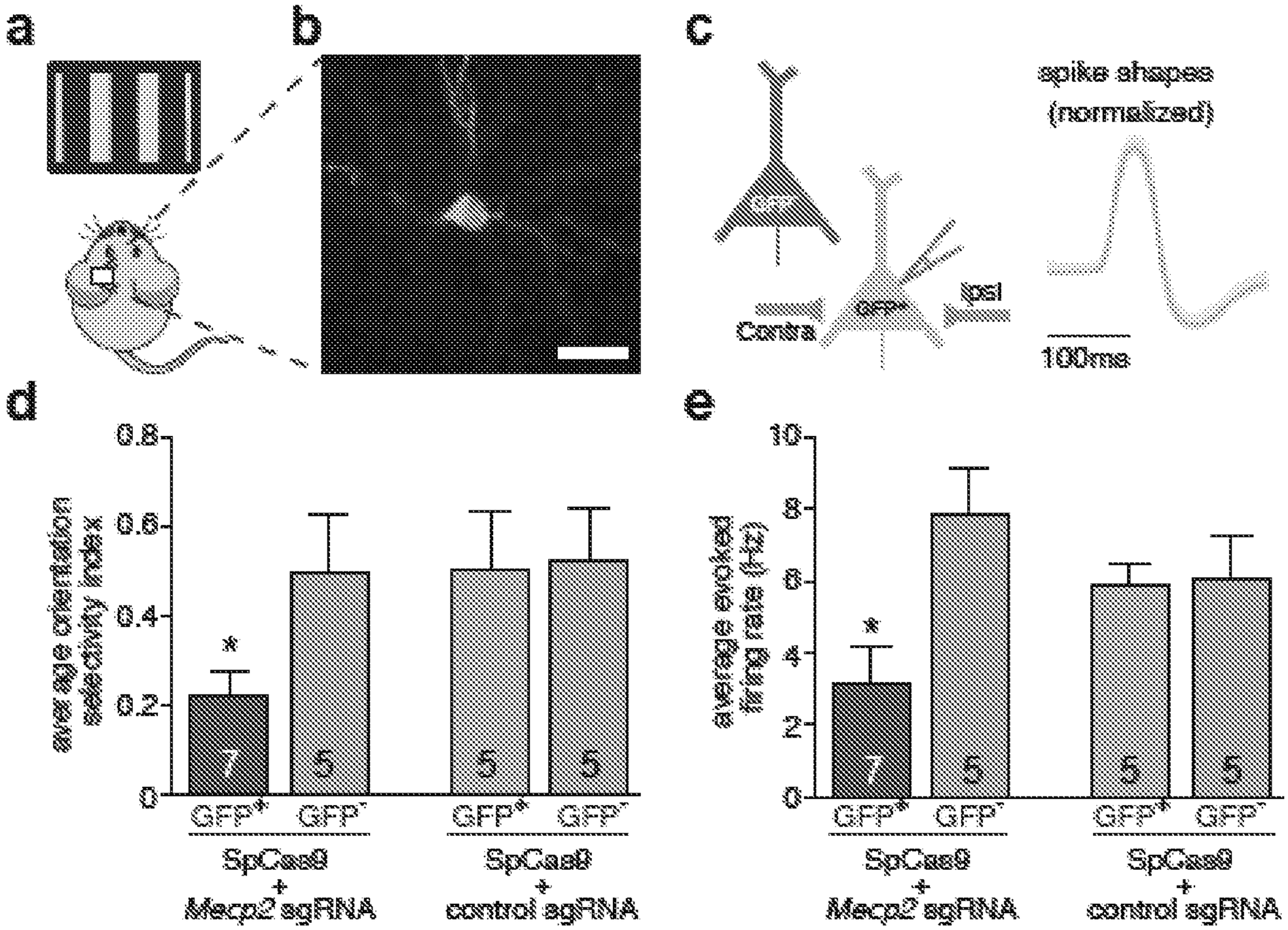

a
b
c
spike shapes (normalized)
cre
Contra
cre+
Ipsi
100ms
d
average orientation selectivity index
0.8
0.6
0.4
0.2
0
*
7
5
5
5
GFP+
GFP-
GFP+
GFP-
SpCas9 + Mecp2 sgRNA
SpCas9 + control sgRNA
e
average evoked firing rate (Hz)
10
8
6
4
2
0
*
7
5
5
5
GFP+
GFP-
GFP+
GFP-
SpCas9 + Mecp2 sgRNA
SpCas9 + control sgRNA

FIG. 65

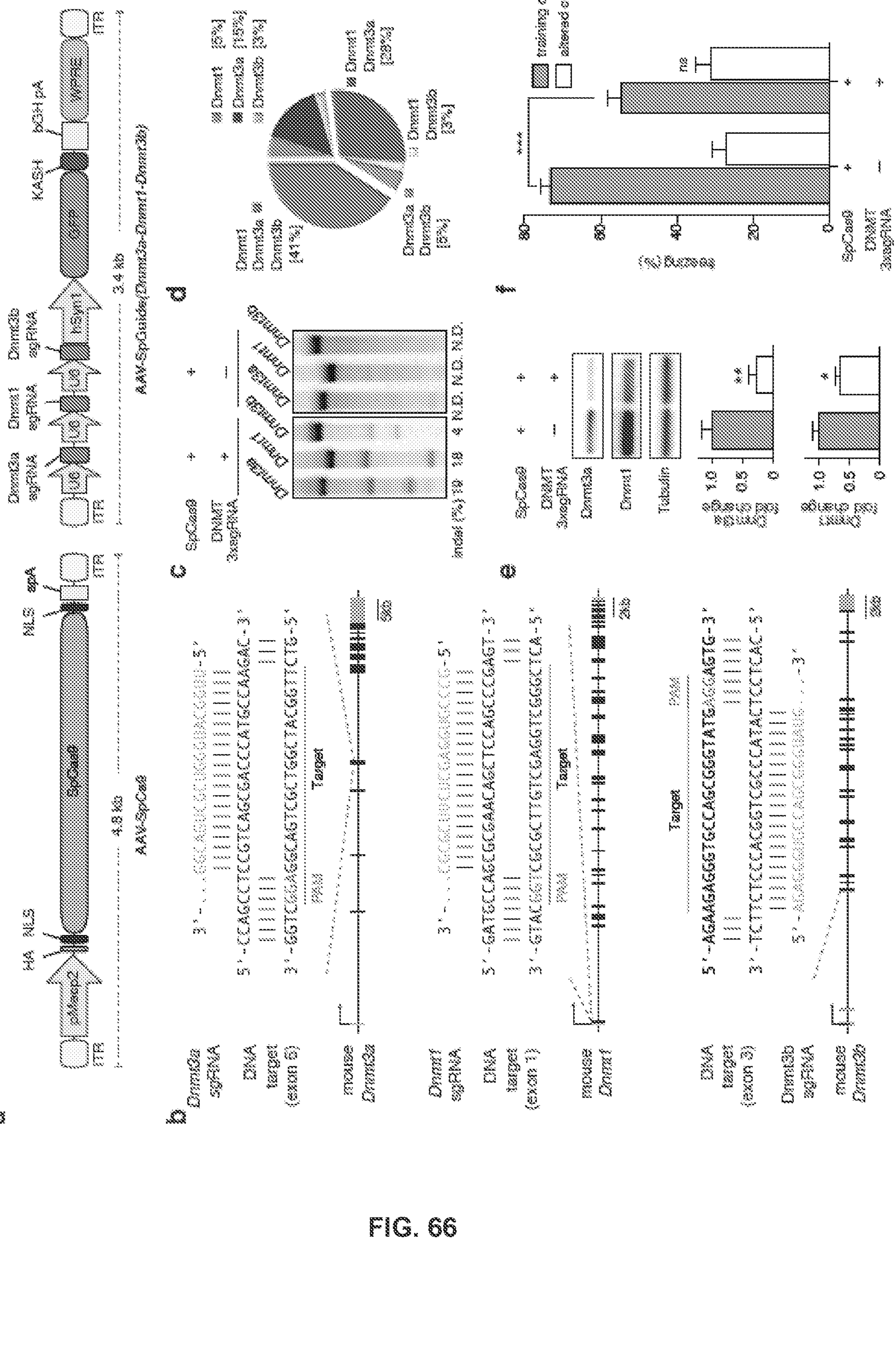

AAV-SpGuides/Dnmt3a-Dnmt1-Dnmt3b
ITR
U6
Dnmt3a sgRNA
U6
Dnmt1 sgRNA
U6
Dnmt3b sgRNA
hSyn1
KASH
3.4 kb
ITR
AAV-SpCas9
HA
NLS
SpCas9
NLS
spA
4.8 kb
ITR
a
b
Dnmt3a sgRNA
DNA target (exon 6)
mouse Dnmt3a
Dnmt1 sgRNA
DNA target (exon 1)
mouse Dnmt1
Dnmt3b sgRNA
DNA target (exon 3)
mouse Dnmt3b
PAM
Target
c
SpCas9
DNMT 3xsgRNA
indel (%)
N.D.
d
Dnmt1 (5%)
Dnmt3a (15%)
Dnmt3b (3%)
Dnmt1 Dnmt3a (28%)
Dnmt1 Dnmt3b (5%)
Dnmt3a Dnmt3b (41%)
Dnmt1 Dnmt3a Dnmt3b (6%)
e
training context
altered context
ns
***
(%) Freezing
Dnmt3a
Dnmt1
Tubulin
Dnmt3a/Tubulin
Dnmt1/Tubulin
**
*

FIG. 66

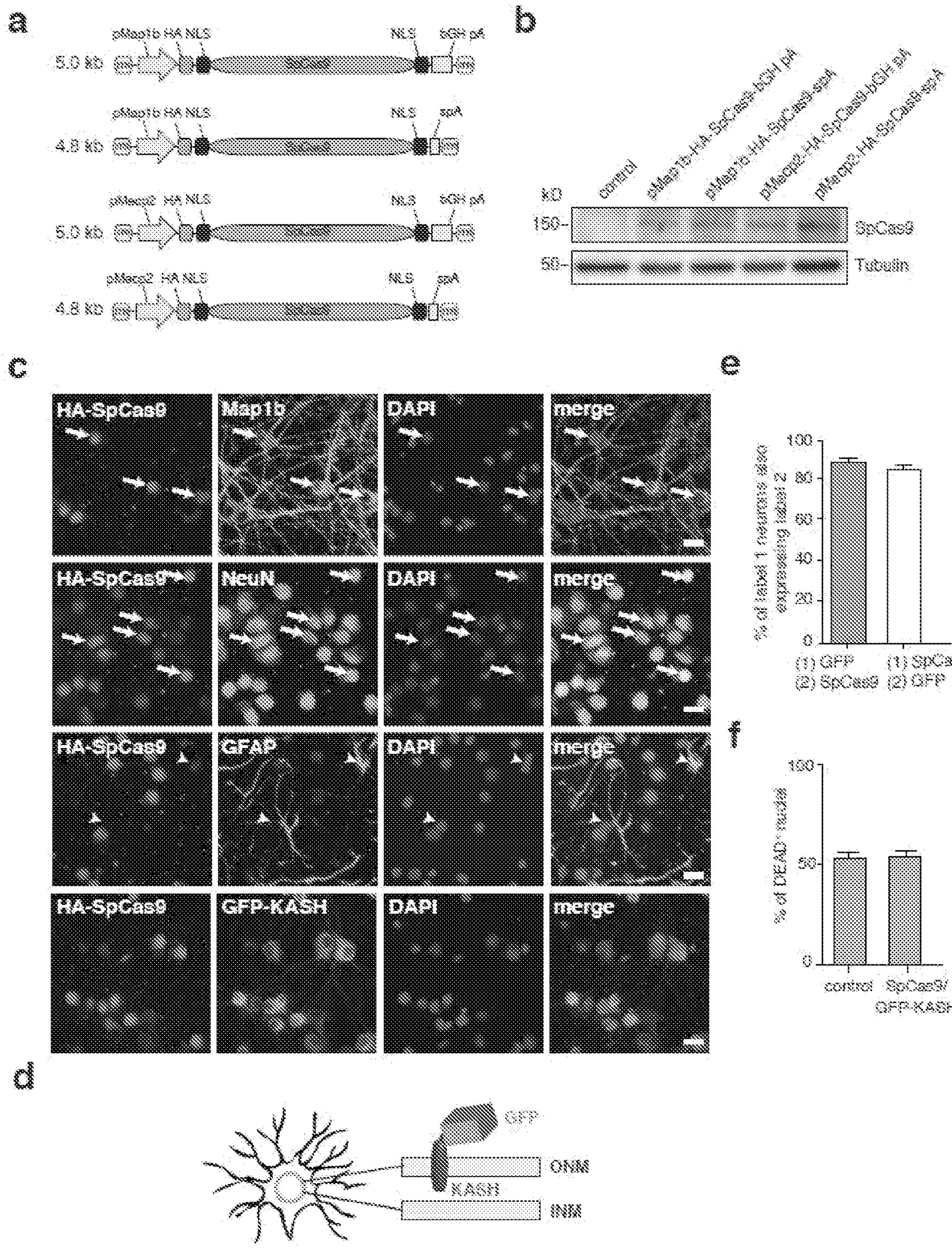
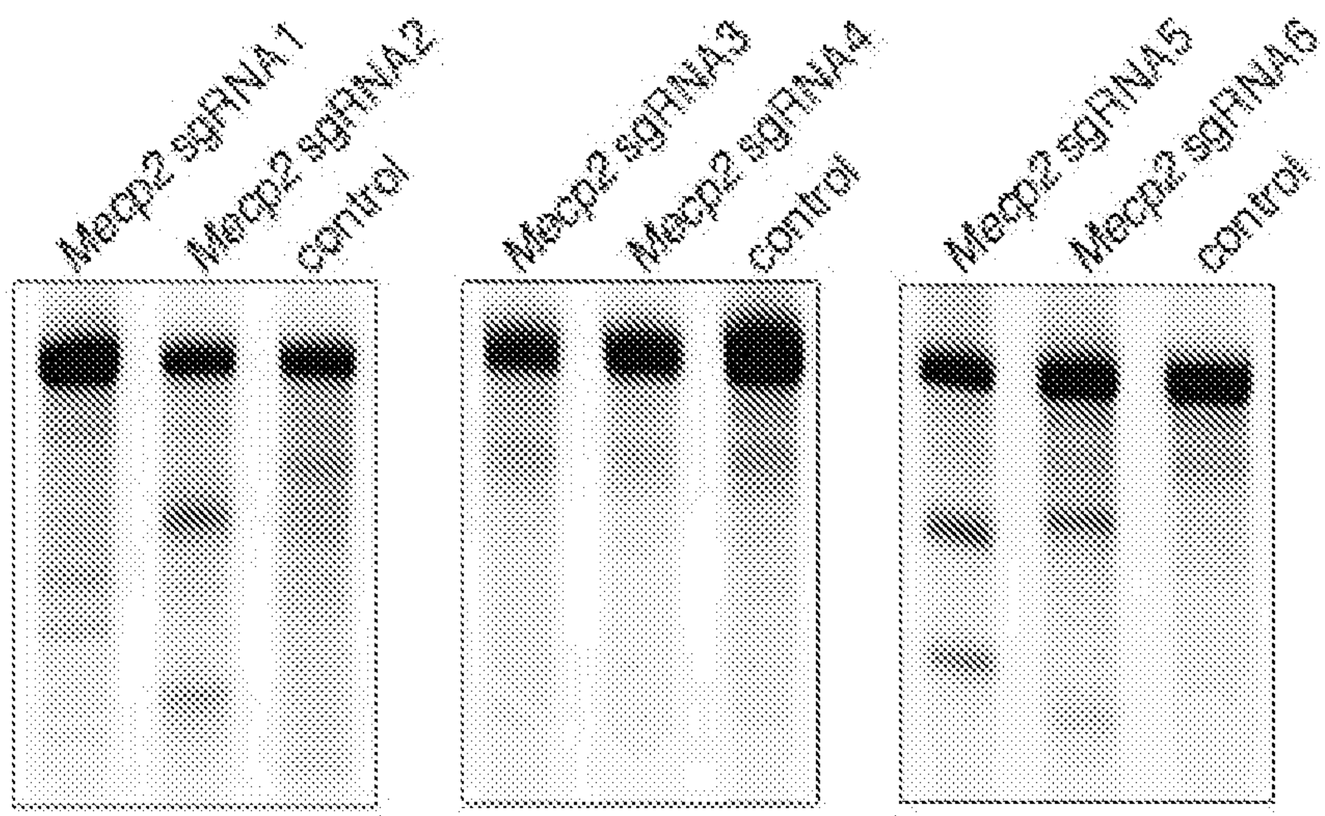

a
pMecp1b HA NLS
NLS
bGH pA
5.0 kb
SpCas9
pMecp1b HA NLS
NLS
spA
4.8 kb
SpCas9
pMecp2 HA NLS
NLS
bGH pA
5.0 kb
SpCas9
pMecp2 HA NLS
NLS
spA
4.8 kb
SpCas9
b
kD
control
pMecp1b-HA-SpCas9-bGH pA
pMecp1b-HA-SpCas9-spA
pMecp2-HA-SpCas9-bGH pA
pMecp2-HA-SpCas9-spA
150-
SpCas9
50-
Tubulin
c
HA-SpCas9
Map1b
DAPI
merge
HA-SpCas9
NeuN
DAPI
merge
HA-SpCas9
GFAP
DAPI
merge
HA-SpCas9
GFP-KASH
DAPI
merge
d
GFP
ONM
KASH
INM
e
% of labeled 1 neurons also expressing label 2
100
80
60
40
20
0
(1) GFP
(1) SpCas9
(2) SpCas9
(2) GFP
f
% of DEAD nuclei
100
50
0
control
SpCas9/
GFP-KASH

FIG. 67

a

	Mecp2 target sequence	PAM
Target 1	CTGGGAGAGGGAGCCCCTCC	AGG
Target 2	AAAGGTGGGAGACACCTCCT	TGG
Target 3	TCCAACCTTCAGGCAAGGTG	GGG
Target 4	AGGAAGTCTGGCCGATCTGC	TGG
Target 5	CCATTCTGCAGAGCCAGCAG	AGG
Target 6	CTCTGAGGCCCTGGAGATCC	TGG

b



indel (%)	5	21	N.D.	N.D.	N.D.	N.D.	23	13	N.D.

FIG. 68

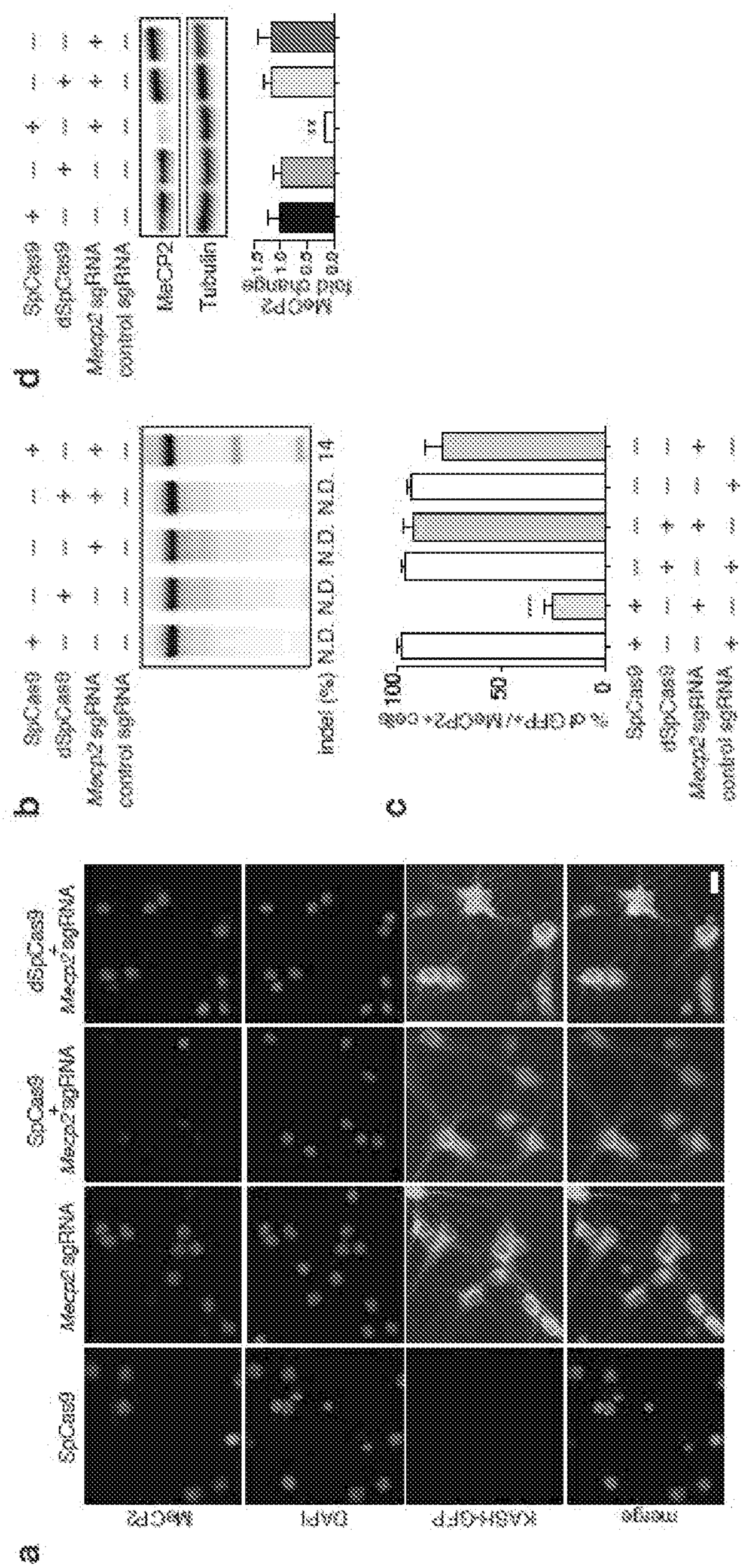

a
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
MeCP2
Tubulin
%
MeCP2+ cells
b
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
Indel (%) N.D. N.D. N.D. N.D. 14
c
% of GFP+/MeCP2+ cells
100
50
0
SpCas9
dSpCas9
Mecp2 sgRNA
control sgRNA
d
SpCas9
+
Mecp2 sgRNA
dSpCas9
+
Mecp2 sgRNA
MeCP2
DAPI
KASH-GFP
merge
SpCas9

FIG. 69

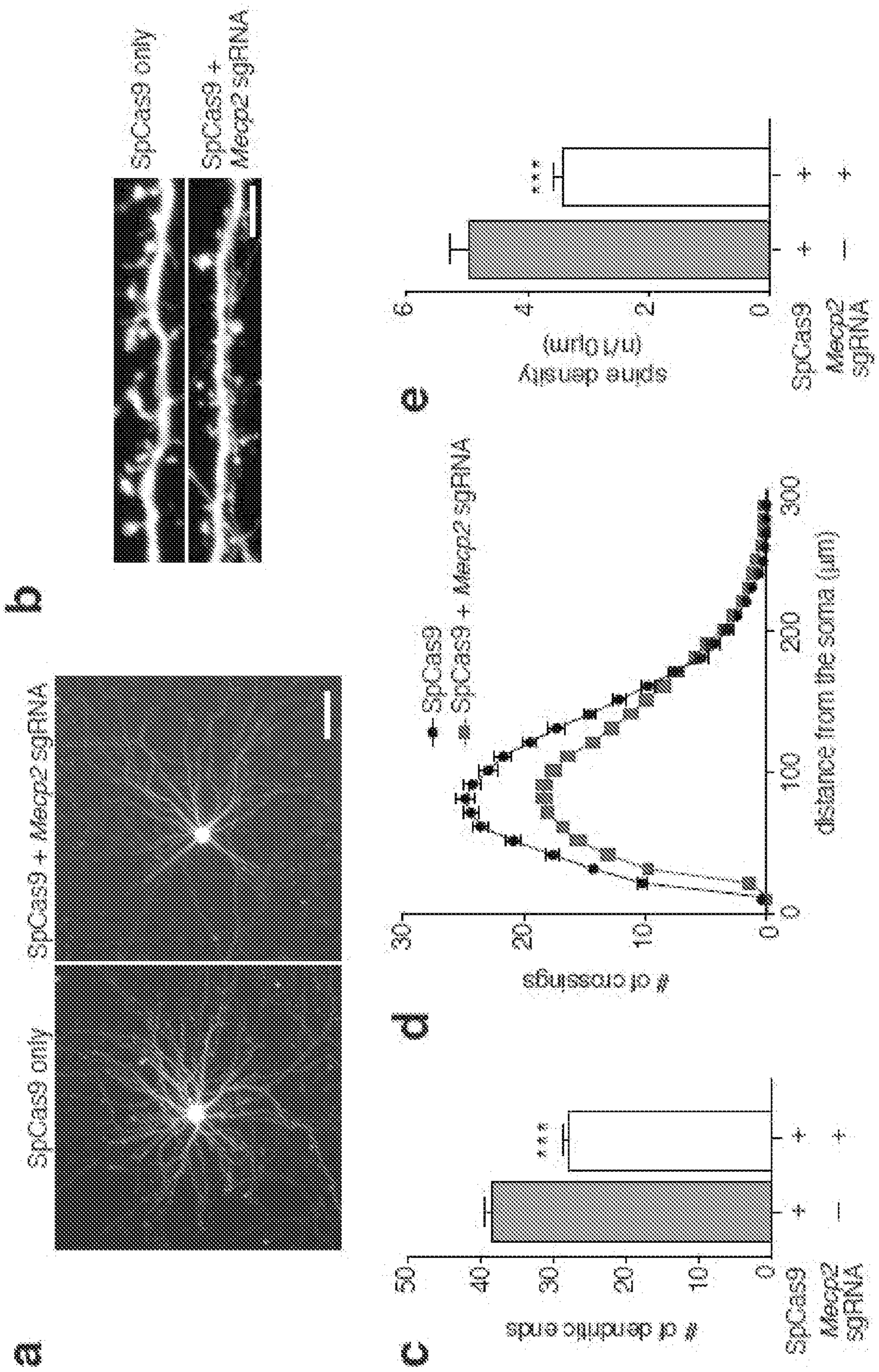

a
SpCas9 only
SpCas9 + Mecp2 sgRNA
spine density (#/10 μm)
6
4
2
0
SpCas9
Mecp2 sgRNA
b
SpCas9 only
SpCas9 + Mecp2 sgRNA
c
# of dendritic ends
60
40
30
20
10
0
SpCas9
Mecp2 sgRNA
d
# of crossings
30
20
10
0
SpCas9
SpCas9 + Mecp2 sgRNA
distance from the soma (μm)
0
100
200
300
e
spine density (#/10 μm)
6
4
2
0
SpCas9
Mecp2 sgRNA

FIG. 70

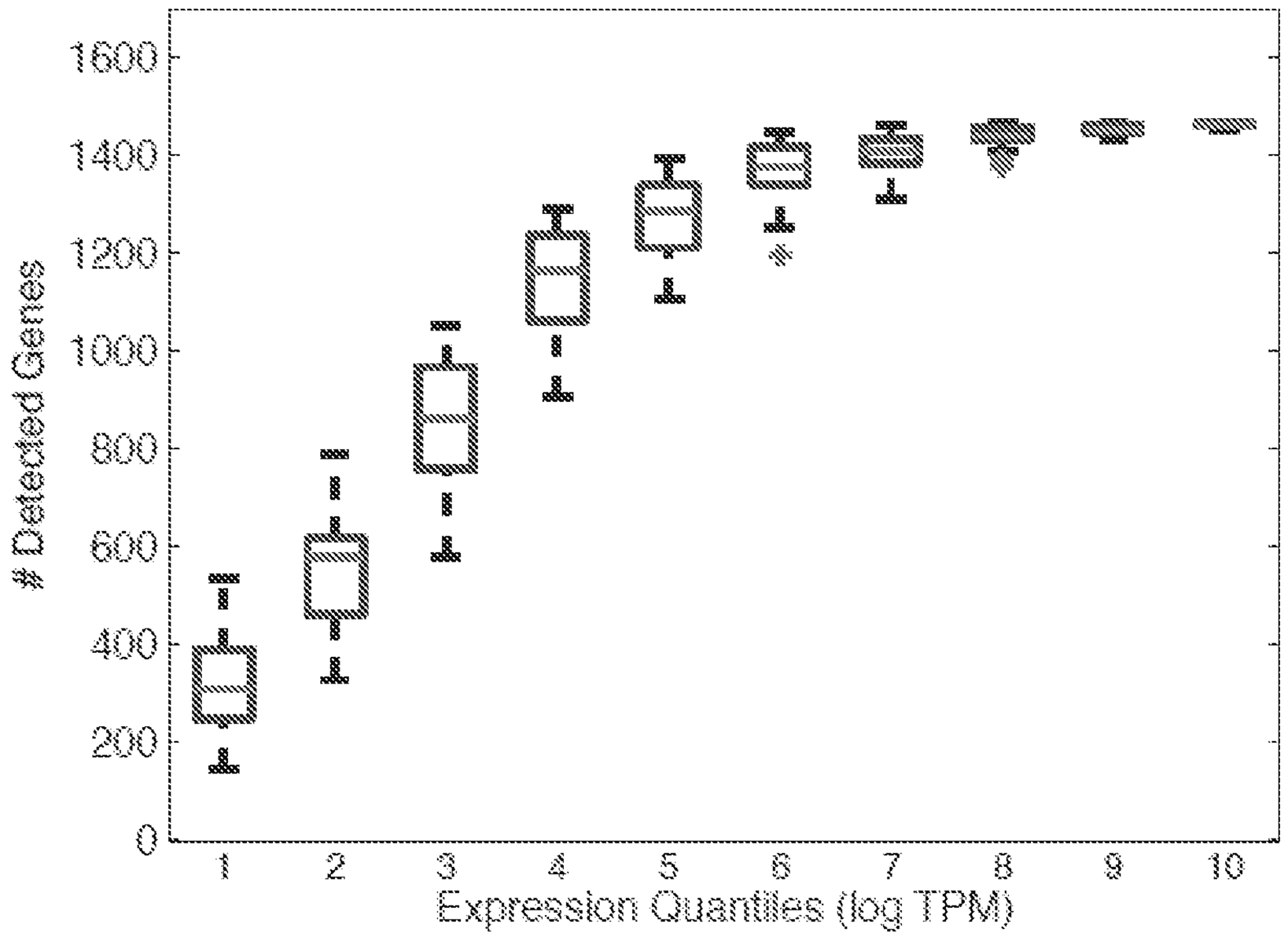

Detected Genes
0
200
400
600
800
1000
1200
1400
1600
1
2
3
4
5
6
7
8
9
10
Expression Quantiles (log TPM)

FIG. 71

a

target	target sequence	PAM
Dnmt3a	TTGGCATGGGTCGCTGACGG	AGG
Dnmt1	CGGGCTGGAGCTGTTCGGCGC	TGG
Dnmt3b	AGAGGGTGCCAGCGGGTATG	AGG

b

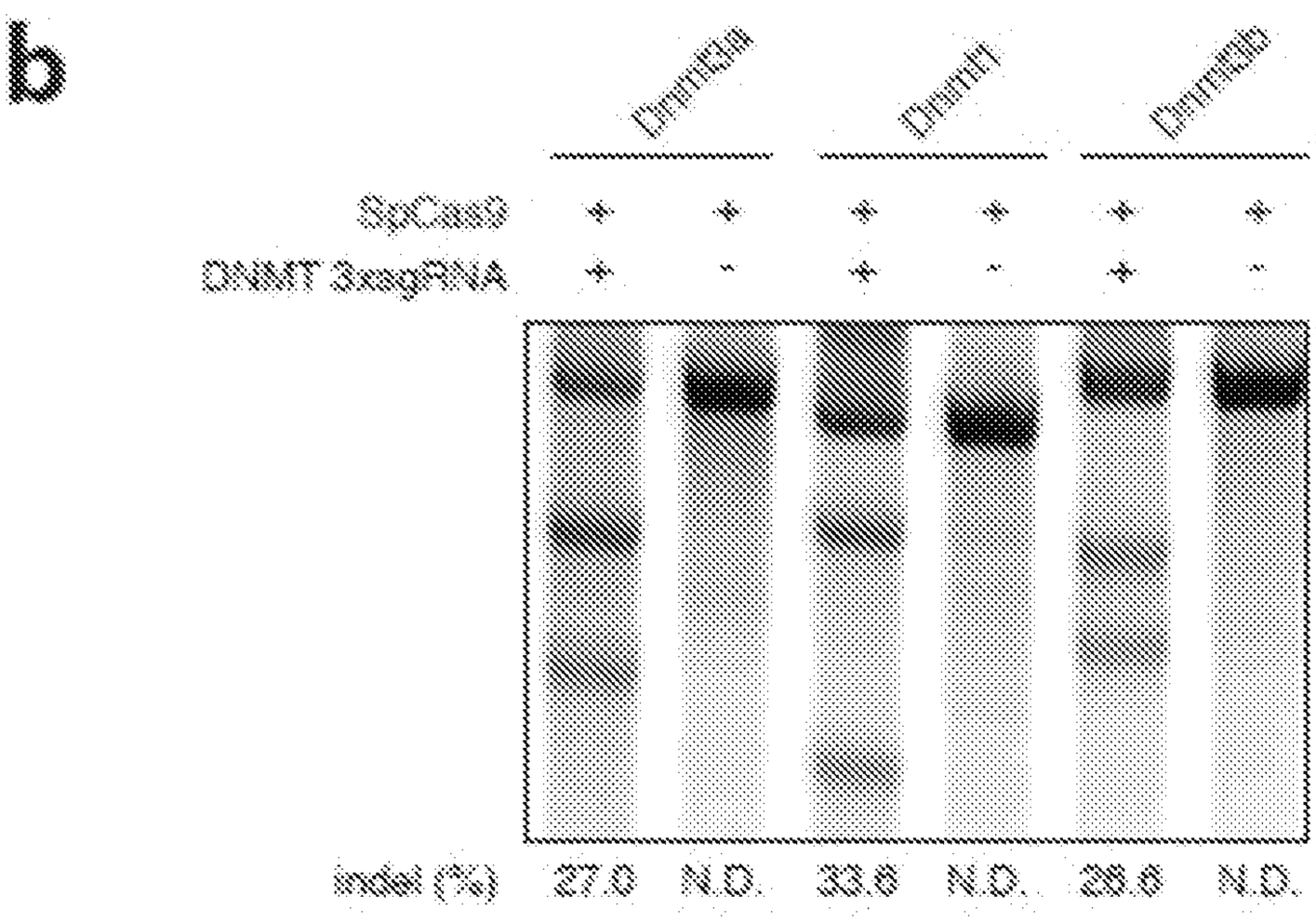


FIG. 72

a *Dnmt3a* locus

PAM

CCTCCG--TCAGCGACCCATGCCAA
CCTCCG-ATCAGCGACCCATGCCAA
CCTCCGTGTCAGCGACCCATGCCAA
CCTCCG-TTCAGCGACCCATGCCAA
CCTCCG-CTCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG---CAGCGACCCATGCCAA
CCTC----TCAGCGACCCATGCCAA
CCTCC---TCAGCGACCCATGCCAA
CCTCCG------CGACCCATGCCAA
CCTC------AGCGACCCATGCCAA
C--------TCAGCGACCCATGCCAA

b *Dnmt1* locus

PAM

CCAGCG-CGAACAGCTCCAGCCCG
CCAGCGCCGAACAGCTCCAGCCCG
CCAGCGTCGAACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCG--G-ACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG------AGCTCCAGCCCG
CCAG-----AACAGCTCCAGCCCG
CCAGCG---AACAGCTCCAGCCCG
CCAGCGC---ACAGCTCCAGCCCG
CCAGCGC--------TCCAGCCCG

c *Dnmt3b* locus

PAM

AGAGGGTGCCAGCGGG--TATGAGG
AGAGGGTGCCAGCGGG-TTATGAGG
AGAGGGTGCCAGCGGGTATATGAGG
AGAGGGTGCCAGCGGG-TAATGAGG
AGAGGGTGCCAGCGGG--T-19bp-
AGAGGGTGCCAG------TATGAGG
AGAGGGTGCCAGCGGG--T--GAGG
AGAGGGTGCCAGCG--------AGG
AGAGGGTGCCAGCGGG-----GAGG

FIG. 13

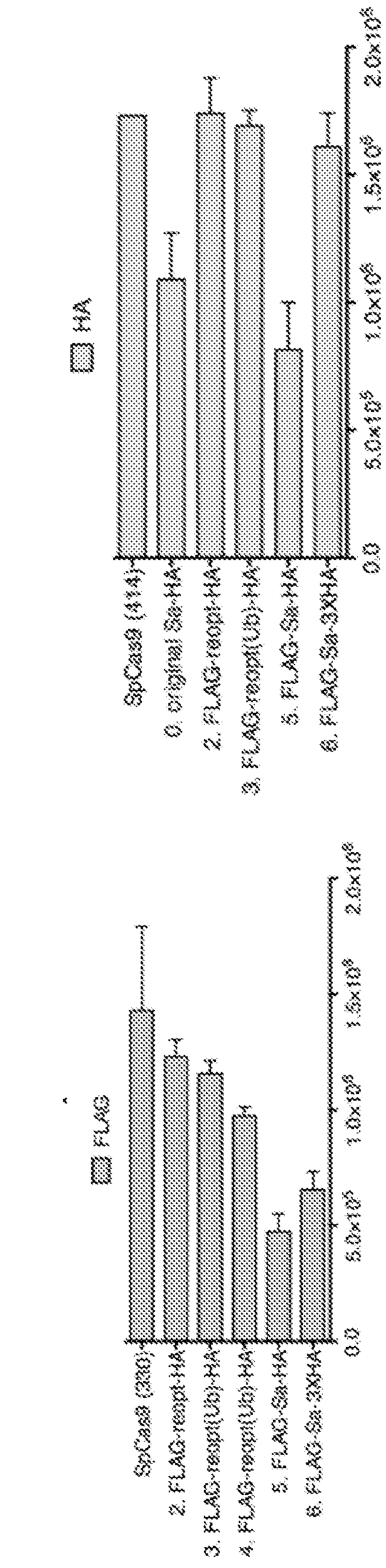

HA
SpCas9 (1368)
0. original Se-HA
2. FLAG-nopt-HA
3. FLAG-nopt(Ub)-HA
5. FLAG-Se-HA
6. FLAG-Se-3XHA
0.0
5.0x10^5
1.0x10^6
1.5x10^6
2.0x10^6
FLAG
SpCas9 (1368)
2. FLAG-nopt-HA
3. FLAG-nopt(Ub)-HA
4. FLAG-nopt(Ub)-HA
5. FLAG-Se-HA
6. FLAG-Se-3XHA
0.0
5.0x10^5
1.0x10^6
1.5x10^6
2.0x10^6

FIG. 74

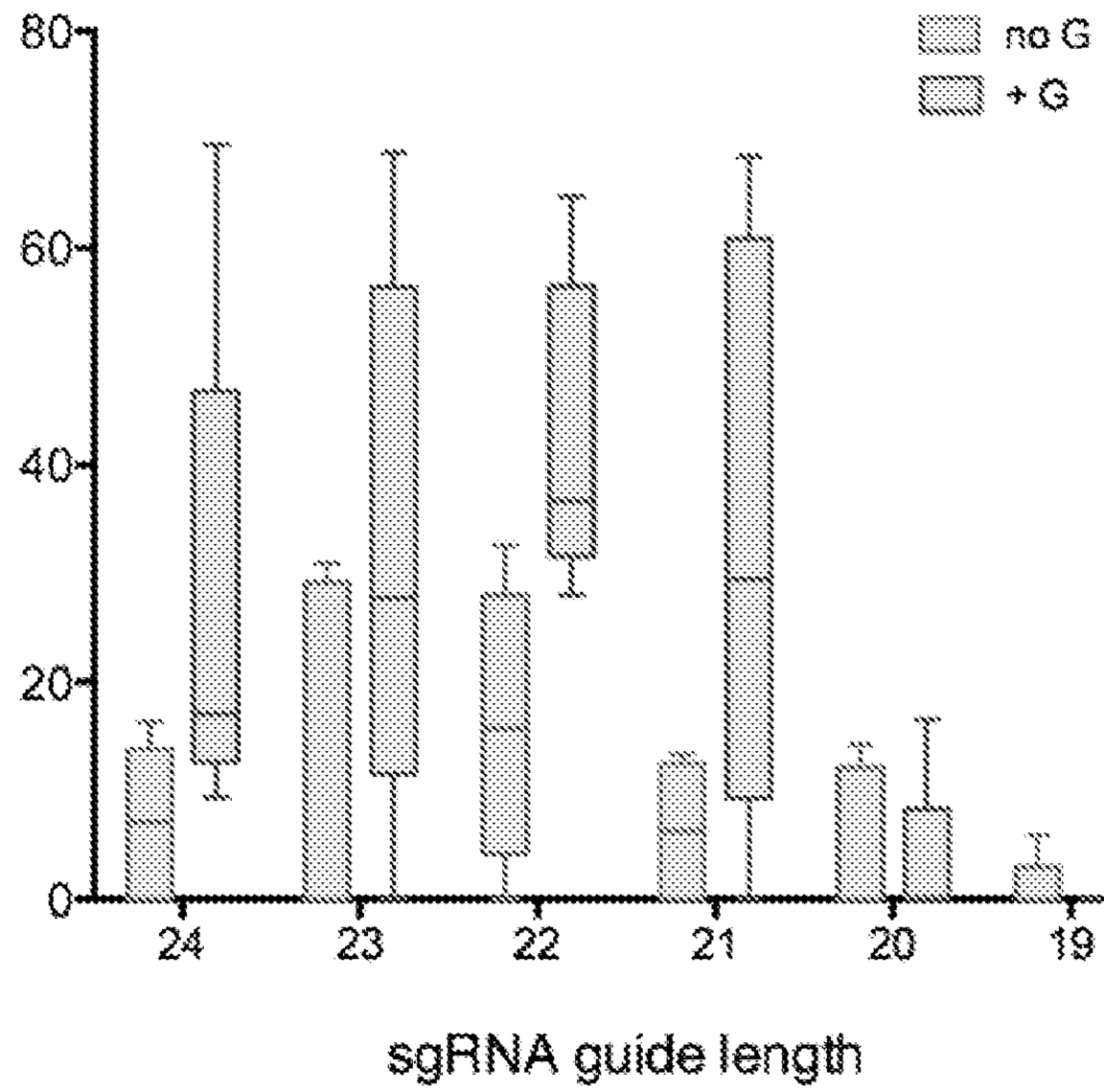


FIG. 75

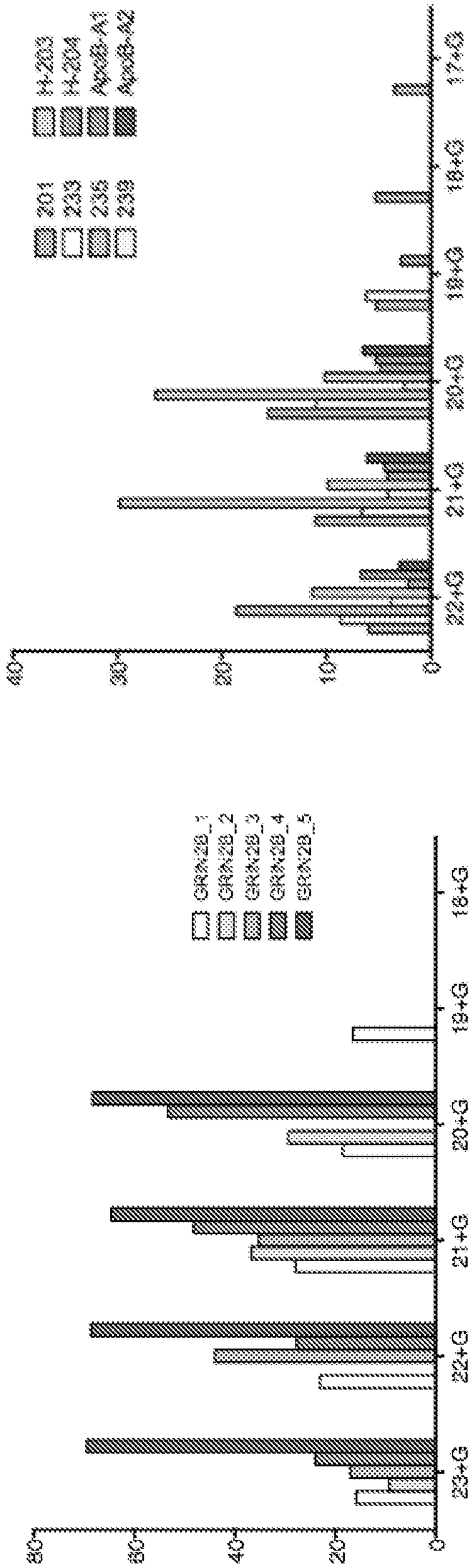

14-203
14-204
hpx28-A1
hpx28-A2
201
233
235
239
17+G
16+G
18+G
20+G
21+G
22+G
40
30
20
10
0
GRHx28_1
GRHx28_2
GRHx28_3
GRHx28_4
GRHx28_5
19+G
23+G
60
40
20

FIG. 76

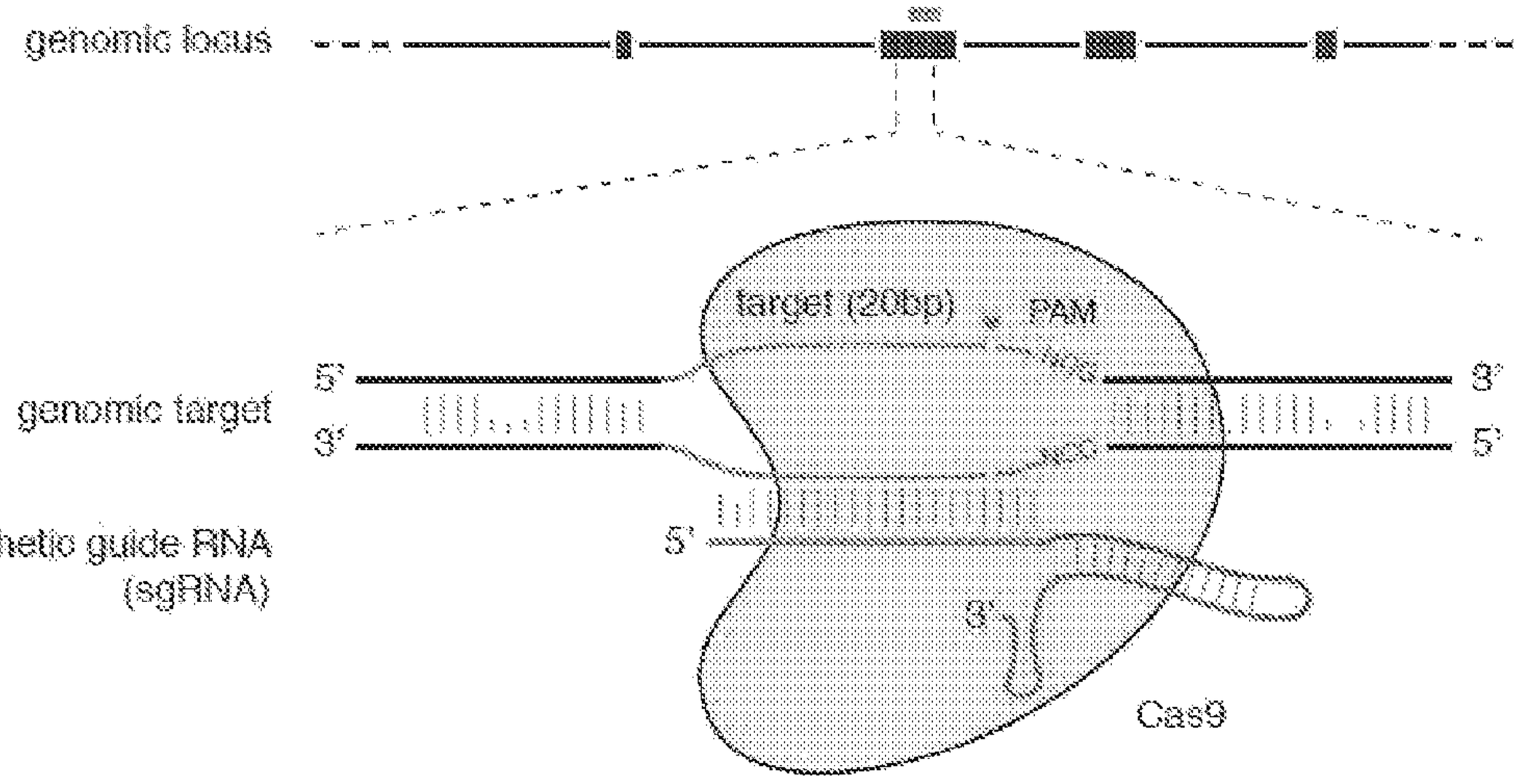

genomic locus
genomic target
synthetic guide RNA
(sgRNA)
target (20bp)
PAM
5'
3'
3'
5'
5'
3'
Cas9

FIG. 1