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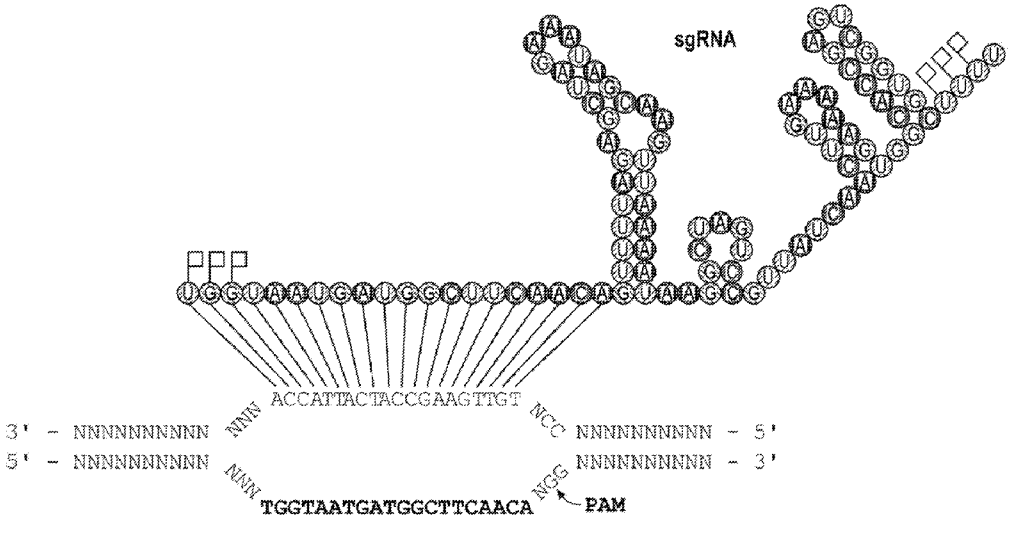

FIG. 1

(57) Abstract: The present invention relates to guide RNAs having chemical modifications and their use in CRISPR-Cas systems. The chemically modified guide RNAs have enhanced specificity for target polynucleotide sequences. The present invention also relates to methods of using chemically modified guide RNAs for cleaving or nicking polynucleotides, and for high specificity genome editing.

[Continued on next page]

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HIGH SPECIFICITY GENOME EDITING USING CHEMICALLY MODIFIED GUIDE RNAS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/347553, filed June 8, 2016 and of U.S. Nonprovisional Application No. 15/493,129, filed April 20, 2017, the contents of which are incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of molecular biology. In particular, the present invention relates to the clusters of regularly interspaced short palindromic repeats (CRISPR) technology.

BACKGROUND OF THE INVENTION

[0003] The native prokaryotic CRISPR-Cas system comprises an array of short repeats with intervening variable sequences of constant length (*i.e.*, clusters of regularly interspaced short palindromic repeats, or "CRISPR"), and CRISPR-associated ("Cas") proteins. The RNA of the transcribed CRISPR array is processed by a subset of the Cas proteins into small guide RNAs, which generally have two components as discussed below. There are at least six different systems: Type I, Type II, Type III, Type IV, Type V and Type VI. The enzymes involved in the processing of the RNA into mature crRNA are different in the six systems. In the native prokaryotic Type II system, the guide RNA ("gRNA") comprises two short, non-coding RNA species referred to as CRISPR RNA ("crRNA") and trans-acting RNA ("tracrRNA"). In an exemplary system, the gRNA forms a complex with a Cas nuclease. The gRNA:Cas nuclease complex binds a target polynucleotide sequence having a protospacer adjacent motif ("PAM") and a protospacer, which is a sequence complementary to a portion of the gRNA. The recognition and binding of the target polynucleotide by the gRNA:Cas nuclease complex induces cleavage of the target polynucleotide. The native CRISPR-Cas system functions as an immune system in prokaryotes, where gRNA:Cas nuclease complexes recognize and silence exogenous genetic elements in a manner analogous to RNAi in eukaryotic organisms, thereby conferring resistance to exogenous genetic elements such as plasmids and phages.

[0004] It has been demonstrated that a single-guide RNA ("sgRNA") where the crRNA and tracrRNA are covenlently linked can replace the complex formed between the naturally-existing crRNA and tracrRNA.

[0005] Considerations relevant to developing a gRNA, including a sgRNA, include specificity, stability, and functionality. Specificity refers to the ability of a particular gRNA:Cas nuclease complex to

bind, nick, and/or cleave a desired target sequence, whereas less or no binding, nicking, and/or cleavage of polynucleotides different in sequence and/or location from the desired target occurs. Thus, specificity refers to minimizing off-target effects of the gRNA:Cas nuclease complex. There is a need for providing gRNA, including sgRNA, having desired binding affinity for the target polynucleotide with reduced off-target effects while, nonetheless, having desired gRNA functionality. There is also a need for improved ways to make and use gRNA, including sgRNA, having enhanced specificity, with desired binding affinity for the target polynucleotide and/or reduced off-target binding.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] FIG. 1 is an illustration of an exemplary CRISPR-Cas system. A complex is formed by a single-guide RNA and a Cas protein, and the complex recognizes and binds a target polynucleotide. The Cas nuclease is the *S. pyrogenes* Cas9 nuclease. The *S. pyrogenes* Cas9 nuclease recognizes a PAM sequence (here, the PAM sequence is a 3-nucleotide sequence of NGG, where N is A, G, C or T, but other PAM sequences are known to exist, such as NAG and others). The sgRNA includes a guide sequence, a crRNA sequence or segment, and tracrRNA sequence or segment. The guide sequence of the sgRNA hybridizes with the DNA target directly upstream of the PAM sequence.

[0007] FIG. 2A shows an exemplary guide RNA 201 comprising a crRNA segment 203 and a tracrRNA segment 205. FIG. 2B shows an exemplary single-guide RNA 207 comprising a crRNA segment 209 and a tracrRNA segment 211, wherein the crRNA segment and the tracrRNA segment are linked through a loop 213.

[0008] FIG. 3 shows the structures of some of the various chemical modifications that can be included in a guide RNA; of course, FIG. 3 is not intended to be limiting and many other modifications as described herein can be employed.

[0009] FIG. 4 is a graph demonstrating how ultraviolet light (UV) absorbance increases as an oligonucleotide duplex separates into separate strands by heating. A sigmoidal curve reflects the dissociation of the duplex into separate strands, and the center of the sigmoidal curve is the T_m of the duplex. The curve indicates that the melting temperature of a 20-nucleotide RNA/DNA duplex at physiological salt concentrations is around 50°C.

[0010] FIG. 5A illustrates a single-guide RNA (sgRNA) or a two-piece dual-guide RNA ("dgRNA") wherein a crRNA segment and a tracrRNA segment form a hybridized duplex and shows the guide RNA's extension region, locking region, sampling region, seed region (typically a 10-mer), a dual-guide stem or a single-guide stem-loop, and a tracrRNA region. FIG. 5B illustrates how, after the initial

formation of a seed duplex between the seed portion of a guide sequence and a complementary DNA sequence, the binding of the nucleotides proceeds sequentially through the sampling region and locking region to form a RNA/DNA duplex having a melting temperature. FIG. 5C illustrates that chemical modifications in nucleotides of the seed region can decrease the binding energy of individual base pairs while retaining a high level of cooperativity, thereby extending the sampling region and lowering the melting temperature of the RNA/DNA duplex. FIG. 5C also illustrates chemical modification in the sampling region.

[0011] FIG. 6A illustrates experimental crRNA polynucleotides with 20-nucleotide guide sequences and modified by various types of chemical modifications incorporated at various positions in the guide sequence. FIG. 6B shows the melting curve for an RNA/DNA duplex comprising an unmodified crRNA. FIGs. 6C through 6F show melting curves for RNA/DNA duplexes comprising chemically modified crRNAs comprising different types of modifications at nucleotides 6 through 9 in distinct guide sequences.

[0012] FIG. 7 is a graph showing change in melting temperature of a 20–base pair duplex of guide sequence (in a crRNA) hybridized to a complementary DNA strand after incremental 5′ truncation or 5′ extension of the guide sequence as indicated on the x-axis by negative or positive integers, respectively.

[0013] FIG. 8A shows the impact of chemical modifications in gRNAs targeted to the "*CLTA1*" sequence in the human *CLTA* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of two different off-target polynucleotides called "OFF1" and "OFF3," representing *CLTA1* ON-target, *CLTA1* OFF1-target, and *CLTA1* OFF3-target, respectively.

[0014] FIG. 8B is derived from the cleavage results in FIG. 8A by calculating a ratio of cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed. Also calculated is a "Specificity Score" obtained by multiplying each ratio by the respective ON-target cleavage percentage per guide RNA assayed. The shaded ratios and Specificity Scores are notable for their larger values with respect to the others shown in FIG. 8B.

[0015] FIG. 9A shows the impact of 2′-O-methyl-3'-PACE ("MP") modifications at various locations in gRNAs targeted to the "*CLTA4*" sequence in the human *CLTA* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of three different off-target polynucleotides called "OFF1", "OFF2", and "OFF3," representing *CLTA4* ON-target, *CLTA4* OFF1-target, *CLTA4* OFF2-target, and *CLTA4* OFF3-target, respectively.

[0016] FIG. 9B is derived from the cleavage results in FIG. 9A by calculating a ratio of cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed. Specificity Scores are calculated as described for FIG. 8B, and the scores greater than or equal to 1.5 are shaded. The three highest scores per off-target polynucleotide are indicated by darker shading.

[0017] FIG. 10 shows the impact of MP modifications at various locations in gRNAs targeted to a sequence in the human *IL2RG* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of an off-target polynucleotide called "OFF3" in this figure representing *IL2RG* ON-target and *IL2RG* OFF3-target, respectively. A ratio is calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed. Specificity Scores are calculated as described for FIG. 8B, and the scores greater than 2.0 are shaded. The three highest scores are indicated by darker shading.

[0018] FIG. 11A shows the impact of MP modifications at various locations in gRNAs targeted to a sequence in the human *HBB* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of an off-target polynucleotide called "OFF1" in this figure representing *HBB* ON-target and *HBB* OFF1-target, respectively. A ratio is calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed. Specificity Scores are calculated as described for FIG. 8B, and the scores greater than 2.0 are shaded. The three highest scores are indicated by darker shading.

[0019] FIG. 11B shows the impact of various types of modifications in gRNAs targeted to a sequence in the human *HBB* gene in human K562 cells transfected with synthetic sgRNA and Cas9-expressing plasmid with regard to cleavage of a target genomic locus called "ON" versus concurrent cleavage of three different off-target genomic loci called "OFF1", "OFF2" and "OFF3" in this figure representing endogenous *HBB* ON-target, *HBB* OFF1-target, *HBB* OFF2-target and *HBB* OFF3-target sites, respectively. "Unmodif" indicates an sgRNA that was not chemically modified. "3xM" indicates that 2'-O-methyl ("M") nucleotide was incorporated at the very first three and the very last three nucleotides of an sgRNA strand, namely at its 5' and 3' termini respectively. Similarly, "3xMS" indicates that 2′-O-methyl-3'-phosphorothioate ("MS") nucleotide was incorporated likewise at the 5' and 3' termini of an sgRNA, whereas "3xMSP" indicates that 2′-O-methyl-3'-thioPACE ("MSP") nucleotide was incorporated likewise at the 5′ and 3′ termini of an sgRNA. All sgRNAs were assayed for editing of the indicated loci in transfected cells.

[0020] FIG. 12A shows the same results in entries 1–17 as shown in FIG. 11A, with the entries ranked according to Specificity Score from highest to lowest. Entries 18–64 show the impact of additional MP modifications at various locations in gRNAs targeted to a sequence in the human *HBB* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of an off-target polynucleotide called "OFF1," representing *HBB* ON-target and *HBB* OFF1-target, respectively. "1xMP" indicates that the terminal nucleotides at both the 5′ and 3′ ends have been modified with MP. A ratio is calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed. Specificity Scores are calculated as described for FIG. 8B. The highest scores are shaded.

[0021] FIG. 12B shows the impact of MP modifications at various locations in gRNAs targeted to a sequence in the human *HBB* gene in transfected cells with regard to cleavage of a target genomic locus called "ON" and concurrent cleavage of an off-target genomic locus called "OFF1" in this figure representing endogenous *HBB* ON-target and *HBB* OFF1-target sites respectively. The percentage of cleavage yielded at either or both sites in cultured cells transfected with a complex of synthetic sgRNA and recombinant Cas9 protein is determined 48 hours post-transfection by PCR amplification of the on-target and off-target loci in split samples of purified genomic DNA, followed by next-generation sequencing of pooled amplicons and bioinformatic partitioning of the sequence reads according to the presence versus absence of an indel near the on-target or off-target cleavage site being evaluated. Indels generated in each sample of transfected cells are normalized relative to a control sample of mock-transfected cells treated with buffer instead of sgRNA:Cas9 complex. A ratio is calculated for the number of sequence reads showing a target site indel versus the number of reads showing an off-target site indel for each sgRNA transfected separately. Specificity Scores are calculated as described for FIG. 8B. "1xMP" indicates that the terminal nucleotides at both the 5′ and 3′ ends have been modified with MP. Entries 1–21 show results obtained by transfecting and culturing K562 cells, whereas entries 22–42 show results obtained by transfecting and culturing induced pluripotent stem cells (also known as iPS cells or iPSCs). Entries 1–12 are ranked according to Specificity Score from highest to lowest. Likewise entries 13–19, entries 22–33, and entries 34–40 are ranked by Specificity Score per grouping. Specificity Scores greater than 2.0 are shaded.

[0022] FIG. 12C shows an alternative organization of the results in FIG. 12B according to the measured ratios, sorted from highest to lowest ratio per grouping.

[0023] FIG. 13 shows a comparison of the results presented in FIGs. 9A, 9B, 10 and 11A. FIG. 13 shows several trends from studies of chemically modified guide RNAs with respect to target specificity

when used in a Cas system for cleaving target polynucleotides. The concepts supported by the trends are especially useful when off-target polynucleotides are also present in the Cas system.

[0024] FIG. 14 shows the impact of various types of modifications in IL2RG sgRNAs and VEGFA sgRNAs in K562 cells.

DETAILED DESCRIPTION OF THE INVENTION

[0025] This invention is based, at least in part, on an unexpected discovery that certain chemical modifications to gRNAs are tolerated by the CRISPR-Cas system and decrease the off-target effects of Cas:gRNA complexation without substantially compromising the efficacy of Cas:gRNA binding to, nicking of, and/or cleavage of the target polynucleotide.

[0026] This invention provides synthetic guide RNAs comprising at least one specificity-enhancing modification. In certain embodiments, the at least one specificity-enhancing modification weakens or strengthens the association of at least one nucleotide pair between the synthetic guide RNA and the target polynucleotide and/or weakens the association of at least one nucleotide pair between the synthetic guide RNA and at least one off-target polynucleotide such that at least one of the off-target weakenings is greater than the on-target weakening if present. The synthetic guide RNA has gRNA functionality. The specificity-enhancing modification(s) can be located in the guide sequence, for example, in the locking region, sampling region, and/or seed region. In certain embodiments, the specificity-enhancing modification(s) lowers melting temperatures of duplexes formed by the gRNA and a target polynucleotide sequence and off-target polynucleotide sequence(s), or raises melting temperature of the gRNA/target duplex and lowers melting temperature of at least one gRNA/off-target duplex. This invention also provides gRNA:Cas protein complex comprising these synthetic guide RNAs, methods for cleaving, nicking or binding target polynucleotides using the synthetic guide RNAs, and sets, libraries, kits and arrays comprising the synthetic guide RNAs. This invention also provides method of preparing synthetic guide RNAs.

I. Definitions

[0027] As used herein, the term "guide RNA" generally refers to an RNA molecule (or a group of RNA molecules collectively) that can bind to a Cas protein and aid in targeting the Cas protein to a specific location within a target polynucleotide (e.g., a DNA or an mRNA molecule). A guide RNA can comprise a crRNA segment and a tracrRNA segment. In some embodiments, a guide RNA comprises a crRNA but not a tracrRNA. As used herein, the term "crRNA" or "crRNA segment" refers to an RNA molecule or portion thereof that includes a polynucleotide-targeting guide sequence, a stem sequence (for additional clarity: the stem sequence encompasses a stem-forming sequence which, in a single guide

RNA, forms a stem with a corresponding part of tracrRNA), and, optionally, a 5′-overhang sequence. As used herein, the term "tracrRNA" or "tracrRNA segment" refers to an RNA molecule or portion thereof that includes a protein-binding segment (e.g., the protein-binding segment is capable of interacting with a CRISPR-associated protein, such as a Cas9). The tracrRNA aslo includes a segment that hybridizes partially or completely to the crRNA. The term "guide RNA" encompasses a single-guide RNA (sgRNA), where the crRNA segment and the tracrRNA segment are located in the same RNA molecule or strand. The term "guide RNA" also encompasses, collectively, a group of two or more RNA molecules, where the crRNA segment and the tracrRNA segment are located in separate RNA molecules. The term "guide RNA" also encompasses an RNA molecule or suitable group of molecular segments that binds a Cas protein other than Cas9 (e.g., Cpf1 protein) and that possesses a guide sequence within the single or segmented strand of RNA comprising the functions of a guide RNA which include Cas protein binding to form a gRNA:Cas protein complex capable of binding, nicking and/or cleaving a complementary sequence (or "target sequence") in a target polynucleotide.

[0028] The term "guide sequence" refers to a contiguous sequence of nucleotides in a guide RNA which has partial or complete complementarity to a target sequence in a target polynucleotide and can hybridize to the target sequence by base pairing facilitated by a Cas protein. As illustrated in the example shown in Fig. 1, a target sequence is adjacent to a PAM site (the PAM sequence, and its complementary sequence on the other strand, together constitute a PAM site). Immediately upstream of the PAM sequence (NGG for cas9) is a sequence (bolded, bottom strand in Fig. 1) complementary to the target sequence. The target sequence, which hybridizes to the guide sequence, is immediately downstream from the complement (CCN for cas9) of the PAM sequence. Nucleotide 1 of the guide sequence (first nucleotide at the 5') is complementary to the last nucleotide of the target sequence, while the last nucleotide of the guide sequence (nucleotide 20 of the guide sequence in Fig. 1) is complementary to the first nucleotide of the target sequence, which is next to the PAM site (and immediately downstream from the complement of the PAM sequence). In other examples such as Cpf1, the location of the target sequence, which hybridizes to the guide sequence, may be upstream from the complement of the PAM sequence.

[0029] A guide sequence can be as short as about 10 nucleotides and as long as about 30 nucleotides. Typical guide sequences are 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24 nucleotides long. Synthetic guide sequences are usually 20 nucleotides long, but can be longer or shorter. When a guide sequence is shorter than 20 nucleotides, it is typically a deletion from the 5'-end compared to a 20-nucleotide guide sequence. By way of example, a guide sequence may consist of 20 nucleotides complementary to a target sequence. In other words, the guide sequence is identical to the 20 nucleotides

upstream of the PAM sequence, except the A/U difference between DNA and RNA. If this guide sequence is truncated by 3 nucleotides from the 5'-end, nucleotide 4 of the 20-nucleotide guide sequence now becomes nucleotide 1 in the 17-mer, nucleotide 5 of the 20-nucleotide guide sequence now becomes nucleotide 2 in the 17-mer, etc. The new position is the original position minus 3. Similarly, a guide sequence may hybridize to more than 20 nucleotides at the targt site, and the additional nucleotides are located at the 5'-end of the guide sequence, because the 3'-end of the guide sequence is complementary to the target next to the PAM site.. Again by way of example, in a 22-nucleotide guide sequence, the original nucleotide 1 in the 20-mer now becomes nucleotide 3, the original nucleotide 2 in the 20-mer becomes nucleotide 4, etc. The new position is the original position plus 2, or minus (-2). Thus, a guide sequence consists of nucleotides 1 through "20 minus N" (20-N) counting from the 5'-end, wherein N is a positive or negative integer between -10 and 10 (optionally between -10 and 6). A given nucleotide position within the guide sequence will be noted as "Position Number Minus N" (number-N). For example, the nucleotide at position 5 will be noted as "5-N" (5 minus N), to indicate the shift of position 5 from the reference position obtained from a 20 nucleotides guide sequence that occurs when the guide sequence is truncated or extended by N nucleotides at the 5'-end. Nucleotide positions are positive integers. Thus, any (Number-N) position that is negative or zero is moot, and should be ignored. A guide sequence can be positioned anywhere within the strand or strands that constitute a gRNA. Typical guide sequences are located at or near the 5' end or the 3' end of a gRNA strand.

[0030] The term "scaffold" refers to the portions of guide RNA molecules comprising sequences which are substantially identical or are highly conserved across natural biological species. Scaffolds include the tracrRNA segment and the portion of the crRNA segment other than the polynucleotide-targeting guide sequence (repeat portion) at or near the 3′ end of the crRNA segment.

[0031] The term "nucleic acid", "polynucleotide" or "oligonucleotide" refer to a DNA molecule, an RNA molecule, or analogs thereof. As used herein, the terms "nucleic acid", "polynucleotide" and "oligonucleotide" include, but are not limited to DNA molecules such as cDNA, genomic DNA, plasmid or vector DNA or synthetic DNA and RNA molecules such as a guide RNA, messenger RNA or synthetic RNA. Moreover, as used herein, the terms "nucleic acid" and "polynucleotide" include single-stranded and double-stranded forms. A standard convention in the art is that oligonucleotides, polynucleotides, RNA molecules, distinct strands of DNA molecules, and various nucleic acids comprising 2 or more nucleotides are generally numbered from their 5′ ends, and this convention is used throughout, including instances of 5′ extensions or "overhangs" covalently linked to such molecules.

[0032] As used herein, "modification" or "chemical modification" refers to a chemical moiety, or portion of a chemical structure, which differs from that found in the four most common natural ribonucleotides: adenosine, guanosine, cytidine, and uridine ribonucleotides. Thus the term "modification" refers to a molecular change in, or on, the most common natural molecular structure of adenosine, guanosine, cytidine, or uridine ribonucleotide. The term "modification" may refer to a change in or on a nucleobase, in or on a sugar, and/or in an internucleotide phosphodiester linkage. The term "modification" may refer to a chemical structural change in a ribonucleotide that occurs in nature such as a chemical modification that occurs in natural transfer RNAs (tRNAs), for example but not limited to 2′-O-Methyl, 1-Methyladenosine, 2-Methyladenosine, 1-Methylguanosine, 7-Methylguanosine, 2-Thiocytosine, 5-Methylcytosine, 5-Formylcytosine, Pseudouridine, Dihydrouridine, Ribothymidine, or the like. The term "modification" may refer to a chemical modification that is not typically found in nature, for example but not limited to 2′-Fluoro, 2′-O-Methoxyethyl, 2′-O-Phenyl, or the like. The term "same modification" refers to the same type of chemical modification in or on a sugar, or in an internucleotide linkage; for example, a 2′-O-Methyl may be attached to the 2′ position of an adenosine, guanosine, cytidine, and/or uridine ribonucleotide, and such modifications may be referred to as the same type of modification or the "same modification." Conversely, modifications to nucleobases would only be identified as the "same modification" if the modified nucleobases were composed of the same molecular structure. To illustrate the distinction by example, 1-Methylguanosine and 7-Methylguanosine both have a methyl group modification of the most common natural guanosine but they are not the "same modification" because the modified nucleobases have different molecular structures. In further examples, a strand of RNA may comprise three modified nucleotides, for example a guanosine and two cytidines, each modified by 2′-O-Methyl-3′-Phosphonoacetate, and such nucleotides may accurately be described as modified in an identical manner or modified with the same modification. Conversely, a different strand of RNA may comprise a 5-Methylcytidine as well as a cytidine that lacks a 5-methyl substituent, and both cytidine nucleotides may be modified by 2′-O-Methyl-3′-Phosphonoacetate, nonetheless these two cytidine nucleotides comprise different modifications and would not be referred to as modified in an identical manner.

[0033] The term "modification" in the context of an oligonucleotide or polynucleotide includes but is not limited to (a) end modifications, e.g., 5′ end modifications or 3′ end modifications, (b) nucleobase (or "base") modifications, including replacement or removal of bases, (c) sugar modifications, including modifications at the 2′, 3′, and/or 4′ positions, and (d) backbone modifications, including modification or replacement of the phosphodiester linkages. The term "modified nucleotide" generally refers to a nucleotide having a modification to the chemical structure of one or more of the base, the

sugar, and the phosphodiester linkage or backbone portions, including nucleotide phosphates. Chemical modifications to guide RNA are disclosed in U.S. Patent Application No. 14/757,204, filed December 3, 2015, the entire contents of which are incorporated by reference herein.

[0034] The terms "xA", "xG", "xC", "xT", "xU", or "x(A,G,C,T,U)" and "yA", "yG", "yC", "yT", "yU", or "y(A,G,C,T,U)" refer to nucleotides, nucleobases, or nucleobase analogs as described by Krueger et al., "Synthesis and Properties of Size-Expanded DNAs: Toward Designed, Functional Genetic Systems", (2007) *Acc. Chem. Res. 40*, 141–50, the contents of which is hereby incorporated by reference in its entirety.

[0035] The term "Unstructured Nucleic Acid" or "UNA" refers to nucleotides, nucleobases, or nucleobase analogs as described in US Patent 7,371,580, the contents of which is hereby incorporated by reference in its entirety. An unstructured nucleic acid, or UNA, modification is also referred to as a "pseudo-complementary" nucleotide, nucleobase or nucleobase analog (see *e.g.*, Lahoud et al. (1991) *Nucl. Acids Res. 36*:10, 3409–19).

[0036] The terms "PACE" and "thioPACE" refer to internucleotide phosphodiester linkage analogs containing phosphonoacetate or thiophosphonoacetate groups, respectively. These modifications belong to a broad class of compounds comprising phosphonocarboxylate moiety, phosphonocarboxylate ester moiety, thiophosphonocarboxylate moiety and thiophosphonocarboxylate ester moiety. These linkages can be described respectively by the general formulae $P(CR^1R^2)_nCOOR$ and $(S)\text{-}P(CR^1R^2)_nCOOR$ wherein n is an integer from 0 to 6 and each of R^1 and R^2 is independently selected from the group consisting of H, an alkyl and substituted alkyl. Some of these modifications are described by Yamada, Dellinger, et al., "Synthesis and Biochemical Evaluation of Phosphonoformate Oligodeoxyribonucleotides" (2006) *J. Am. Chem. Soc. 128*:15, 5251–61, the contents of which is hereby incorporated by reference in its entirety.

[0037] In some places of the present disclosure, particularly in figures disclosing structures and sequences of synthetic guide RNAs and experimental results with such synthetic guide RNAs certain abbreviations are used to indicate certain modifications. "M" is used herein to indicate a 2′-O-methyl modification; "S" is used herein to indicate a 3'-phosphorothioate internucleotide linkage modification; "P" is used herein to indicate a 3'-phosphonoacetate (or PACE) internucleotide linkage modification; "MS" is used herein to indicate a 2′-O-methyl-3'-phosphorothioate internucleotide linkage modification; "MP" is used herein to indicate an 2′-O-methyl-3'-phosphonoacetate (or 2′-O-methyl-3'-PACE)

internucleotide linkage modification; and "MSP" is used herein to indicate a 2′-O-methyl-3'-thiophosphonoacetate internucleotide linkage modification.

[0038] "Sugar pucker" refers to a sugar ring that is non-planar due to steric forces causing one or two atoms of a 5-membered sugar ring to be out of plane. In ribofuranose, the plane C1′-O4′-C4′ is fixed. Endo-pucker means that C2′ or C3′ are turned out of this plane into the direction of O5′. Exo-pucker describes a shift in the opposite direction. C2′-endo and C3′-endo are naturally in equilibrium, but chemical modification can drive the sugar to a preferred pucker. In RNA C3′-endo conformation is predominant. DNA may adjust and is able to take on both conformations.

[0039] The term "seed region" refers to the region of the guide sequence that is complementary to a target nucleic acid sequence which initiates hybridization of the guide sequence to the target nucleic acid sequence. In some cases, the seed region forms a quasi-stable duplex that is aided by a protein, peptide, or protein complex. In general, the term seed region in a guide sequence of a gRNA consists of nucleotides 11 through 20 in a 20-nucleotide guide sequence, counted from the 5′ end of the guide sequence, but the region can run shorter or longer depending on the nucleotide sequence and on chemical modifications on the RNA nucleotides in this region or through modification of the associated peptides, proteins or protein complexes.

[0040] The term "sampling region" refers to the region adjacent to the seed region, and the binding of these nucleotides proceeds until the binding energy of the duplex is equivalent to the temperature at which the binding is occurring. In general, the term sampling region in a gRNA consists of nucleotides 5 through 10 in a 20-nucleotide guide sequence, counted from the 5′ end of the guide sequence, unless otherwise indicated, as may be the case when one or more modifications functionally extend the sampling region, thereby encompassing nucleotides 1 through 10, alternatively 2 through 10, alternatively 3 through 10, alternatively 4 through 10, alternatively 1 through 11, alternatively 2 through 11, alternatively 3 through 11, alternatively 4 through 11, alternatively 5 through 11, alternatively 1 through 12, alternatively 2 through 12, alternatively 3 through 12, in the guide sequence.

[0041] The term "locking region" refers to the region adjacent to the sampling region in which the binding energy of the duplex formed is above the temperature at which the binding is occurring. In general, the term locking region in a gRNA consists of nucleotides 1 through 4 in a 20-nucleotide guide sequence, counted from the 5′ end of the guide sequence, unless otherwise indicated, as may be the case when one or more modifications functionally shorten the locking region to nucleotide 1, alternatively nucleotides 1 through 2, alternatively nucleotides 1 through 3, at the 5′ end of a guide sequence. The

locking region can extend beyond the 20-nucleotide length of typical guide sequences of CRISPR-Cas9 systems if one or more nucleotides are covalently linked to the 5′ end to extend the guide sequence from the typical 20 nucleotides to 21 nucleotides, alternatively to 22 nucleotides, alternatively to 23 nucleotides, alternatively to 24 nucleotides, alternatively to 25 nucleotides or more.

[0042] As used herein, the term "target polynucleotide" or "target" refers to a polynucleotide containing a target nucleic acid sequence. A target polynucleotide may be single-stranded or double-stranded, and, in certain embodiments, is double-stranded DNA. In certain embodiments, the target polynucleotide is single-stranded RNA. A "target nucleic acid sequence" or "target sequence," as used herein, means a specific sequence or the complement thereof that one wishes to bind to, nick, or cleave using a CRISPR system. In certain embodiments, two or more target sequences may be selected to be bound, nicked or cleaved in the same reaction, for example to replace the sequence between two particular target sequences for homologous recombination purposes. Alternatively, two or more target sequences are also useful when multiple targets are to be bound and enriched at the same time. Thus, where two mor more target sequences are used, the respective target polynucleotides may or may not be in the same gene, depending on the purpose.

[0043] An "off-target polynucleotide" or "off-target" refers to a polynucleotide containing a partially homologous acid sequence to the intended target nucleic acid. An off-target polynucleotide may be single-stranded or double-stranded, and, in certain embodiments, is double-stranded DNA. An "off-target nucleic acid sequence" or "off-target sequence," as used herein, means a specific sequence or the complement thereof that one does not wish to bind to, nick, or cleave using a CRISPR system and that has substantial sequence identity with, but is not identical to, a target nucleic acid sequence. For example, an off-target nucleic acid sequence has substantial sequence identity with a target nucleic acid sequence when it has at least about 60%, at least about 75%, at least about 85%, at least about 90%, at least about 90–95%, at least about 97%, or more nucleotide (or amino acid) sequence identity.

[0044] The terms "HBB polynucleotide, "VEGFA polynucleotide," "IL2RG polynucleotide," "CLTA1 "polynucleotide," and "CLTA4 polynucleotide" refer to any polynucleotide that comprises at least a portion of the genes *HBB, VEGFA, IL2RG, CLTA1* or *CLTA4*, respectively. Such polynucleotides include naturally occurring, recombinant, or synthetic polynucleotides. Such polynucleotides may include polynucleotide sequences found at the locus in the genome associated with such genes, and accordingly encompasses alleles and variants of such genes.

[0045] The term "specificity" refers to how well a guide RNA is able to distinguish between ON target polynucleotides and one or more OFF target polynucleotides. The specificity of a guide RNA can be determined by, for example, calculating an ON target cleaving, binding, or nicking percentage as well as an OFF target cleaving, binding, or nicking percentage; calculating an ON:OFF ratio; and/or a specificity score derived from comparable ON and OFF target percentages (see Examples of this disclosure). The term "ON target percentage" refers to the percentage of cleaving, nicking or binding of a target polynucleotide within an assay; by way of example, a guide RNA having a 90% ON target percentage if it leads to the cleavage, nicking or binding of 90% of the target polynucleotides present in an assay. The term "ON:OFF ratio" refers to the ratio of ON target percentage and OFF target percentage per guide RNA assayed; by way of example, a guide RNA having a 80% ON target percentage and a 8% OFF target percentage has an ON:OFF ratio of 10. The term "specificity score" refers to a number obtained by multiplying an ON:OFF ratio by its respective ON-target percentage per guide RNA assayed; by way of example, a guide RNA having a 80% ON target percentage and a 8% OFF target percentage yields an ON:OFF ratio of 10 and has a specificity score of 8. In some assays, binding or nicking is assessed using cleaving as a surrogate; for example, in an assay where indel formation at a target site is quantified by sequencing to assess cleavage, such assay can be used to assess binding or nicking activity of the gRNA as well.

[0046] The term "consecutive specificity-enhancing modifications" refers to two or more specificity-enhancing modifications in a guide RNA that are adjacent to each other. The guide RNA may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 consecutive specificity-enhancing modifications. In widely used CRISPR-Cas9 systems, the guide RNA comprises a guide sequence comprising nucleotides 1 through 20, counted from the 5′ end of the guide sequence. The one or more consecutive specificity-enhancing modifications may comprise modification at, for example, nucleotides 1 and 2, nucleotides 1 through 3, nucleotides 1 through 4, nucleotides 1 through 5, nucleotides 1 through 6, nucleotides 1 through 7, nucleotides 1 through 8, nucleotides 1 through 9, nucleotides 1 through 10, nucleotides 2 and 3, nucleotides 2 through 4, nucleotides 2 through 5, nucleotides 2 through 6, nucleotides 2 through 7, nucleotides 2 through 8, nucleotides 2 through 9, nucleotides 2 through 10, nucleotides 3 and 4, nucleotides 3 through 5, nucleotides 3 through 6, nucleotides 3 through 7, nucleotides 3 through 8, nucleotides 3 through 9, nucleotides 3 through 10, and so on.

[0047] The term "hybridization" or "hybridizing" refers to a process where completely or partially complementary polynucleotide strands come together under suitable hybridization conditions to form a double-stranded structure or region in which the two constituent strands are joined by hydrogen bonds. As

used herein, the term "partial hybridization" includes where the double-stranded structure or region contains one or more bulges or mismatches. Although hydrogen bonds typically form between adenine and thymine or adenine and uracil (A and T or A and U) or cytosine and guanine (C and G), other noncanonical base pairs may form (see, *e.g.*, Adams *et al.*, "The Biochemistry of the Nucleic Acids," 11th ed., 1992). It is contemplated that modified nucleotides may form hydrogen bonds that allow or promote hybridization in a non-cannonical way.

[0048] The term "cleavage" or "cleaving" refers to breaking of the covalent phosphodiester linkage in the phosphodiester backbone of a polynucleotide. The terms "cleavage" or "cleaving" encompass both single-stranded breaks and double-stranded breaks. Double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. Cleavage can result in the production of either blunt ends or staggered ends.

[0049] The term "CRISPR-associated protein" or "Cas protein" refers to a wild type Cas protein, a fragment thereof, or a mutant or variant thereof. The term "Cas mutant" or "Cas variant" refers to a protein or polypeptide derivative of a wild type Cas protein, *e.g.*, a protein having one or more point mutations, insertions, deletions, truncations, a fusion protein, or a combination thereof. In certain embodiments, the "Cas mutant" or "Cas variant" substantially retains the nuclease activity of the Cas protein. In certain embodiments, the "Cas mutant" or "Cas variant" is mutated such that one or both nuclease domains are inactive. In certain embodiments, the "Cas mutant" or "Cas variant" has nuclease activity. In certain embodiments, the "Cas mutant" or "Cas variant" lacks some or all of the nuclease activity of its wild-type counterpart. The term "CRISPR-associated protein" or "Cas protein" also includes a wild type Cpf1 protein of various species of prokaryotes (and named for Clustered Regularly Interspaced Short Palindromic Repeats from Prevotella and Francisella 1 ribonucleoproteins or CRISPR/Cpf1 ribonucleoproteins), a fragment thereof, or a mutant or variant thereof.

[0050] The term "nuclease domain" of a Cas protein refers to the polypeptide sequence or domain within the protein which possesses the catalytic activity for DNA cleavage. Cas9 typically catalyzes a double-stranded break upstream of the PAM sequence. A nuclease domain can be contained in a single polypeptide chain, or cleavage activity can result from the association of two (or more) polypeptides. A single nuclease domain may consist of more than one isolated stretch of amino acids within a given polypeptide. Examples of these domains include RuvC-like motifs (amino acids 7–22, 759–766 and 982–989 in SEQ ID NO: 1) and HNH motif (amino acids 837–863); see Gasiunas et al. (2012) *Proc. Natl. Acad. Sci. USA 109*:39, E2579–E2586 and WO2013176772.

[0051] A synthetic guide RNA that has "gRNA functionality" is one that has one or more of the functions of naturally occurring guide RNA, such as associating with a Cas protein, or a function performed by the guide RNA in association with a Cas protein. In certain embodiments, the functionality includes binding a target polynucleotide. In certain embodiments, the functionality includes targeting a Cas protein or a gRNA:Cas protein complex to a target polynucleotide. In certain embodiments, the functionality includes nicking a target polynucleotide. In certain embodiments, the functionality includes cleaving a target polynucleotide. In certain embodiments, the functionality includes associating with or binding to a Cas protein. For example, the Cas protein may be engineered to be a "dead" Cas protein (dCas) fused to one or more proteins or portions thereof, such as a transcription factor enhancer or repressor a deaminase protein etc., such that the one or more functions is/are performed by the fused protein(s) or portion(s) thereof. In certain embodiments, the functionality is any other known function of a guide RNA in a CRISPR-Cas system with a Cas protein, including an artificial CRISPR-Cas system with an engineered Cas protein. In certain embodiments, the functionality is any other function of natural guide RNA. The synthetic guide RNA may have gRNA functionality to a greater or lesser extent than a naturally occurring guide RNA. In certain embodiments, a synthetic guide RNA may have greater functionality as to one property and lesser functionality as to another property in comparison to a similar naturally occurring guide RNA.

[0052] A Cas protein having a single-strand "nicking" activity refers to a Cas protein, including a Cas mutant or Cas variant, that has reduced ability to cleave one of two strands of a dsDNA as compared to a wild type Cas protein. For example, in certain embodiments, a Cas protein having a single-strand nicking activity has a mutation (*e.g.*, amino acid substitution) that reduces the function of the RuvC domain (or the HNH domain) and as a result reduces the ability to cleave one strand of the target DNA. Examples of such variants include the D10A, H839A/H840A, and/or N863A substitutions in *S. pyogenes* Cas9, and also include the same or similar substitutions at equivalent sites in Cas9 enzymes of other species.

[0053] A Cas protein having "binding" activity or that "binds" a target polynucleotide refers to a Cas protein which forms a complex with a guide RNA and, when in such a complex, the guide RNA hybridizes with another polynucleotide, such as a target polynucleotide sequence, via hydrogen bonding between the bases of the guide RNA and the other polynucleotide to form base pairs. The hydrogen bonding may occur by Watson Crick base pairing or in any other sequence specific manner. The hybrid may comprise two strands forming a duplex, three or more strands forming a multi-stranded triplex, or any combination of these.

[0054] A "CRISPR function" means any function or effect that can be achieved by a CRISPR system, including but not limited to gene editing, DNA cleavage, DNA nicking, DNA binding, regulation of gene expression, CRISPR activation (CRISPRa), CRISPR interference (CRISPRi), and any other function that can be achieved by linking a cas protein to another effector, thereby achieving the effector function on a target sequence recognized by the cas protein. For example, a nuclease-free cas protein can be fused to a transcription factor, a deaminase, a methylase, etc. The resulting fusion protein, in the presence of a guide RNA for the target, can be used to regulate the transcription of, deaminate, or methylate, the target.

[0055] As used herein, the term "portion" or "fragment" of a sequence refers to any portion of the sequence (*e.g.*, a nucleotide subsequence or an amino acid subsequence) that is smaller than the complete sequence. Portions of polynucleotides can be any length, for example, at least 5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 150, 200, 300 or 500 or more nucleotides in length. A portion of a guide sequence can be about 50%, 40%, 30%, 20%, 10% of the guide sequence, *e.g.,* one-third of the guide sequence or shorter, *e.g.*, 7, 6, 5, 4, 3, or 2 nucleotides in length.

[0056] The term "derived from" in the context of a molecule refers to a molecule isolated or made using a parent molecule or information from that parent molecule. For example, a Cas9 single-mutant nickase and a Cas9 double-mutant null-nuclease (also known as deactivated Cas9, "dead Cas9", or dCas9) are derived from a wild-type Cas9 protein.

[0057] The term "substantially identical" in the context of two or more polynucleotides (or two or more polypeptides) refers to sequences or subsequences that have at least about 60%, at least about 70%, at least about 80%, at least about 90%, about 90–95%, at least about 95%, at least about 98%, at least about 99% or more nucleotide (or amino acid) sequence identity, when compared and aligned for maximum correspondence using a sequence comparison algorithm or by visual inspection. Preferably, the "substantial identity" between polynucleotides exists over a region of the polynucleotide at least about 20 nucleotides in length, at least about 50 nucleotides in length, at least about 100 nucleotides in length, at least about 200 nucleotides in length, at least about 300 nucleotides in length, at least about 500 nucleotides in length, or over the entire length of the polynucleotide. Preferably, the "substantial identity" between polypeptides exists over a region of the polypeptide at least about 50 amino acid residues in length, at least about 100 amino acid residues in length, or over the entire length of the polypeptide.

[0058] As disclosed herein, a number of ranges of values are provided. It is understood that each intervening value, to the tenth of the unit of the lower limit, between the upper and lower limits of that

range is also specifically contemplated. Each smaller range or intervening value encompassed by a stated range is also specifically contemplated. The term "about" generally refers to plus or minus 10% of the indicated number. For example, "about 10%" may indicate a range of 9% to 11%, and "about 20" may mean from 18–22. Other meanings of "about" may be apparent from the context, such as rounding off, so, for example "about 1" may also mean from 0.5 to 1.4.

II. CRISPR-mediated Sequence-Specific Binding and/or Cleavage or Nicking

[0059] Shown in FIG. 1 is a diagram of CRISPR-Cas9–mediated sequence-specific cleavage of DNA. The guide RNA is depicted as sgRNA with an exemplary 20-nucleotide (or 20-nt; nucleotide is often abbreviated as "nt") guide sequence (other guide sequences may be, for example, from about 15 to about 30 nts in length) within the 5′ domain, an internally positioned base-paired stem, and a 3′ domain. The guide sequence is complementary to an exemplary 20-nt target sequence in a DNA target. The stem corresponds to a repeat sequence in crRNA and is complementary to a sequence in the tracrRNA. The 3′ domain of the guide RNA corresponds to the 3′ domain of the tracrRNA that binds a Cas9 nuclease. The Cas9:gRNA complex binds and cleaves a target DNA sequence or protospacer directly upstream of a PAM sequence recognized by Cas9. In FIG. 1, a 3-nt PAM sequence is exemplified; however, other PAM sequences including 4-nt, 5-nt and even longer PAM sequences are known.

[0060] Guide RNAs, for CRISPR-Cas genome editing, function in RNA-protein complexes where the RNA acts both as a scaffold for the protein and as sequence recognition for the duplex DNA target. The complex recognizes genomic DNA through first scanning for the nucleotide PAM sequence. Once a PAM sequence is identified the RNA-protein complex attempts to form an RNA/DNA duplex between the genomic DNA target and the guide sequence of the guide RNA. This duplex is first initiated by a Cas protein–mediated base pairing of the "seed region" of the gRNA, in which the seed region is thought to be around ten nucleotides in length. After the binding of the seed region, a stable RNA/DNA duplex is formed by the hybridization of the remaining nucleotides on the 5′ end of the guide RNA; this typically results in a 20-nucleotide DNA/RNA duplex and proceeds to a double-stranded cleavage of the target DNA by the protein complex.

[0061] An important aspect to enable the utility of CRISPR-Cas RNA-protein complexes for genome editing is sequence specificity. CRISPR-Cas RNA-protein complexes cleave genomic DNA as a first step in the process to inactivate or modify a gene through repair or recombination. In this process the cleavage of genomic DNA at unintentional "off-target" sites can have unwanted consequences, such as the creation of sequence mutations elsewhere in the genome. Currently, these off-target cleavage events are either being detected by screening techniques, removed by breeding techniques, or ignored. CRISPR-

Cas RNA-protein complexes evolved as an adaptive immune system in prokaryotes; an improvement in sequence specificity of these complexes would constitute a significant advance and innovation toward CRISPR-Cas RNA-protein complexes having wide utility as a tool in eukaryotic genomics.

[0062] Sequence specificity starts by a gRNA:Cas protein complex being able to scan, detect, and bind to a target site, or string of contiguous nucleotides within the entire genome of an organism. In order to do this the target sequence needs to be long enough that its string or sequence of contiguous nucleotides exists only once in the entire genome of the organism of interest and is located at the site of the desired genome editing. The length of the string of contiguous nucleotides, or polynucleotide, necessary to impart uniqueness within a genome is determined by the "information content" of that particular polynucleotide. For most eukaryotic cells and organisms, the target polynucleotide needs to be in the range of 18 to 22 nucleotides in length to have enough information content to be unique (J. Mol. Biol. (1986), 188, 415–431) in the entire genome. In general, the longer the target polynucleotide the more information content and the more likely its sequence will exist only once in a genome; a 19-nucleotide sequence has more information content than an 18-nucleotide sequence, a 20-nucleotide sequence has more information content than a 19-nucleotide sequence and so on. However, a unique 20-nucleotide sequence could match the sequence of all but 1 nucleotide in a different 20-nt sequence elsewhere in the genome, and the sequence containing a single mismatch comprises an off-target site. The relative binding of a guide sequence to the off-target sequence versus the 20-nucleotide target sequence is controlled by the respective binding energies and kinetic equilibria between the guide sequence and the on-target sequence as well as the off-target sequence.

[0063] The differential binding energies of oligonucleotides are controlled and can be maximized by the cooperative effect of DNA and RNA binding. Cooperativity has been defined for DNA and RNA binding during hybridization in two ways. First, when an oligonucleotide begins to bind its individual nucleotide subunits, the binding of nucleotide subunits to complementary nucleobases has a positive effect on the subsequent binding of the next adjacent nucleotide in the oligonucleotide sequence. At the same time the unbinding of an individual nucleotide has a negative effect on the binding of the next adjacent nucleotide. When a mismatch attempts to base pair, the unbinding of that mismatched pair has a negative effect on the binding of the adjacent nucleotide pair and likewise when a matched nucleotide pair binds successfully it has a positive effect on the binding of the adjacent nucleotide pair. From an overall energy perspective when an oligonucleotide begins to bind as individual nucleotide subunits that bind and unbind in multiple incremental steps, the intermediate states are statistically underrepresented relative to a system where the steps occur independently of each other. In other words, there are limited degrees of freedom and a limited number of kinetic states other than bound or unbound. From a molecular

perspective the string of nucleotides is somewhat rigid and once a nucleotide binds, the adjacent nucleotide has very limited energetic confirmations it can adopt other than the one that leads to binding. The need for molecular rigidity to retain DNA and RNA cooperative binding was first demonstrated almost 30 years ago by Z. A. Shabarova (1988) Bioorg. Khim. 14:12,1656–62. DNA oligonucleotides comprising 14 nucleotides in length were constructed from two oligonucleotides, each 7 nucleotides in length, which were covalently connected by 1,3-diaminopropane or 1,3-propanediol linkages by using chemical ligation. The flexible oligonucleotide was bound to a complementary 14-nucleotide DNA oligonucleotide and the binding energy measured. Without the rigidity of the natural DNA backbone the flexible 14-nucleotide DNA single strand bound with a significantly lower binding energy, as if it were two independent 7-nucleotide DNA oligonucleotides lacking cooperativity. The observed phenomenon known as cooperativity allows a higher degree of match verses mismatch distinction than would be seen in a non-cooperative system and is an important component for increasing the specificity of nucleotide sequences. The Cas protein has been shown by crystal structure to pre-order the 10 nucleotides of the seed region into a single-stranded portion of an A-form helix. The preordering of the RNA into an A-form helix limits the number of kinetic states that the guide sequence of the gRNA can adopt, thus increasing the cooperativity of the DNA/RNA hybridization in the seed region.

[0064] The overall binding energy of a string of nucleotides to a complementary string of nucleotides is typically defined by the melting temperature (Tm). The melting temperature is the temperature required to dissociate two bound nucleotide strings or strands (i.e., bound by base pairing or hybridization) to the point that they are 50% bound and 50% unbound. The melting temperature is measured by a phenomenon known as hyperchromicity. The UV absorption is increased when the two bound oligonucleotide strands are being separated by heat. Heat denaturation of oligonucleotides causes the double helix structure to unwind to form single-stranded oligonucleotides. When two bound oligonucleotides in solution are heated above their melting temperature, the duplex unwinds to form two single strands that absorb more light than the duplex. When the UV absorbance is graphed as a function of the temperature, a sigmoidal curve is obtained at the point where the duplex begins to dissociate and the center of the sigmoidal curve is defined as the Tm.

[0065] FIG. 4 is a graph demonstrating how UV absorbance increases as an oligonucleotide duplex separates into separate strands by heating. A sigmoidal curve reflects the dissociate of the duplex into separate strands, and the center of the sigmoidal curve is the T_m of the duplex. This curve indicates that the melting temperature of a 20-nucleotide DNA/RNA duplex at physiological salt concentrations is around 50°C.

[0066] An oligonucleotide duplex has the greatest match verses mismatch specificity at the melting temperature where only 50% of the duplex is formed. At this temperature a single mismatch at an off-target polynucleotide will block hybridization of the oligonucleotide if the binding and unbinding of the duplex has a high degree of cooperativity or shows a steep sigmoidal curve. If a gene editing experiment is performed at 37°C., then the best discrimination of match verses mismatch would be obtained using a guide RNA whose binding to its target would have a Tm of 37°C. The issue here is that this aspect is based only on the thermodynamics of single-stranded nucleic acids, and by contrast a guide RNA with a 37°C Tm would only partially bind to its double-stranded target, thereby giving low overall activity or gene editing. Nonetheless, lowering the overall Tm of the guide RNA to its target incrementally while monitoring activity should increase the specificity. This principle was demonstrated by Yanfang et al. (2014) Nat. Biotechnol. 32, 279–284, by truncating the guide RNA from 20 nucleotides to 17 nucleotides in length; they claimed a 5,000-fold increase in specificity at certain off-target sites without sacrificing on-target genome editing efficiencies. At physiological salt conditions, truncation of an RNA in a RNA/DNA duplex decreases the Tm of that duplex by about 2°C per base pair. Truncation of the guide sequence in a gRNA to 17 nucleotides would decrease the binding energy by around 6°C resulting in a duplex Tm of around 42°C. However, when the guide sequence is truncated from 20 nucleotides to 17 nucleotides, significant information content is lost such that it can more readily find an increased number of off-target sites identical or similar to the 17-nt guide sequence across the entire genome. A more useful approach would be to decrease the binding energy of the 20-nucleotide RNA/DNA duplex through chemical modification while retaining cooperativity of binding and unbinding.

[0067] Guide RNAs for CRISPR-Cas genome editing, or for target polynucleotide cleaving or nicking, exist as either single-guide RNAs or two-piece dual-guide RNAs where the two pieces are referred to as the crRNA (clustered repeat RNA) and the tracrRNA (trans-activating clustered repeat RNA). See Figs. 2A and 2B above. Fig. 5A also illustrates a single-guide RNA or two-piece dual-guide RNA 501 (wherein a crRNA segment and a tracrRNA segment form a hybridized duplex). Moving from left to right (i.e., from the 5′ end to the 3′ end of the guide RNA), Fig. 5A generally shows an extension 503 (sometimes referred to as an overhang) on the guide RNA, a sampling and locking region 505, a Cas protein-–binding seed region 507 (typically a 10-nt portion), a dual-guide stem 509 or a single-guide stem-loop 511, and a tracrRNA region 513. Cas9 protein can bind any or all of these gRNA regions except perhaps 503. The guide sequence comprises the locking, sampling, and seed regions. For both the single-guide RNA and the dual-guide RNA, the guide sequence of about 20 nucleotides on the 5′ end of the guide RNA is what binds and forms a stable duplex with the target DNA. This hybridization binding relative to competing hybridization with other sites of similar sequence determines the overall specificity

of the gRNA for the target and thus the specificity of genome editing or gene inactivation by a gRNA:Cas protein complex.

[0068] The binding of guide RNAs to their target polynucleotides occurs via Cas9-mediated formation of a seed RNA/DNA duplex initiated by the 3′ end of the guide sequence. Once the initial seed duplex is formed, the gRNA should continue to hybridize toward its 5′ end like a zipper.

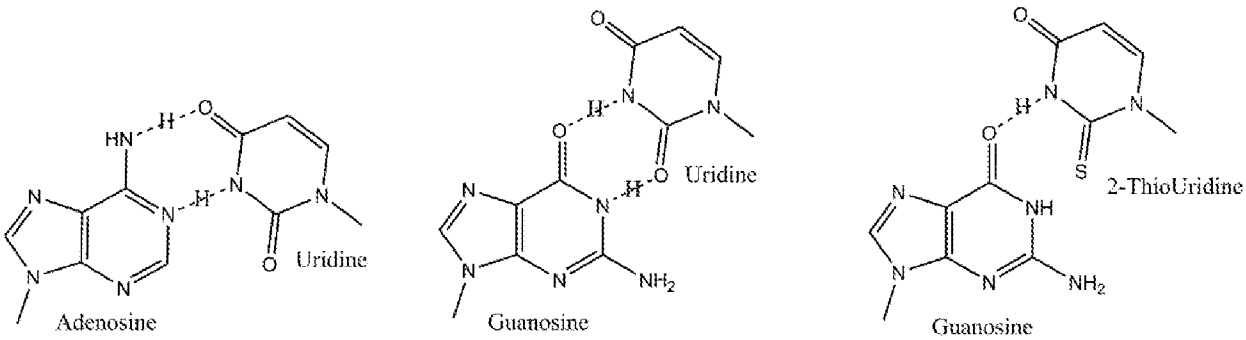
[0069] FIG. 5B illustrates how, after the initial formation of a seed duplex 517 from gRNA 501 and genomic DNA 515, the binding of the nucleotides proceeds sequentially through the sampling region. The sampling region is the region adjacent to the seed region, and the hybridization binding of these nucleotides proceeds until the point where the binding energy of the duplex is approximately equivalent to the temperature at which the binding is occurring. The rate of binding and unbinding in this region is controlled by the larger equilibrium of the bound versus unbound RNA (e.g., 501 versus 519 or 501 versus 521) which is also controlled by interaction of the Cas9 protein. In the case of guide RNAs forming an RNA/DNA duplex with genomic DNA at physiological salt concentrations and at 37°C, the typical sampling region spans the 5 to 6 nucleotides just 5′ of the 10-nucleotide seed region, based on the fact that a 15- to 16-nucleotide RNA/DNA duplex has a Tm of approximately 37°C. Once the number of bound nucleotides surpasses the Tm threshold, the binding continues in a sequential fashion through the locking region to the point where 20 nucleotides of the guide sequence are bound, and the binding may proceed farther if a target-complementary extension or 5′ overhang is present on the 5′ end of the guide sequence. Once the binding reaches the locking region, the equilibrium between bound RNA versus unbound RNA (e.g., 501 versus 523) changes such that its unbinding or release of a target polynucleotide is negatively impacted by the overall equilibrium of the bound versus unbound RNA which now lies significantly in the direction of the bound or hybridized state. The effect that the larger equilibrium of the bound versus unbound RNA has on specificity was shown by Slaymaker et al. (2016), "Rationally engineered Cas9 nucleases with improved specificity", Science 351, 84–8. Slaymaker et al. reported that a Cas9 protein was engineered that decreased off-target indel formation by converting positively charged amino acids in the nucleic acid–binding groove (or nt groove) of the protein to neutrally charged alanine residues. These changes decrease the overall Cas9-mediated affinity of the guide RNA for the double-stranded target DNA and force the affinity to depend more on the RNA/DNA hybridization including base-pair recognition.

[0070] FIG. 5C illustrates how chemical modifications of nucleotides that decrease the binding energy of individual base pairs yet retain a high level of cooperativity can be utilized in the seed and sampling regions of a gRNA 525 to extend the sampling region beyond 5 or 6 nucleotides. This effect can

significantly increase the specificity of binding of a guide RNA 525 to genomic DNA 515, as the chemical modifications increase the number of nucleotides required to achieve a hybridization binding energy equivalent to the temperature at which the binding is occurring. In a similar effect, the overall number of nucleotides required to shift the larger equilibrium of the bound versus unbound RNA (525 versus 527, 531, or 533) is increased, and this is calculated to result in an overall increase in sequence specificity.

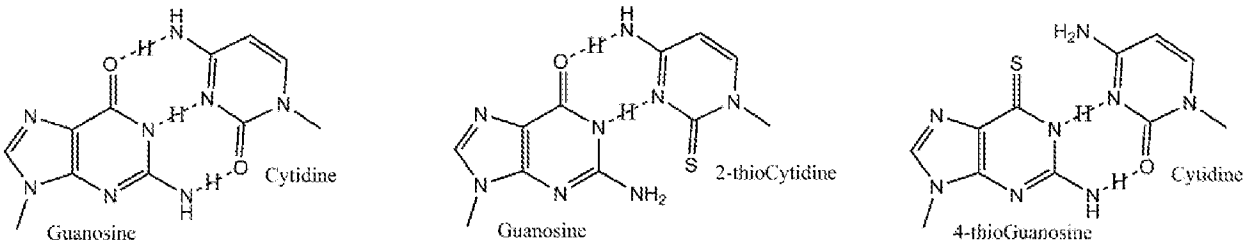
[0071] It was surprising and unexpected to find that nucleotide modifications can be used to decrease the activity of gRNA:Cas protein complexes toward partially complementary off-target polynucleotides through any of three different motifs in the guide sequence portion: the heterocyclic nucleobase, the sugar, and the internucleotide phosphate linkage. In gRNAs having chemical modification(s), it is important that the modification does not significantly increase non-specific binding or significantly decrease the cooperativity of hybridization, as either or both can promote off-target binding, nicking or cleaving by the Cas protein, which is undesirable.

[0072] Among the nucleobases in a guide sequence, it is possible to decrease the number of atoms accessible for base pairing and thus decrease the hydrogen-bonding potential that drives hybridization. However, decreasing the hydrogen bonding potential can also increase recognition of off-target polynucleotide sequences through alternative base pairing. To avoid this, it is important to utilize high-specificity base pairing in a guide sequence. An example of modifying the nucleobase to promote high specificity is to install a 2-thiouridine in place of a uridine in a guide sequence. Uridine nucleotides, which normally bind to adenosine nucleotides by two hydrogen bonds, can alternatively bind to guanosine nucleotides to yield a rather weak base pair often referred to as a G-U wobble pair. If 2-thiouridine is used instead of uridine, the 2-thiouridine can form only a less stable G-U wobble pair if any, because the sulfur substituent on the C2 position of the uracil base cannot serve as a hydrogen bond acceptor.



Wobble Base Pair

[0073] This same strategy can be used to decrease the hydrogen bonding potential of a typical Guanosine/Cytidine base pair by reducing the number of potential hydrogen bonds from three to two, for example by using either 2-thiocytidine or 4-thioguanosine. Their diminished potential for forming hydrogen bonds in modified G-C base pairs is illustrated here.



[0074] Modifications of the sugar moiety of a ribonucleotide can also be used to decrease the affinity of a guide sequence to a complementary DNA strand. This can be done in two ways, the first is to alter the sugar pucker, and the second is to deform the RNA/DNA duplex by steric crowding. Generally, the sugar pucker is described to be in either one of two states: the 2′-endo (south, DNA-like) sugar pucker, or the 3′-endo (north, RNA-like) sugar pucker. The 2′-endo pucker is thought to have a destabilizing effect on base pairing, and this is thought result from changes in the torsional angle of the glycosidic bond, thus preventing formation of the highest-affinity base-stacking geometry. Examples of modifications to RNA that can result in a a destabilizing effect on base pairing are deoxyribose, 2′-deoxy-2′-fluoroarabinofuranosyl, 2′-deoxy-2′-fluororibofuranosyl, 2′-O-phenyl, 2′-thiophenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino and 2′-O-substituted phenyl substituents.

[0075] Modification of an internucleotide bond can also decrease the binding affinity of nucleotides while maintaining the cooperativity of base pair hybridization. Examples of these are phosphonoacetates, thiophosphonoacetates, phosphonocarboxylates, thiophosphonocarboxylates, phosphonopropionates, thiophosphonopropionates, methylphosphonates, methylthiophosphonates, and boranophosphonates.

[0076] In certain embodiments, a sugar modification or nucleobase modification that increases or decreases the binding energy of the overall guide RNA can be added to modulate or further tune the binding energy from incorporation of other modifications to the guide RNA. As an example, incorporation of a 2′-O-methyl-thiophosphonoacetate (MSP) will decrease the binding energy of the guide RNA by ≅1.5 degrees as compared to the unmodified guide RNA. If a 2′-O-methyl nucleotide is incorporated elsewhere in the guide sequence it will increase the overall binding energy by ≅0.2 degrees and the resulting guide RNA will have a decreased overall binding energy of ≅1.3 degrees as compared to the unmodified guide RNA. In certain embodiments, the sugar modification comprises 2′-O-C_{1-3}alkyl-O-

C_{1-3}alkyl, such as 2′-methoxyethoxy (2′-O-$CH_2CH_2OCH_3$) also known as 2′-O-(2-methoxyethyl) or 2′-MOE. In certain embodiments, the sugar modification comprises 2′-halo, such as 2′-F, 2′-Br, 2′-Cl, or 2′-I. In certain embodiments, the sugar modification comprises 2′-NH_2. In certain embodiments, the sugar modification comprises 2′-H (*e.g.*, a 2′-deoxynucleotide). In certain embodiments, the sugar modification comprises 2′-arabino or 2′-F-arabino. In certain embodiments, the sugar modification comprises 2′-LNA or 2′-ULNA. In certain embodiments, the sugar comprises a 4′-thioribosyl.

[0077] In certain embodiments, a nucleotide sugar modification or nucleobase modification that increases or decreases the binding energy of the overall guide RNA can be incorporated on the same nucleotide where a phosphodiester linkage is modified to modulate the binding energy of the modified nucleotide. As an example, 3'-phosphonocarboxylate linkages can be used with sugar modifications such as 2′-O-methyl, 2′-F, or 2′-O-(2-methoxyethyl). In certain embodiments, the sugar comprises 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl, such as 2′-methoxyethoxy (2′-O-$CH_2CH_2OCH_3$) also known as 2′-O-(2-methoxyethyl) or 2′-MOE. In certain embodiments, the sugar comprises 2′-halo, such as 2′-F, 2′-Br, 2′-Cl, or 2′-I. In certain embodiments, the sugar comprises 2′-NH_2. In certain embodiments, the sugar comprises 2′-H (*e.g.*, a 2′-deoxynucleotide). In certain embodiments, the sugar comprises 2′-arabino or 2′-F-arabino. In certain embodiments, the sugar comprises 2′-LNA or 2′-ULNA. In certain embodiments, the sugar comprises a 4′-thioribosyl.

III. Guide RNAs

[0078] In at least one aspect, the present invention comprises a chemically modified guide RNA that has guide RNA functionality. The chemically modified guide RNA comprises at least one specificity-enhancing modification and may comprise other chemical modifications having more functions or different functions than specificity enhancement.

[0079] A guide RNA that comprises any nucleotide other than the four canonical ribonucleotides, namely A, C, G, and U, whether unnatural or natural (e.g., a pseudouridine, inosine or a deoxynucleotide), is a chemically modified guide RNA. Likewise, a guide RNA that comprises any backbone or internucleotide linkage other than a natural phosphodiester internucleotide linkage possesses a chemical modification and therefore is a chemically modified guide RNA. In certain embodiments, the retained functionality includes binding a Cas protein. In certain embodiments, the retained functionality includes binding a target polynucleotide. In certain embodiments, the retained functionality includes targeting a Cas protein or a gRNA:Cas protein complex to a target polynucleotide. In certain embodiments, the retained functionality includes nicking a target polynucleotide by a gRNA:Cas protein complex. In certain embodiments, the retained functionality includes cleaving a target polynucleotide by a

gRNA:Cas protein complex. In certain embodiments, the retained functionality is any other known function of a guide RNA in a CRISPR-Cas system with a Cas protein, including an artificial CRISPR-Cas system with an engineered Cas protein. In certain embodiments, the retained functionality is any other function of a natural guide RNA.

A. Exemplary Modifications

[0080] In certain embodiments, the specificity-enhancing modification is a deoxyribose nucleotide, a 2′-deoxy-2′-fluoroarabinofuranosyl nucleotide, a 2′-deoxy-2′-fluororibofuranosyl nucleotide, a sugar having a 2′-O-phenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino, or 2′-O-substituted phenyl, or combinations thereof. In certain embodiments, the specificity-enhancing modification is a phosphonoacetate, thiophosphonoacetate, phosphonopropionate, thiophosphonopropionate, methylphosphonate, methylthiophosphonate, or boranophosphonate; or combinations of any of the foregoing.

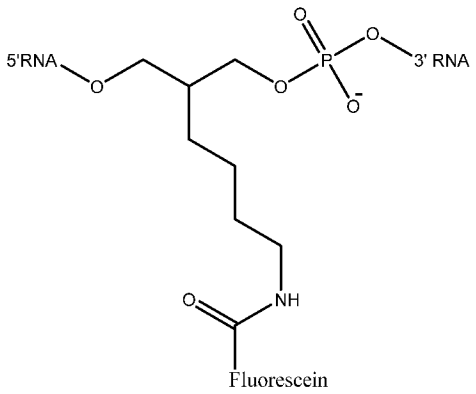
[0081] In certain embodiments, a nucleotide sugar modification incorporated into the guide RNA is selected from the group consisting of deoxyribose, 2′-deoxy-2′-fluoroarabinofuranosyl, 2′-deoxy-2′-fluororibofuranosyl, and sugars having 2′-O-phenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino, and 2′-O-substituted phenyl. In certain embodiments, an internucleotide linkage modification incorporated into the guide RNA is selected from the group consisting of: phosphorothioate "P(S)" (P(S)), phosphonocarboxylate ($P(CH_2)_nCOOR$) such as phosphonoacetate "PACE" ($P(CH_2COO^-)$), thiophosphonocarboxylate ($(S)P(CH_2)_nCOOR$) such as thiophosphonoacetate "thioPACE" ($(S)P(CH_2COO^-)$), alkylphosphonate ($P(C_{1-3}alkyl)$ such as methylphosphonate $-P(CH_3)$, boranophosphonate ($P(BH_3)$), and phosphorodithioate ($P(S)_2$). In certain embodiments, an internucleotide linkage modification incorporated into the guide RNA is selected from the group consisting of phosphonoacetates, thiophosphonoacetates, phosphonopropionates, thiophosphonopropionates, methylphosphonates, methylthiophosphonates, and boranophosphonates.

[0082] In certain embodiments, a nucleobase ("base") modification incorporated into the guide RNA is selected from the group consisting of: 2-thiouracil ("2-thioU"), 2-thiocytosine ("2-thioC"), 4-thiouracil ("4-thioU"), 6-thioguanine ("6-thioG"), 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylcytosine ("5-methylC"), 5-methyluracil ("5-methylU"), 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-ethynylcytosine, 5-aminoallyluracil ("5-aminoallylU"), 5-aminoallyl-cytosine ("5-

aminoallylC"), an abasic nucleotide, Unstructured Nucleic Acid ("UNA"), isoguanine ("isoG"), isocytosine ("isoC") [as described in "Enzymatic Incorporation of a New Base pair into DNA and RNA Extends the Genetic Alphabet." Piccirilli, J. A.; Krauch, T.; Moroney, S. E.; Benner, S. A. (1990) *Nature*, *343*, 33], 5-methyl-2-pyrimidine (as described in Rappaport, H. P. (1993) *Biochemistry, 32*, 3047), x(A,G,C,T,U) and y(A,G,C,T,U).

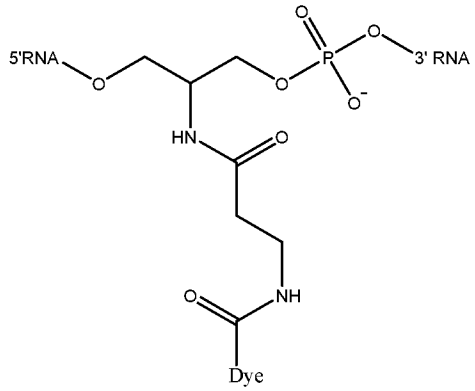
[0083] In certain embodiments, one or more isotopic modifications are introduced on the nucleotide sugar, the nucleobase, the phosphodiester linkage and/or the nucleotide phosphates. Such modifications include nucleotides comprising one or more ^{15}N, ^{13}C, ^{14}C, Deuterium, ^{3}H, ^{32}P, ^{125}I , ^{131}I atoms or other atoms or elements used as tracers.

[0084] In certain embodiments, an "end" modification incorporated into the guide RNA is selected from the group consisting of: PEG (polyethyleneglycol), hydrocarbon linkers (including: heteroatom (O,S,N)–substituted hydrocarbon spacers; halo-substituted hydrocarbon spacers; keto-, carboxyl-, amido-, thionyl-, carbamoyl-, thionocarbamaoyl-containing hydrocarbon spacers), spermine linkers, dyes including fluorescent dyes (for example fluoresceins, rhodamines, cyanines) attached to linkers such as for example 6-fluorescein-hexyl, quenchers (for example dabcyl, BHQ) and other labels (for example biotin, digoxigenin, acridine, streptavidin, avidin, peptides and/or proteins). In certain embodiments, an "end" modification comprises a conjugation (or ligation) of the guide RNA to another molecule comprising an oligonucleotide (comprising deoxynucleotides and/or ribonucleotides), a peptide, a protein, a sugar, an oligosaccharide, a steroid, a lipid, a folic acid, a vitamin and/or other molecule. In certain embodiments, an "end" modification incorporated into the guide RNA is located internally in the guide RNA sequence via a linker such as for example 2-(4-butylamidofluorescein)propane-1,3-diol bis(phosphodiester) linker (depicted below), which is incorporated as a phosphodiester linkage and can be incorporated anywhere between two nucleotides in the guide RNA.



2-(4-butylamidofluorescein)propane-1,3-diol bis(phosphodiester) linker

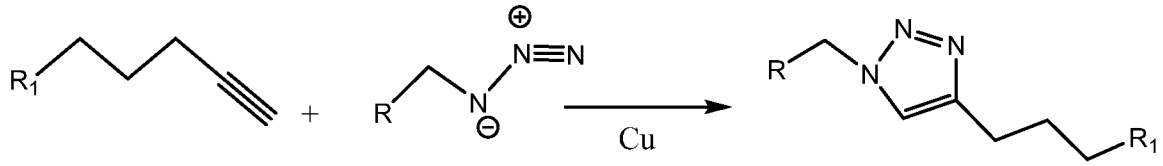
[0085] Other linkers include for example by way of illustration, but are not limited to:



2-(3-(dye-amido)propanamido)propane-1,3-diol bis(phosphodiester) linker

[0086] In certain embodiments, the end modification comprises a terminal functional group such as an amine, a thiol (or sulfhydryl), a hydroxyl, a carboxyl, carbonyl, thionyl, thiocarbonyl, a carbamoyl, a thiocarbamoyl, a phoshoryl, an alkene, an alkyne, an halogen or a functional group-terminated linker, either of which can be subsequently conjugated to a desired moiety, for example a fluorescent dye or a non-fluorescent label or tag or any other molecule such as for example an oligonucleotide (comprising deoxynucleotides and/or ribonucleotides, including an aptamer), an amino acid, a peptide, a protein, a sugar, an oligosaccharide, a steroid, a lipid, a folic acid, a vitamin. The conjugation employs standard chemistry well-known in the art, including but not limited to coupling via N-hydroxysuccinimide, isothiocyanate, DCC (or DCI), and/or any other standard method.

[0087] In certain embodiments, the label or dye is attached or conjugated to a modified nucleotide in the gRNA. The conjugation of a fluorescent dye or other moiety such as a non-fluorescent label or tag (for example biotin, avidin, streptavidin, or moiety containing an isotopic label such as ^{15}N,^{13}C, ^{14}C, Deuterium, ^{3}H, ^{32}P, ^{125}I and the like) or any other molecule such as for example an oligonucleotide (comprising deoxynucleotides and/or ribonucleotides including an aptamer), an amino acid, a peptide, a protein, a sugar, an oligosaccharide, a steroid, a lipid, a folic acid, a vitamin or other molecule can be effectuated using the so-called “click” chemistry or the so-called “squarate” conjugation chemistry. The “click” chemistry refers to the [3+2] cycloaddition of an alkyne moiety with an azide moiety, leading to a triazolo linkage between the two moieties as shown in the following scheme:

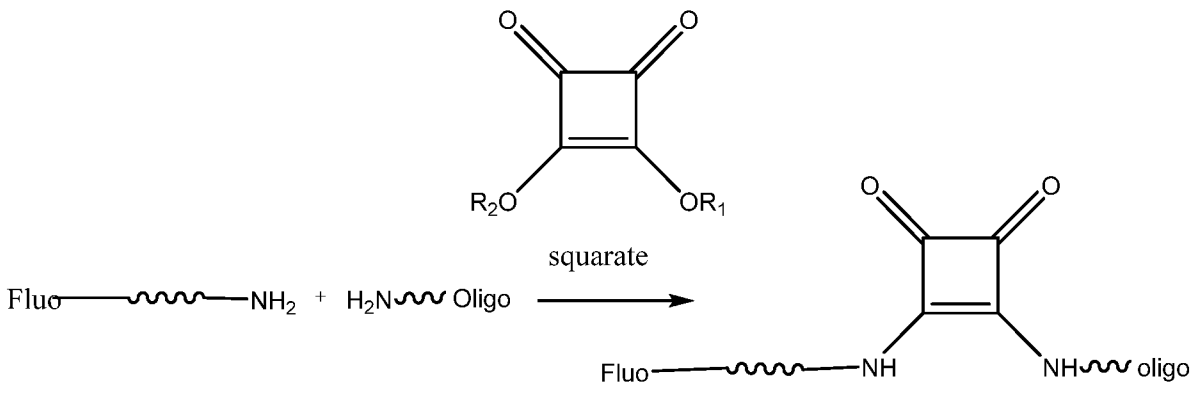


Alkyne azide triazolo conjugate

as described for example in El-Sagheer, A.H and Brown, T. "Click chemistry with DNA", *Chem. Soc. Rev.*, 2010, *39*, 1388–1405 and Mojibul, H.M. and XiaoHua, P., DNA-associated click chemistry, *Sci. China Chem.*, 2014, *57*:2, 215–31, the contents of which are hereby incorporated by reference in their entirety.

[0088] In certain embodiments, the conjugation can be effectuated by alternative cycloaddition such as Diels-Alder [4+2] cycloaddition of a π-conjugated diene moiety with an alkene moiety.

[0089] The "squarate" conjugation chemistry links two moieties each having an amine via a squarate derivative to result in a squarate conjugate that contains a squarate moiety (see *e.g.*, Tietze et al. (1991) *Chem. Ber.*, *124*, 1215–21, the contents of which are hereby incorporated by reference in their entirety). For example, a fluorescein containing a linker amine is conjugated to an oligoribonucleotide containing an amine through a squarate linker as described in the scheme below. An example of the squarate linker is depicted in the following scheme:



Squarate conjugate

B. Guide RNA with at least one specificity-enhancing modification

[0090] In one aspect, the present technology provides a guide RNA having at least one specificity-enhancing modification, constituting a modified gRNA and optionally a stability-enhancing modification.

[0091] In certain embodiments, at least one specificity-enhancing modification is within the guide sequence or crRNA segment of the guide RNA. In certain embodiments, the modification is within the guide sequence of the crRNA. In certain embodiments, the modification is within the first five (5)

nucleotides of the 5′ end of the guide sequence or crRNA segment. In certain embodiments, the modification is within the first four (4) nucleotides of the guide sequence or crRNA segment. In certain embodiments, the modification is within the first three (3) nucleotides of the guide sequence or crRNA segment. In certain embodiments, the modification is also within a 5′-overhang on the crRNA segment. In certain embodiments, where the guide sequence consists of nucleotides 1 through 20-N, counted from the 5′ end of the guide sequence, where N is a positive or negative integer between -10 and 10 (optionally between -10 and 6), at least one specificity-enhancing modification is within nucleotides 4-N to 20-N, alternatively within nucleotides 5-N to 20-N, alternatively within nucleotides 10-N to 20-N, alternatively within nucleotides 13-N to 20-N, alternatively within nucleotides 13-N to 14-N or 16-N to 19-N, alternatively within nucleotides 13-N to 14-N or 16-N to 18-N. In certain embodiments, the modification is at nucleotides 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, 16–N, or any combination thereof.

[0092] In certain embodiments, a modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 specificity-enhancing modified nucleotides in the guide sequence portion of the gRNA and up to 100 additional modified nucleotides in the other portions or segments of the gRNA. In other embodiments, the modified gRNA comprises a 5′ extension or overhang on the guide sequence portion, and the extension is 1 to 20 nucleotides in length comprising 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 specificity-enhancing modified nucleotides in addition to the 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 specificity-enhancing modified nucleotides in the guide sequence portion of the gRNA and optionally comprising up to 100 additional modified nucleotides in the other portions of the gRNA. In certain embodiments, all nucleotides in a gRNA are modified. In certain embodiments, all the modifications are the same. In certain embodiments, all the modified nucleotides have the same type of modification. In certain embodiments, the modified gRNA comprises a combination of differently modified nucleotides. In certain embodiments, the modified gRNA comprises two or more modified nucleotides. In certain embodiments, the modified gRNA comprises three or more modified nucleotides. In certain embodiments, the modified nucleotides are arranged contiguously. In certain embodiments, the modified gRNA comprises at least one contiguous stretch of modified nucleotides. In certain embodiments, the modified gRNA comprises a contiguous stretch of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 modified nucleotides. Each modified nucleotide may independently comprise one or more types of modifications. In certain embodiments, no modified nucleotides are contiguous, or some but not all are contiguous, in the sequence of the modified gRNA.

[0093] In certain embodiments, a chemical modification is within the 5′ portion of the guide RNA. When a guide RNA is a dual guide RNA, a chemical modification within a 5′ portion refers to a modification within the 5′ portion of the crRNA segment of the guide RNA, and not to a modification within a 5′ portion of a tracrRNA segment. When a guide RNA is a single guide RNA, it has one 5′ portion, located in the crRNA segment. In certain embodiments, the modification is within the first five (5) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, the modification is within the first three (3) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, the modification is within the 3′ portion of the guide RNA. In certain embodiments, the modification is within the last five (5) nucleotides of the 3′ portion of the guide RNA. In certain embodiments, the modification is within the last three (3) nucleotides of the 3′ portion of the guide RNA. In certain embodiments, the modification is within the internal region (*i.e.*, between the 5′ end and the 3′ end) of the guide RNA.

[0094] In certain embodiments, a chemical modification is incorporated in the 5′ portion or the 3′ portion of the guide RNA, particularly within the first 5 or 10 nucleotides of the 5′ portion or within the last 5 or 10 nucleotides of the 3′ portion to, for example, protect the RNA from degradation by nucleases or for other purposes. In some other embodiments, the modification is in both the 5′ portion and the 3′ portion of the guide RNA, particularly within the first 5 or 10 nucleotides of the 5′ portion and within the last 5 or 10 nucleotides of the 3′ portion to, for example, protect the RNA from degradation by nucleases or for other purposes. In certain embodiments, more than one type of modification is present in both the 5′ portion and the 3′ portion of the guide RNA. In certain embodiments, the modifications are located at the 5′ end, at the 3′ end, and within the internal sequence of the guide RNA. In certain embodiments, a guide RNA comprises 40 or fewer, alternatively 20 or fewer, alternatively 15 or fewer, alternatively 10 or fewer, alternatively 5 or fewer, alternatively 3 or fewer deoxyribonucleotide residues in the 5′ or 3′ portion of the guide RNA. Where the guide RNA is a dual guide, each RNA molecule may comprise modification(s) at the 5'-end, 3'-end, or both. In certain embodiments, consecutive nucleotides at the end (5', 3', or both) are modified, such as 2, 3, 4, 5 or more consecutive nucleotides..

[0095] In general, the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10 (optionally between -10 and 6). N can be selected from -10, -9, -8, -7, -6, -5, -4, -3, -2, -1, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. For example, the guide sequence may be 20 nucleotides long (N=0), 19 nucleotides long (N=1), 21 nucleotides long (N= -1), or the like. In certain embodiments, the guide sequence comprises at least one specificity-enhancing modification at nucleotide position (starting from the 5'-end of the guide sequence) 4-N, 5-N, 7-N, 9-N, 11-N, 14–N, or 16–N, or a combination thereof. A few examples are described below.

[0096] In certain embodiments, the guide sequence consists of nucleotides 1 through 20, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one nucleotide selected from positions 4, 5, 7, 9, 10, and 11. In certain embodiments, the chemical modification is at nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0097] In certain embodiments, the guide sequence consists of nucleotides 1 through 19, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one nucleotide selected from positions 3, 4, 6, 8, 9, and 10. In certain embodiments, the chemical modification is at nucleotide 4 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0098] In certain embodiments, the guide sequence consists of nucleotides 1 through 18, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one nucleotide selected from positions 2, 3, 5, 7, 8, and 9. In certain embodiments, the chemical modification is at nucleotide 3 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0099] In certain embodiments, the guide sequence consists of nucleotides 1 through 17, counted from the 5' end of the guide sequence, and comprises a chemical modification of at lest one nucleotide

selected from positions 1, 2, 4, 6, 7, and 8. In certain embodiments, the chemical modification is at nucleotide 2 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 1 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[00100] In certain embodiments, the guide sequence consists of nucleotides 1 through 16, counted from the 5' end of the guide sequence, and comprises a chemical modification of at lest one nucleotide selected from positions 1, 3, 5, 6, and 7. In certain embodiments, the chemical modification is at nucleotide 1 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0100] In certain embodiments, the guide sequence consists of nucleotides 1 through 15, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one nucleotide selected from positions 2, 4, 5, and 6. In certain embodiments, the chemical modification is at nucleotide 2 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0101] In certain embodiments, the guide sequence consists of nucleotides 1 through 21, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one of nucleotides 5, 6, 8, 10, 11, and 12. In certain embodiments, the chemical modification is at nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 of the guide sequence. In certain embodiments,

the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0102] In certain embodiments, the guide sequence consists of nucleotides 1 through 22, counted from the 5' end of the guide sequence, and comprises a chemical modification at at least one of nucleotides 6, 7, 9, 11, 12, and 13. In certain embodiments, the chemical modification is at nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0103] In certain embodiments, the guide sequence consists of nucleotides 1 through 20-N, wherein N is a positive or negative integer between -10 and 10 (optionally between -10 and 6), counted from the 5′ end of the guide sequence, and comprises at least one chemical modifications at any nucleotides from 4-N through 20-N. In certain embodiments, the guide sequence comprises modifications at at least two nucleotides selected from nucleotides 4-N throught 20-N. In certain embodiments, the guide sequence comprises at least one modification at nucleotide 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, or 16–N, as well as at least another modification at a nucleotide selected from 4-N through 20-N (but not 15-N). In certain embodiments, the nucleotide selected from 4-N through 20-N is 5-N, 6-N, 7-N, 8-N, 9-N, 10-N, 16-N, or 17-N. In certain embodiments, the guide RNA further comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0104] In certain embodiments, the guide sequence consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 5, 6, 7, 8, 9, 10, 16, and 17. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 17 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 10 and nucleotide 17 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 16 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 10 and nucleotide 16 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 and nucleotide 16 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 and nucleotide

17 of the guide sequence. In certain embodiments, the guide RNA comprises at least one end modification. In certain embodiments, the guide RNA comprises a 5' extension or overhang upstream of the guide sequence.

[0105] In certain embodiments, the guide sequence consists of nucleotides 1 through 19, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 4, 5, 6, 7, 8, 9, 15, and 16. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 9 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 16 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 and nucleotide 16 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 15 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 9 and nucleotide 15 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 and nucleotide 15 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 and nucleotide 16 of the guide sequence.

[0106] In certain embodiments, the guide sequence consists of nucleotides 1 through 18, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 3, 4, 5, 6, 7, 8, 14, and 15. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 8 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 15 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 and nucleotide 15 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 14 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 8 and nucleotide 14 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 and nucleotide 14 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 15 of the guide sequence.

[0107] In certain embodiments, the guide sequence consists of nucleotides 1 through 17, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 2, 3, 4, 5, 6, 7, 13, and 14. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 7 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 14 of the guide sequence. In certain embodiments, the

chemical modification is at nucleotide 3 and nucleotide 4 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 and nucleotide 14 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 7 and nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 14 of the guide sequence.

[0108] In certain embodiments, the guide sequence consists of nucleotides 1 through 16, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 1, 2, 3, 4, 5, 6, 12, and 13. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 6 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 1 and nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 3 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 13 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 1 and nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 6 and nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 1 and nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 13 of the guide sequence.

[0109] In certain embodiments, the guide sequence consists of nucleotides 1 through 15, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 1, 2, 3, 4, 5, 11, and 12. In certain embodiments, the chemical modification is at nucleotide 1 and nucleotide 5 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 1 and nucleotide 2 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 12 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 5 and nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 12 of the guide sequence.

[0110] In certain embodiments, the guide sequence consists of nucleotides 1 through 14, counted from the 5' end of the guide sequence, and comprises at least two chemical modifications at nucleotides selected from positions 1, 2, 3, 4, 10, and 11. In certain embodiments, the chemical modification is at nucleotide 4 and nucleotide 11 of the guide sequence. In certain embodiments, the chemical modification

is at nucleotide 4 and nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 3 and nucleotide 10 of the guide sequence. In certain embodiments, the chemical modification is at nucleotide 2 and nucleotide 11 of the guide sequence.

[0111] In certain embodiments, a chemical modification comprises an end modification, such as a 5′ end modification or a 3′ end modification. Examples of end modifications include, but are not limited to phosphorylation (as natural phosphate or polyphosphate or as modified phosphonate groups such as for example, alkylphosphonate, phosphonocarboxylate, phosphonoacetate, boranophosphonate, phosphorothioate, phosphorodithioate and the like), biotinylation, conjugating or conjugated molecules, linkers, dyes, labels, tags, functional groups (such as for example but not limited to 5′-amino, 5′-thio, 5′-amido, 5′carboxy and the like), inverted linkages, or hydrocarbon moieties which may comprise ether, polyethylene glycol (PEG), ester, hydroxyl, aryl, halo, phosphodiester, bicyclic, heterocyclic or other organic functional group. In certain embodiments, the end modification comprises dimethoxytrityl.

[0112] In certain embodiments, a chemical modification comprises a modified base. As used herein, "unmodified" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Examples of modified bases include, but are not limited to, synthetic and natural bases such as 2-thioU, 2-thioC, 4-thioU, 6-thioG, 2-aminoA, 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylC, 5-methylU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-allylU, 5-allylC, 5-aminoallyl-uracil, and 5-aminoallyl-cytosine. In certain embodiments, the modification comprises an abasic nucleotide. In certain embodiments, the modification comprises a nonstandard purine or pyrimidine structure, such as Z or P, isoC or isoG, UNA, 5-methylpyrymidine, x(A,G,C,T,U) or y(A,G,C,T,U). In certain embodiments, the modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 modified bases. In other embodiments, the modified gRNA comprises at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130 or 140 modified bases. In certain embodiments, all bases in a gRNA are modified.

[0113] In certain embodiments, the modification comprises a modified sugar. Examples of modified sugars include, but are not limited to, sugars having modifications at the 2′ position or modifications at the 4′ position. For example, in certain embodiments, the sugar comprises 2′-O-C_{1-4}alkyl, such as 2′-O-methyl (2′-OMe). In certain embodiments, the sugar comprises 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl, such as 2′-methoxyethoxy (2′-O-$CH_2CH_2OCH_3$) also known as 2′-O-(2-methoxyethyl) or 2′-MOE. In certain embodiments, the sugar comprises 2′-halo, such as 2′-F, 2′-Br, 2′-Cl, or 2′-I. In certain embodiments, the sugar comprises 2′-NH_2. In certain embodiments, the sugar comprises 2′-H (*e.g.*, a 2′-deoxynucleotide). In

certain embodiments, the sugar comprises 2′-arabino or 2′-F-arabino. In certain embodiments, the sugar comprises 2′-LNA or 2′-ULNA. In certain embodiments, the sugar comprises a 4′-thioribosyl. In certain embodiments, the modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 modified sugars. In other embodiments, the modified gRNA comprises at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130 or 140 modified sugars. In certain embodiments, all sugars in a gRNA are modified.

[0114] In certain embodiments, the modification comprises a modified backbone (*i.e.*, an internucleotide linkage other than a natural phosphodiester). Examples of modified internucleotide linkages include, but are not limited to, a phosphorothioate internucleotide linkage, a chiral phosphorothioate internucleotide linkage, a phosphorodithioate internucleotide linkage, a boranophosphonate internucleotide linkage, a C_{1-4}alkyl phosphonate internucleotide linkage such as a methylphosphonate internucleotide linkage, a boranophosphonate internucleotide linkage, a phosphonocarboxylate internucleotide linkage such as a phosphonoacetate internucleotide linkage, a phosphonocarboxylate ester internucleotide linkage such as a phosphonoacetate ester internucleotide linkage, a thiophosphonocarboxylate internucleotide linkage such as for example a thiophosphonoacetate internucleotide linkage, a thiophosphonocarboxylate ester internucleotide linkage such as a thiophosphonoacetate ester internucleotide linkage. Various salts, mixed salts and free acid forms are also included. In certain embodiments, the modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 modified internucleotide linkages. In other embodiments, the modified gRNA comprises at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130 or 140 modified internucleotide linkages. In certain embodiments, all internucleotide linkages in a gRNA are modified.

[0115] In certain embodiments, the modification is or comprises a 2′-O-C_{1-4}alkyl, 2′-H, 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl, 2′-F, 2′-NH_2, 2′-arabino, 2′-F-arabino, 2′-LNA, 2′-ULNA, 4′-thioribosyl, 2-thioU, 2-thioC, 4-thioU, 6-thioG, 2-aminoA, 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-MeC, 5-MeU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-allylU, 5-allylC, 5-aminoallyl-uracil, 5-aminoallyl-cytosine, an abasic nucleotide, Z nucleotide, P nucleotide, UNA, isoC, isoG, 5-methyl-pyrimidine, x(A,G,C,T,U) nucleotide, y(A,G,C,T,U) nucleotide, a 3’-phosphorothioate group, a 3’-phosphonoacetate group, a 3’-phosphonoacetate ester group, a 3’-thiophosphonoacetate group, a 3’-thiophosphonoacetate ester group, a 3’-methylphosphonate group, a 3’-boranophosphonate group or a 3’-phosphorodithioate group, or combinations thereof.

[0116] In certain embodiments, the modified nucleotide comprises a 2′-O-methyl-3’-phosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-O-methyl-3’-phosphorothioate. In certain embodiments, the modified nucleotide comprises a 2′-O-methyl-3’-thiophosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-O-methyl-3’-phosphonocarboxylate. In certain embodiments, the modified nucleotide comprises a 2′-deoxy-3’-phosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-deoxy-3’-phosphorothioate. In certain embodiments, the modified nucleotide comprises a 2′-deoxy-3’-thiophosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-deoxy-3’-phosphonocarboxylate. In certain embodiments, the modified nucleotide comprises a 2′-halo-3’-phosphorothioate. In certain embodiments, the modified nucleotide comprises a 2′-halo-3’-phosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-halo-3’-thiophosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-halo-3’-phosphonocarboxylate. In certain embodiments, the modified nucleotide comprises a 2′-fluoro-3’-phosphorothioate. In certain embodiments, the modified nucleotide comprises a 2′-fluoro-3’-phosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-fluoro-3’-thiophosphonoacetate. In certain embodiments, the modified nucleotide comprises a 2′-fluoro-3’-phosphonocarboxylate. In certain embodiments, the modified nucleotide comprises a Z base. In certain embodiments, the modified nucleotide comprises a P base.

[0117] In certain embodiments, the guide RNA comprises an oligonucleotide represented by Formula (I):

W-Y or Y-W (I)

[0118] wherein W represents a nucleotide or a stretch of nucleotides of the oligonucleotide comprising at least one stability-enhancing modification and Y represents an unmodified portion of the oligonucleotide.

[0119] In certain embodiments, W is within the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within the first five (5) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within the first four (4) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within the first three (3) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within nucleotides 4 to 20, alternatively within nucleotides 5 to 20 of the guide sequence, alternatively within nucleotides 10 to 20 of the guide sequence, alternatively within nucleotides 13 to 20 of the guide sequence, alternatively within nucleotides 13-14 or 16–19 of the guide sequence, alternatively within nucleotides 13–14 or 16–18 of the guide sequence.

[0120] In certain embodiments, W comprises a 2′-O-C_{1-4}alkyl, 2′-H, 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl, 2′-F, 2′-NH_2, 2′-arabino, 2′-F-arabino, 2′-LNA, 2′-ULNA, 4′-thioribosyl, 2-thioU, 2-thioC, 4-thioU, 6-thioG, 2-aminoA, 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-MeC, 5-MeU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-allylU, 5-allylC, 5-aminoallyl-uracil, 5-aminoallyl-cytosine, abasic nucleotides, Z nucleotide, P nucleotide, UNA, isoC, isoG, 5-methyl-pyrimidine, x(A,G,C,T,U), y(A,G,C,T,U), a phosphorothioate internucleotide linkage, a phosphonoacetate internucleotide linkage, a phosphonoacetate ester internucleotide linkage, a thiophosphonoacetate internucleotide linkage, a thiophosphonoacetate ester internucleotide linkage a methylphosphonate internucleotide linkage, a boranophosphonate internucleotide linkage, a phosphorodithioate internucleotide linkage, or combinations thereof.

[0121] In certain embodiments, W comprises a 2′-O-methyl and a 3’-phosphonoacetate group on the same nucleotide. In certain embodiments, W comprises a 2′-O-methyl and a 3’-phosphorothioate group on the same nucleotide. In certain embodiments, W comprises a 2′-O-methyl and 3’-thiophosphonoacetate group on the same nucleotide. In certain embodiments, W comprises a 2′-F and a 3’-phosphorothioate group on the same nucleotide. In certain embodiments, W comprises a 2′-F and a 3’-phosphonoacetate group on the same nucleotide. In certain embodiments, W comprises a 2′-F and 3’-thiophosphonoacetate group on the same nucleotide.

[0122] In certain embodiments, W comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 modified nucleotides. In certain embodiments, each of the modified nucleotides comprises the same modification. In certain embodiments, W comprises a combination of variously modified nucleotides. In certain embodiments, W comprises two or more modified nucleotides. In certain embodiments, W comprises three or more modified nucleotides. In certain embodiments, the modified nucleotides are not arranged contiguously in the sequence, or at least not entirely, as one or more unmodified nucleotides may intercede. In certain embodiments, the modified nucleotides are arranged contiguously. In certain embodiments, W comprises at least one contiguous stretch of modified nucleotides. In certain embodiments, W comprises a contiguous stretch of at least three (3) modified nucleotides. In certain embodiments, W comprises a contiguous stretch of at least four (4) modified nucleotides. In certain embodiments, W comprises a contiguous stretch of at least five (5) modified nucleotides.

[0123] In certain embodiments, the modification is a stability-altering modification. Stability refers to the ability of the gRNA to resist degradation by enzymes, such as nucleases, and other substances that exist in intra-cellular and extra-cellular environments. In certain embodiments, the modification increases nuclease resistance of the guide RNA relative to a guide RNA without the modification, thus it enhances

the guide RNA stability. In certain embodiments, the stability-altering modification is a stability-enhancing modification. For example, in certain embodiments, the stability-enhancing modification comprises a 2′-O-methyl or a 2′-O-C_{1-4}alkyl nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-halo nucleotide, such as 2′-F, 2′-Br, 2′-Cl, or 2′-I. In certain embodiments, the stability-enhancing modification comprises a 2′-MOE or a 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl. In certain embodiments, the stability-enhancing modification comprises a 2′-NH_2 nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-H (or 2′-deoxy) nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-arabino or a 2′–F-arabino. In certain embodiments, the stability-enhancing modification comprises a 4′-thioribosyl sugar moiety. In certain embodiments, the stability-enhancing modification comprises a 3’-phosphorothioate group. In certain embodiments, the stability-enhancing modification comprises a 3’-phosphonoacetate group. In certain embodiments, the stability-enhancing modification comprises a nucleotide containing a 3’-thiophosphonoacetate group. In certain embodiments, the stability-enhancing modification comprises a nucleotide containing a 3’-methylphosphonate group. In certain embodiments, the stability-enhancing modification comprises a nucleotide containing a 3’-boranophosphate group. In certain embodiments, the stability-enhancing modification comprises a nucleotide containing a 3’-phosphorodithioate group. In certain embodiments, the stability-enhancing modification comprises an unlocked nucleic acid (“ULNA”) nucleotide.

[0124] In certain embodiments, the stability-enhancing modification comprises a 2′-O-methyl and a 3’-phosphorothioate group on the same nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-O-methyl and a 3’-phosphonoacetate group on the same nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-O-methyl and a 3’-thiophosphonoacetate group on the same nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-fluoro and a 3’-phosphorothioate group on the same nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-fluoro and a 3’-phosphonoacetate group on the same nucleotide. In certain embodiments, the stability-enhancing modification comprises a 2′-fluoro and a 3’-thiophosphonoacetate group on the same nucleotide.

[0125] In certain embodiments, the modification is a specificity-altering modification. In some embodiments, specificity enhancement may be achieved by enhancing on-target binding and/or cleavage, or reducing off-target binding and/or cleavage, or a combination of both. In some other embodiments, specificity reduction may be achieved, for example, by reducing on-target binding and/or cleavage, or increasing off-target binding and/or cleavage, or a combination of both.

[0126] In certain embodiments, the specificity-altering modification comprises a 2′-O-methyl. In certain embodiments, the specificity-altering modification comprises a 2′-halo, such as 2′-fluoro.

[0127] In certain embodiments, the specificity-altering modification comprises a 2-thiouracil base (2-thioU). In certain embodiments, the specificity-altering modification comprises 2-thioC. In certain embodiments, the specificity-altering modification comprises 4-thioU. In certain embodiments, the specificity-altering modification comprises 6-thioG. In certain embodiments, the specificity-altering modification comprises 2-aminoA. In certain embodiments, the specificity-altering modification comprises a 2-aminopurine. In certain embodiments, the specificity-altering modification comprises pseudouracil. In certain embodiments, the specificity-altering modification comprises hypoxanthine. In certain embodiments, the specificity-altering modification comprises 7-deazaguanine. In certain embodiments, the specificity-altering modification comprises 7-deaza-8-azaguanine. In certain embodiments, the specificity-altering modification comprises 7-deazaadenine. In certain embodiments, the specificity-altering modification comprises 7-deaza-8-azaadenine. In certain embodiments, the specificity-altering modification comprises 5-methylC. In certain embodiments, the specificity-altering modification comprises 5-methylU. In certain embodiments, the specificity-altering modification comprises 5-hydroxymethylcytosine. In certain embodiments, the specificity-altering modification comprises 5-hydroxymethyluracil. In certain embodiments, the specificity-altering modification comprises 5,6-dehydrouracil. In certain embodiments, the specificity-altering modification comprises 5-propynylcytosine. In certain embodiments, the specificity-altering modification comprises 5-propynyluracil. In certain embodiments, the specificity-altering modification comprises 5-ethynylcytosine. In certain embodiments, the specificity-altering modification comprises 5-ethynyluracil. In certain embodiments, the specificity-altering modification comprises 5-allylU. In certain embodiments, the specificity-altering modification comprises 5-allylC. In certain embodiments, the specificity-altering modification comprises 5-aminoallylU. In certain embodiments, the specificity-altering modification comprises 5-aminoallylC. In certain embodiments, the specificity-altering modification comprises an abasic nucleotide. In certain embodiments, the specificity-altering modification comprises a Z base. In certain embodiments, the specificity-altering modification comprises P base. In certain embodiments, the specificity-altering modification comprises a UNA base. In certain embodiments, the specificity-altering modification comprises isoC. In certain embodiments, the specificity-altering modification comprises isoG. In certain embodiments, the specificity-altering modification comprises 5-methyl-pyrimidine. In certain embodiments, the specificity-altering modification comprises x(A,G,C,T,U). In certain embodiments, the specificity-altering modification comprises y(A,G,C,T,U).

[0128] In certain embodiments, the specificity-altering modification comprises a phosphorothioate internucleotide linkage. In certain embodiments, the specificity-altering modification comprises a phosphonoacetate internucleotide linkage. In certain embodiments, the specificity-altering modification comprises a thiophosphonoacetate internucleotide linkage. In certain embodiments, the specificity-

altering modification comprises a methylphosphonate internucleotide linkage. In certain embodiments, the specificity-altering modification comprises a boranophosphate internucleotide linkage. In certain embodiments, the specificity-altering modification comprises a phosphorodithioate internucleotide linkage. In certain embodiments, the specificity-altering modification comprises a ULNA. In certain embodiments, the specificity-altering modification comprises an LNA.

[0129] In certain embodiments, the modification alters RNA base pairing by, for example, altering the melting temperature (T_m) of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification lowers the T_m of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification raises the T_m of the guide RNA relative to a guide RNA without the modification.

[0130] In certain embodiments, a gRNA comprises a guide sequence capable of hybridizing to a target polynucleotide, and the guide sequence comprises one or more modifications that alter base pairing of the guide sequence with the target polynucleotide by altering the melting temperature (T_m) of the gRNA:target polynucleotide duplex relative to a similar duplex without the modification. In certain embodiments, the modification lowers the T_m of the gRNA:target polynucleotide duplex relative to a similar duplex without the modification.

[0131] In certain embodiments, the specificity-altering modification lowers the T_m of a base pairing interaction. In certain embodiments, the specificity-enhancing modification lowers the Tm of a first DNA/RNA duplex comprising the guide RNA and target polynucleotide by at least about 1°C, alternatively at least about 2°C, at least about 3°C, at least about 4°C, at least about 5°C, and/or up to about 6°C, alternatively up to about 8°C, alternatively up to about 10°C, alternatively up to about 13°C, for example by lowering the Tm from about 1°C to about 13°C, alternatively from about 1°C to about 6°C. In certain embodiments, the specificity-enhancing modification lowers the Tm of a second DNA/RNA duplex comprising the guide RNA and an off-target polynucleotide by at least about 1°C, alternatively at least about 2°C, at least about 3°C, at least about 4°C, at least about 5°C, and/or up to about 6°C, alternatively up to about 8°C, alternatively up to about 10°C, alternatively up to about 13°C, for example by lowering the Tm from about 1°C to about 13°C, alternatively from about 1°C to about 6°C.

[0132] In certain embodiments, the synthetic guide RNA comprises a chemical modification that alters transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification increases transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification decreases transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification neutralizes the anionic charge on phosphate to allow passive diffusion into cells. In certain embodiments, the charge-neutralizing modification comprises a phosphonoacetate alkyl ester

internucleotide linkage, such as a phosphonoacetate methyl ester internucleotide linkage. Further considerations relevant to developing a gRNA include transfectability and immunostimulatory properties. In certain embodiments, the synthetic guide RNA comprises a chemical modification that promotes efficient and titratable transfectability into cells, especially into the nuclei of eukaryotic cells, and reduces immunostimulatory properties in transfected cells. In certain embodiments, the synthetic guide RNA comprises a chemical modification that promotes effective delivery into and maintaining in an intended cell, tissue, bodily fluid or organism for a duration sufficient to allow the desired gRNA functionality. In certain embodiments, the synthetic guide RNA comprises a chemical modification that alters the immunostimulatory effect of the guide RNA relative to a guide RNA without the modification.

[0133] In certain embodiments, the synthetic guide RNA comprises a chemical modification that enhances both stability and specificity of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification enhances both stability and transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification enhances both specificity and transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, the modification enhances the overall efficacy of the guide RNA relative to a guide RNA without the modification.

[0134] In certain embodiments, a guide RNA having a chemical modification of the present application has a specificity score of greater than 1. In certain embodiments, a guide RNA having a chemical modification of the present application has a specificity score of at least about 1.1. Thus, in certain embodiments, a guide RNA having a chemical modification of the present application has a specificity score of at least about 1.1, at least about 1.5, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 11, at least about 12, at least about 13, at least about 14, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, or at least about 55. In certain embodiments, a guide RNA having a chemical modification of the present application has a specificity score of from about 2 to about 60. In certain embodiments, a guide RNA having a chemical modification of the present application has a specificity score of from about 10 to about 60.

[0135] In certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of at least about 1%. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of at least about 5%. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of at least about 10%. In certain embodiments, a gRNA:Cas protein complex comprising a guide RNA having a chemical modification of the present application has an ON target

cleavage of at least about 30%. Thus, in certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, or at least about 90%, at least about 95%, or at least about 99%. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of from about 25% to about 99.9%. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON target cleavage of from about 50% to about 99.9%.

[0136] In certain embodiments, a guide RNA having a chemical modification of the present application has an ON:OFF ratio of greater than 1. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON:OFF ratio of at least about 1.1:1. Thus, in certain embodiments, a guide RNA having a chemical modification of the present application has an ON:OFF ratio of at least about 1.1:1, at least about 1.5:1, at least about 2:1, at least about 3:1, at least about 4:1, at least about 5:1, at least about 6:1, at least about 7:1, at least about 8:1, at least about 9:1, at least about 10:1, at least about 15:1, at least about 20:1, at least about 25:1, at least about 30:1, at least about 35:1, at least about 40:1, at least about 45:1, at least about 50:1, at least about 60:1, at least about 70:1, at least about 80:1, at least about 90:1, at least about 95:1, or at least about 99:1. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON:OFF ratio of at least about 1.5:1 to about 99.9:1. In certain embodiments, a guide RNA having a chemical modification of the present application has an ON:OFF ratio of at least about 10:1 to about 99.9:1.

C. Guide RNA with a combination of modifications

[0137] In one aspect, the present technology provides a guide RNA having a combination of two or more modifications. In certain embodiments, the two modifications are on the same nucleotide (for example, one nucleotide comprises a 2′-O-methyl and a 3’-phosphonoacetate moiety). In other embodiments, the two modifications are on two different nucleotides (for example, one nucleotide has a 2′-O-methyl group and another has a 3’-phosphonoacetate moiety).

[0138] In certain embodiments, each modification in the guide RNA is the same. In certain embodiments, at least one modification in the guide RNA is different from at least one other modification in the guide RNA. In certain embodiments, the guide RNA comprises a combination of different types of modifications, and at least one type in the combination exists in multiple places in the guide RNA. In certain embodiments, at least one type in the combination appears 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 times in the guide RNA.

[0139] In certain embodiments, at least one type of the modifications in the combination appears in two or more modified nucleotides. In certain embodiments, at least one type of the modifications in the combination appears in three or more modified nucleotides. In certain embodiments, the modified nucleotides are not arranged contiguously in the sequence, or at least not entirely, as one or more unmodified nucleotides may intercede. In certain embodiments, the modified nucleotides are arranged contiguously. In certain embodiments, the guide RNA comprises a stretch of contiguous modified nucleotides of the same type. In certain embodiments, the stretch has at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 modified nucleotides.

[0140] In certain embodiments, at least one of the modifications in the combination comprises a modified sugar. In certain embodiments, the modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 modified sugars. In other embodiments, the modified gRNA comprises at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130 or 140 modified sugars. In certain embodiments, all sugars in a gRNA are modified.

[0141] In certain embodiments, at least one of the modifications in the combination comprises a modified backbone (*i.e.*, an internucleotide linkage other than a natural phosphodiester). In certain embodiments, the modified gRNA comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 modified internucleotide linkages. In other embodiments, the modified gRNA comprises at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130 or 140 modified internucleotide linkages. In certain embodiments, all internucleotide linkages in a gRNA are modified.

[0142] In certain embodiments, the guide RNA comprises consecutive modifications. In certain embodiments, a guide sequence comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 consecutive chemical modifications. In certain embodiments, the chemical modifications are at nucleotides 1 and 2, 1 through 3, 1 through 4, 1 through 5, 1 through 6, 1 through 7, 1 through 8, 1 through 9, 1 through 10, 2 and 3, 2 through 4, 2 through 5, 2 through 6, 2 through 7, 2 through 8, 2 through 9, or 2 through 10.

[0143] In certain embodiments, at least one of the modifications in the combination comprises a 2′-O-methyl, a 2′-fluoro, a 2′-amino, a 2′-deoxy, a 2′-arabino, a 2′-F-arabino, a 2-thiouracil, a 2-aminoadenine, a 5-methylcytosine, a 5-aminoallyluracil, a Z base, a 3’-phosphorothioate, a 3’-phosphonoacetate, a 3’-phosphonoacetate ester, a 3’-thiophosphonoacetate, a 3’-thiophosphonoacetate ester, a 3’-methylphosphonate, a 3’-boranophosphonate, a 3’-phosphorodithioate, or combinations thereof. In certain embodiments, at least one of the modifications in the combination comprises a 2′-O-

methyl, a 2′-deoxy, a Z base, a phosphorothioate internucleotide linkage, a phosphonoacetate internucleotide linkage, a thiophosphonoacetate internucleotide linkage, or combinations thereof. In certain embodiments, at least one of the modifications in the combination comprises a 2′-F, a 2-thioU, a 4-thioU, a 2-aminoA, a 5-methylC, a 5-methylU, a 5-aminoallylU, or combinations thereof. In certain embodiments, at least one of the modifications in the combination is an "end" modification such as terminal phosphate, a PEG, a terminal amine, a terminal linker such as a hydrocarbon linker, a substituted hydrocarbon linker, a squarate linker, a triazolo linker, an internal linker such as 2-(4-butylamidofluorescein)propane-1,3-diol bis(phosphodiester) linker, a linker conjugated to a dye, a linker conjugated to a non-fluorescent label, a linker conjugated to a tag or a linker conjugated to a solid support such as for example a bead or microarray. In certain embodiments, at least two of the modifications in the combination comprise a 2′-O-methyl nucleotide and phosphorothioate internucleotide linkage, a 2′-O-methyl nucleotide and phosphonoacetate internucleotide linkage, or a 2′-O-methyl nucleotide and thiophosphonoacetate internucleotide linkage. In certain embodiments, at least two of the modifications in the combination comprise a 2′-O-methyl nucleotide and phosphonocarboxylate internucleotide linkage, a 2′-O-methyl nucleotide and phosphonocarboxylate ester internucleotide linkage, a 2′-O-methyl nucleotide and thiophosphonocarboxylate internucleotide linkage, a 2′-O-methyl nucleotide and thiophosphonocarboxylate ester internucleotide linkage, or combinations thereof. In other embodiments, the modifications in the combination further comprise a 2-thiouracil, 2-thiocytosine, 4-thiouracil, 6-thioguanine, 2-aminoadenine, 2-aminopurine, pseudouracil, inosine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylcytosine, 5-methyluracil, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-allyluracil, 5-allylcytosine, 5-aminoallyl-uracil, 5-aminoallyl-cytosine, or an abasic nucleotide.

[0144] In certain embodiments, at least one of the modifications in the combination comprises a 2′-O-methyl-3'-phosphorothioate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-O-methyl-3'-phosphonoacetate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-O-methyl-3'-thiophosphonoacetate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-halo-3'-phosphorothioate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-halo-3'-phosphonoacetate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-halo-3'-thiophosphonoacetate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-fluoro-3'-phosphorothioate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-fluoro-3'-phosphonoacetate. In certain embodiments, at least one of the modifications in the combination comprises a 2′-fluoro-3'-

thiophosphonoacetate. Possible combinations of at least two or three modifications are represented in figure 6 and figure 7 respectively and are incorporated herein by reference.

[0145] In certain embodiments, the guide RNA comprises an oligonucleotide represented by Formula (III) or Formula (IV):

W-Y-Q (III); or

Y-W-X-Q (IV)

[0146] wherein Q and W each independently represent a nucleotide or a stretch of nucleotides of the oligonucleotide comprising at least one specificity-enhancing modification and Y and X each independently represent an unmodified portion of the oligonucleotide.

[0147] In certain embodiments, W is within the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within the first five (5) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, W is at least partially within the first three (3) nucleotides of the 5′ portion of the guide RNA. In certain embodiments, W is within the internal region (*i.e.*, between the 5′ end and the 3′ end) of the guide RNA. In certain embodiments, W is at least partially within nucleotides 4 to 20, alternatively within nucleotides 5 to 20 of the guide sequence, alternatively within nucleotides 10 to 20 of the guide sequence, alternatively within nucleotides 13 to 20 of the guide sequence, alternatively within nucleotides 13-14 or 16-19 of the guide sequence, alternatively within nucleotides 13-14 or 16-18 of the guide sequence.

[0148] In certain embodiments, at least one of the modifications in the combination enhances stability and specificity of the guide RNA relative to a guide RNA without the modification. In certain embodiments, at least one of the modifications in the combination enhances stability and transfection efficiency of the guide RNA relative to a guide RNA without the modification. In certain embodiments, at least one of the modifications in the combination enhances specificity and transfection efficiency of the guide RNA relative to a guide RNA without the modification.

[0149] In certain embodiments, at least one of the modifications in the combination alters the secondary structure of the guide RNA. This modification alters the base-pairing of any of the RNA/RNA internal duplexes in the guide RNA. Some of these modifications increase the base pairing of the RNA/RNA structure or alternatively increase the Tm of the RNA/RNA duplex, whereas other modifications decrease the base pairing (or Tm) of the RNA/RNA duplex or duplexes. Such modifications include base modified nucleotides, particularly UNA nucleotides such as the 2-thiouridine and 2-aminoadenosine pair, the Z/P nucleotide pair, the isoC/isoG pair, the 6-thioG/5-methylpyrimidine pair, and nucleotides with modifications on the sugar or the internucleotide linkages as discussed before.

[0150] In certain embodiments, the combination includes at least one modification or a set of modifications that increases nucleases resistance (i.e., stability) with at least one modification or a set of

modifications that increases specificity (i.e., reduces off-target effects). In certain embodiments, the combination includes at least one modification or a set of modifications that increases nucleases resistance (i.e., stability) with at least one modification or a set of modifications that raises the Tm of some bases pairing in the guide RNA. In certain embodiments, the combination includes at least one modification or a set of modifications that increases nucleases resistance (i.e., stability) with at least one modification or a set of modifications that lowers the Tm of some bases pairing of the guide RNA. In certain embodiments, the combination includes at least one modification or a set of modifications that increases nuclease resistance (i.e., stability), at least one modification or a set of modifications that increases the Tm of some bases paring in the guide RNA, and at least one modification or a set of modifications that decreases the Tm of some base paring elsewhere in the guide RNA. In certain embodiments, the combination includes at least one modification or a set of modifications that increases nuclease resistance (i.e., stability) and at least one modification or a set of modifications that increases the binding of the guide RNA to Cas protein. In certain embodiments, the combination includes at least one modification or a set of modifications that increases nuclease resistance (i.e., stability) and at least one modification or a set of modifications that decreases the binding of the guide RNA to Cas protein. In certain embodiments, the guide RNA comprises a combination of the different types of modifications.

D. Guide RNA Structure

[0151] In certain embodiments, the guide RNA is able to form a complex with a CRISPR-associated-protein. In certain embodiments, the CRISPR-associated protein is provided by or is derived from a CRISPR-Cas type II system, which has an RNA-guided polynucleotide binding and/or nuclease activity. In certain embodiments, the CRISPR-associated protein is Cas9, a Cas9 mutant, or a Cas9 variant. In certain embodiments, the CRISPR-associated protein is the Cas9 nuclease from *Streptococcus pyogenes*. In certain embodiments, the CRISPR-associated protein is the Cas9 nuclease from *Streptococcus thermophilus*. In certain embodiments, the CRISPR-associated protein is the Cas9 nuclease from *Staphylococcus aureus*. In certain embodiments, the synthetic guide RNA or a synthetic guide RNA:CRISPR-associated protein complex maintains functionality of natural guide RNA or a complex that does not have modified nucleotides. In certain embodiments, the functionality includes binding a target polynucleotide. In certain embodiments, the functionality includes nicking a target polynucleotide. In certain embodiments, the functionality includes cleaving a target polynucleotide. In certain embodiments, the target polynucleotide is within a nucleic acid *in vitro*. In certain embodiments, the target polynucleotide is within the genome of a cell *in vivo* or *in vitro* (such as in cultured cells or cells isolated from an organism). In certain embodiments, the target polynucleotide is a protospacer in DNA.

[0152] In certain embodiments, the crRNA segment comprises from 25 to 80 nucleotides. In certain embodiments, the crRNA segment comprises a guide sequence that is capable of hybridizing to a target

sequence. In certain embodiments, the guide sequence is complementary to the target sequence or a portion thereof. In certain embodiments, the guide sequence comprises from 15 to 30 nucleotides. In certain embodiments, the crRNA segment comprises a stem sequence. In certain embodiments, the stem sequence comprises from 10 to 50 nucleotides. In certain embodiments, the crRNA segment comprises a 5′-overhang sequence. In certain embodiments, the 5′-overhang sequence comprises from 1 to 10 nucleotides, alternatively 1 to 5 nucleotides, alternatively 1, 2 or 3 nucleotides. In certain embodiments, the crRNA comprises both (i) a guide sequence that is capable of hybridizing to a target sequence and (ii) a stem sequence. In certain embodiments, the crRNA comprises (i) a 5′-overhang sequence, (ii) a guide sequence that is capable of hybridizing to a target sequence, and (iii) a stem sequence. In certain embodiments wherein the crRNA segment comprises a stem sequence, the tracrRNA segment comprises a nucleotide sequence that is partially or completely complementary to the stem sequence of the crRNA segment. In certain embodiments, the tracrRNA segment comprises at least one more duplex structure.

[0153] In certain embodiments, the guide RNA is a single-guide RNA, wherein the crRNA segment and the tracrRNA segment are linked through a loop L. In certain embodiments, the loop L comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides. In certain embodiments, the loop L comprises a nucleotide sequence of GNRA, wherein N represents A, C, G, or U and R represents A or G. In certain embodiments, the loop L comprises a nucleotide sequence of GAAA. In certain embodiments, the guide RNA comprises more than one loop.

[0154] The guide RNA comprises a 5′ portion (i.e., the 5′ half) and a 3′ portion (i.e., the 3′ half). In certain embodiments, the crRNA segment is 5′ (i.e., upstream) of the tracrRNA segment. In certain embodiments, the tracrRNA segment is 5′ relative to the crRNA segment.

[0155] In certain embodiments, the guide RNA comprises at least two separate RNA strands, for example, a crRNA strand and a separate tracrRNA strand. See, for example, FIG. 2A. In certain embodiments, each of the strands is a synthetic strand comprising one or more modifications. In certain embodiments, at least one of the strands is a synthetic strand comprising one or more modifications. In certain embodiments, the strands function together to guide binding, nicking, or cleaving of a target polynucleotide by a Cas protein, such as Cas9. In certain embodiments, the crRNA sequence and the tracrRNA sequence are on separate stands and hybridize to each other via two complementary sequences to form a stem or duplex.

[0156] In certain embodiments, the guide RNA is a single-guide RNA comprising a crRNA sequence and a tracrRNA sequence. See, for example, FIG. 2B. In certain embodiments, the crRNA sequence and the tracrRNA sequence are connected by a loop sequence or "loop." In certain embodiments, a single-guide RNA comprises a 5′ portion and a 3′ portion, wherein the crRNA sequence

is upstream of the tracrRNA sequence. In certain embodiments, the guide RNA comprises a crRNA without a tracrRNA segment.

[0157] In certain embodiments, the total length of the two RNA pieces can be about 50–220 (*e.g.*, about 55–200, 60–190, 60–180, 60–170, 60–160, 60–150, 60–140, 60–130, and 60–120) nucleotides in length, such as about 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, or 220 nucleotides in length. Similarly, the single-guide RNA (e.g., FIG. 2B) can be about 50–220 (*e.g.*, about 55–200, 60–190, 60–180, 60–170, 60–160, 60–150, 60–140, 60–130, and 60–120) nucleotides in length, such as about 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, or 220 nucleotides in length.

[0158] As shown in Figures 2A and 2B, the synthetic guide RNA comprises (i) a crRNA sequence that comprises (a) a guide sequence (*e.g.*, segment G_1-G_n, where each G represents a nucleotide in the guide sequence) capable of hybridizing to a target sequence in a nucleic acid, (b) a first stem sequence (*e.g.*, segment X_1–X_n, where each X represents a nucleotide in the first stem sequence) capable of hybridizing partially or completely to a second stem sequence, and, optionally (c) a 5′-overhang sequence (e.g., segment O_1–O_n, where each O represents a nucleotide in the overhang sequence), and (ii) a tracrRNA sequence that comprises the second stem sequence (*e.g.*, segment Y_1–Y_n, where each Y represents a nucleotide in the second stem sequence). The tracrRNA sequence further comprises segment T_1-T_n, where each T represents a nucleotide in the tracrRNA sequence. The synthetic guide RNA shown in FIG. 2A includes one or more modifications. Likewise, the synthetic guide RNA shown in FIG. 2B includes one or more modifications. In certain embodiments, the modification is located at any point along the length of the crRNA, the tracrRNA, or the single-guide RNA comprising a crRNA segment, a tracrRNA segment, and, optionally, a loop. In certain embodiments, any nucleotide represented by O, G, X, Y, or T in the synthetic guide RNA shown in FIGs. 2A and 2B may be a modified nucleotide. The guide RNA shown in FIG. 2B represents a single-guide RNA (sgRNA) where the crRNA segment and the tracrRNA segment are connected by a loop having the sequence GNRA, wherein N represents A, C, G, or U, and R represents A or G.

[0159] In certain embodiments, the crRNA segment of the guide RNA is 25–70 (e.g., 30–60, 35–50, or 40–45) nucleotides in length. In certain embodiments, the guide sequence is 12–30 (*e.g.*, 16–25, 17–20, or 15–18) nucleotides in length. In some embodiments, a 5′ portion of the crRNA does not hybridize or only partially hybridizes with the target sequence. For example, there can be a 5′-overhang on the crRNA segment.

[0160] In certain embodiments, the single-guide RNA comprises a central portion including the stem sequence of the crRNA segment, the stem sequence of the tracrRNA segment, and, optionally, a

loop that covalently connects the crRNA segment to the tracrRNA segment. In certain embodiments, the central segment of the single-guide RNA is 8–60 (*e.g.*, 10–55, 10–50, or 20–40) nucleotides in length.

[0161] In certain embodiments, the tracrRNA segment of the guide RNA is 10–130 (*e.g.*, 10–125, 10–100, 10–75, 10–50, or 10–25) nucleotides in length. In certain embodiments, the tracrRNA segment includes one or more hairpin or duplex structures in addition to any hairpin or duplex structure in the central segment.

E. Synthesis of guide RNA

[0162] In certain embodiments, guide RNAs, including single-guide RNAs are produced by chemical synthesis using the art of synthetic organic chemistry. A guide RNA that comprises any nucleotide other than the four predominant ribonucleotides, namely A, C, G, and U, whether unnatural or natural, such as a pseudouridine, inosine or a deoxynucleotide, possesses a chemical modification or substitution at the nucleotide which is chemically/structurally distinct from any of the four predominant nucleotides in RNAs.

[0163] The synthetic guide RNAs described herein can be chemically synthesized using methods well-known in the art (such as TBDMS chemistry, TOM Chemistry, ACE chemistry, etc.). For example, the synthetic guide RNAs can be synthesized using TC chemistry by the method described in Dellinger et al. (2011) *J. Am. Chem. Soc. 133*, 11540; US Patent 8,202,983; and US Patent Application 2010/0076183A1, the contents of which are incorporated by reference in their entireties. "TC chemistry" refers to the composition and methods of using RNA monomeric nucleotide precursors protected on the 2′-hydroxyl moiety by a thionocarbamate protecting group, to synthesize unmodified RNA or modified RNA comprising one or more modified nucleotides. The ability to chemically synthesize relatively long RNAs (as long as 200-mers or more) using TC-RNA chemistry allows one to produce guide RNAs with special features capable of outperforming those enabled by the four predominant ribonucleotides (A, C, G and U). Some synthetic guide RNAs described herein can also be made using methods known in the art that include *in vitro* transcription and cell-based expression. For example, 2′-fluoro NTPs can be incorporated into synthetic guide RNAs produced by cell-based expression.

[0164] Synthesis of guide RNAs can also be accomplished by chemical or enzymatic synthesis of RNA sequences that are subsequently ligated together by enzymes, or chemically ligated by chemical ligation, including but not limited to cyanogen bromide chemistry, "click" chemistry as published by R. Kumar et al. (2007) *J. Am. Chem. Soc. 129*, 6859–64, or squarate conjugation chemistry as described by K. Hill in WO2013176844 titled "Compositions and methods for conjugating oligonucleotides."

[0165] In certain embodiments, methods are provided for preparing a synthetic guide RNA. The methods comprise selecting a target polynucleotide in a genome; identifying one or more off-target polynucleotide in the genome; identifying one or more shared nucleotide residues, wherein the shared

nucleotide residues are present in both the target polynucleotide and the off-target polynucleotide; and designing a synthetic guide RNA, wherein the guide sequence includes a specificity-enhancing modification. The methods can comprise synthesizing the designed guide RNA. In certain embodiments, the off-target polynucleotide is identified by an algorithm to predict off-target sites as well as their severity such as those found at http://www.rgenome.net/Cas-OFFinder; https://cm.jefferson.edu/Off-Spotter; or http://crispr.mit.edu, or other technique for identifying and quantifying the activation of off-target sites in actual cases, as disclosed in Tsai et al. (2015) *Nat. Biotechnol. 33*, 187–97; Ran et al. (2015) *Nature 520*, 186–91; Frock et al. (2015) *Nat. Biotechnol. 33*, 179–86. In certain embodiments, the method further comprises identifying at least one distinguishing position between the sequences of the target polynucleotide and the off-target polynucleotide, wherein the target polynucleotide and the off-target polynucleotide have a different nucleotide residue at the at least one distinguishing position, and including in the synthetic guide RNA a nucleotide matching (i.e., complementary to) the nucleotide at the at least one distinguishing position in the target polynucleotide.

[0166] As further described below, a guide RNA disclosed herein, including those comprising modified nucleotides and/or modified internucleotide linkages, can be used to perform various CRISPR-mediated functions (including but not limited to editing genes, regulating gene expression, cleaving target sequences, and binding to target sequences) *in vitro* or *in vivo*, such as in cell-free assays, in intact cells, or in whole organisms. For *in vitro* or *in vivo* applications, the RNA can be delivered into cells or whole organisms in any manner known in the art.

Libraries and Arrays

[0167] In one aspect, the present invention provides a set or library of multiple guide RNAs. In certain embodiments, the library contains two or more guide RNAs disclosed herein. The library can contain from about 10 to about 10^7 individual members, *e.g.*, about 10 to about 10^2, about 10^2 to about 10^3, about 10^3 to about 10^5, from about 10^5 to about 10^7 members. An individual member of the library differs from other members of the library at least in the guide sequence, *i.e.*, the DNA targeting segment of the gRNA. On the other hand, in certain embodiments, each individual member of a library can contain the same or substantially the same nucleotide sequence for the tracrRNA segment as all the other members of the library. In this way, the library can comprise members that target different polynucleotides or different sequences in one or more polynucleotides.

[0168] In certain embodiments, the library comprises at least 10^2 unique guide sequences. In certain embodiments, the library comprises at least 10^3 unique guide sequences. In certain embodiments, the library comprises at least 10^4 unique guide sequences. In certain embodiments, the library comprises at least 10^5 unique guide sequences. In certain embodiments, the library comprises at least 10^6 unique guide

sequences. In certain embodiments, the library comprises at least 10^7 unique guide sequences. In certain embodiments, the library targets at least 10 different polynucleotides. In certain embodiments, the library targets at least 10^2 different polynucleotides. In certain embodiments, the library targets at least 10^3 different polynucleotides. In certain embodiments, the library targets at least 10^4 different polynucleotides. In certain embodiments, the library targets at least 10^5 different polynucleotides. In certain embodiments, the library targets at least 10^6 different polynucleotides. In certain embodiments, the library targets at least 10^7 different polynucleotides.

[0169] In certain embodiments, the library comprises a collection of guide RNAs having the same sequence and the same modifications in a progressively shifted window that moves across the sequence of the members in the library. In certain embodiments, the windows collectively cover the entire length of the RNA.

[0170] In certain embodiments, the library allows one to conduct high-throughput, multi-target genomic manipulations and analyses. In certain embodiments, only the DNA-targeting segments of the guide RNAs are varied, while the Cas protein-binding segment is the same. In certain embodiments, a first portion of the library comprises guide RNAs possessing a Cas-binding segment that recognizes, binds and directs a particular Cas protein and a second portion of the library comprises a different Cas-binding segment that recognizes, binds and directs a different Cas protein (e.g., a Cas protein from a different species), thereby allowing the library to function with two or more orthogonal Cas proteins. In certain embodiments, induced expression of a first orthogonal Cas protein utilizes the portion of the library which interacts with the first orthogonal Cas protein. In certain embodiments, induced expression of a first and second orthogonal Cas protein utilizes the portions of the library which interact with the first and second orthogonal Cas proteins, respectively. In certain embodiments, induced expression of the first and second orthogonal Cas proteins occur at different times. Accordingly, one can carry out large-scale gene editing or gene regulation by specifically manipulating or modifying multiple targets as specified in the library.

[0171] In certain embodiments, the library is an “arrayed” library, namely a collection of different features or pools of features in an addressable arrangement. For example, features of an array can be selectively cleaved and transferred to a microtiter plate such that each well in the plate contains a known feature or a known pool of features. In some other embodiments, the library is synthesized in a 48- well or in a 96-well microtiter plate format or in a 384-well plate.

[0172] In certain embodiments, synthesis of the guide RNA of this invention may be conducted on a solid support having a surface to which chemical entities may bind. In some embodiments, guide RNAs being synthesized are attached, directly or indirectly, to the same solid support and may form part of an array. An "array" is a collection of separate molecules of known monomeric sequence each arranged on a

solid support in a spatially defined and a physically addressable manner, such that the location of each sequence is known. An "array," or "microarray' used interchangeably herein includes any one-dimensional, two-dimensional or substantially two-dimensional (as well as a three-dimensional) arrangement of addressable regions bearing a particular chemical moiety or moieties (such as ligands, e.g., biopolymers such as polynucleotide or oligonucleotide sequences (nucleic acids), polypeptides (e.g., proteins), carbohydrates, lipids, etc.) associated with that region. An array is "addressable" when it has multiple regions of different moieties (e.g., different polynucleotide sequences) such that a region (i.e., a "feature" of the array) at a particular predetermined location (i.e., an "address") on the array will detect a particular target or class of targets (although a feature may incidentally detect non-targets of that feature). Array features are typically, but need not be, separated by intervening spaces. The number of features that can be contained on an array will largely be determined by the surface area of the substrate, the size of a feature and the spacing between features. Arrays can have densities of up to several hundred thousand or more features per cm^2, such as 2,500 to 200,000 features/cm^2. The features may or may not be covalently bonded to the substrate.

[0173] Suitable solid supports may have a variety of forms and compositions and derive from naturally occurring materials, naturally occurring materials that have been synthetically modified, or synthetic materials. Examples of suitable support materials include, but are not limited to, silicas, silicon and silicon oxides, teflons, glasses, polysaccharides such as agarose (e.g., Sepharose® from Pharmacia) and dextran (e.g., Sephadex® and Sephacyl®, also from Pharmacia), polyacrylamides, polystyrenes, polyvinyl alcohols, copolymers of hydroxyethyl methacrylate and methyl methacrylate, and the like. In some embodiments, the solid support is a plurality of beads.

[0174] The initial monomer of the guide RNAs to be synthesized on the substrate surface can be bound to a linker which in turn is bound to a surface hydrophilic group, e.g., a surface hydroxyl moiety present on a silica substrate. In some embodiments, a universal linker is used. In some other embodiments, the initial monomer is reacted directly with a surface hydroxyl moiety, surface amine or other reactive functional group. Alternatively, guide RNAs can be synthesized first according to the present invention, and attached to a solid substrate post-synthesis by any method known in the art. Thus, the present invention can be used to prepare arrays of guide RNAs wherein the oligonucleotides are either synthesized on the array, or attached to the array substrate post-synthesis. Subsequently, the guide RNAs or a pool or a plurality of pools of guide RNAs can optionally and selectively be cleaved from the array substrate and be used as a library or libraries.

F. crRNA

[0175] The present invention also provides various crRNAs that comprise the chemical modification(s) as described for guide RNAs, as described herein. The crRNAs can function in a multi-

segment guide RNA, such as a dual guide. In certain embodiments, the crRNA functions as a guide RNA without a tracrRNA segment, e.g., in a Cpf1 system. Thus, in certain embodiments, the present invention provides a synthetic crRNA comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, the target polynucleotide comprising a target sequence adjacent to a PAM site, and (ii) a stem sequence; wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10 (optionally between -10 and 6); wherein the guide sequence comprises at least one modification, and the crRNA results in higher specificity for the target polynucleotide or higher gRNA functionality than a corresponding crRNA without the modification. Here, the crRNA results in higher specificity for the target polynucleotide or higher gRNA functionality than a corresponding crRNA without the modification if, when the crRNA is included in a guide RNA or a gRNA:cas protein complex, the guide RNA or gRNA:cas protein has higher specificity for the target polynucleotide or higher gRNA functionality than a corresponding guide RNA or gRNA:cas ptotein complex in which the crRNA lacks the modification.

[0176] The modifications of interest are described elsewhere in this disclosure, including but not limited to at least one modification at nucleotides 4-N to 20-N, or at at least one nucleotide selected from 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, and 16–N of the guide sequence. Various embodiments of the particular modification(s) and target polynucleotides are also described herein.

IV. Cas Proteins

[0177] As mentioned above, a functional CRISPR-Cas system also requires a protein component (*e.g.,* a Cas protein, which may be a Cas nuclease) that provides a desired activity, such as target binding or target nicking/cleaving. In certain embodiments, the desired activity is target binding. In certain embodiments, the desired activity is target nicking or target cleaving. In certain embodiments, the desired activity also includes a function provided by a polypeptide that is covalently fused to a Cas protein, as disclosed herein. In certain embodiments, the desired activity also includes a function provided by a polypeptide that is covalently fused to a nuclease-deficient Cas protein, as disclosed herein. Examples of such a desired activity include a transcription regulation activity (either activation or repression), an epigenetic modification activity, or a target visualization/identification activity, as described below. The Cas protein can be introduced into an *in vitro* or *in vivo* system as a purified or non-purified (i) Cas protein or (ii) mRNA encoded for expression of the Cas protein or (iii) linear or circular DNA encoded for expression of the protein. Any of these 3 methods of providing the Cas protein are well known in the art and are implied interchangeably when mention is made herein of a Cas protein or use of a Cas protein. In certain embodiments, the Cas protein is constitutively expressed from mRNA or DNA. In certain embodiments, the expression of Cas protein from mRNA or DNA is inducible or induced.

[0178] In certain embodiments, the Cas protein is chemically synthesized (see *e.g.*, Creighton, "Proteins: Structures and Molecular Principles," W.H. Freeman & Co., NY, 1983), or produced by recombinant DNA technology as described herein. For additional guidance, skilled artisans may consult Frederick M. Ausubel *et al.*, "Current Protocols in Molecular Biology," John Wiley & Sons, 2003; and Sambrook *et al.*, "Molecular Cloning, A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor, NY, 2001).

[0179] In certain embodiments, the Cas protein is provided in purified or isolated form. In certain embodiments, the Cas protein is provided at about 80%, about 90%, about 95%, or about 99% purity. In certain embodiments, the Cas protein is provided as part of a composition. In certain embodiments, the Cas protein is provided in aqueous compositions suitable for use as, or inclusion in, a composition for an RNA-guided nuclease reaction. Those of skill in the art are well aware of the various substances that can be included in such nuclease reaction compositions.

[0180] In certain embodiments, a Cas protein is provided as a recombinant polypeptide. In certain examples, the recombinant polypeptide is prepared as a fusion protein. For example, in certain embodiments, a nucleic acid encoding the Cas protein is linked to another nucleic acid encoding a fusion partner, *e.g.*, glutathione-S-transferase (GST), 6x-His epitope tag, or M13 Gene 3 protein. Suitable host cells can be used to expresses the fusion protein. In certain embodiments, the fusion protein is isolated by methods known in the art. In certain embodiments, the fusion protein can be further treated, *e.g.*, by enzymatic digestion, to remove the fusion partner and obtain the Cas protein. Alternatively, Cas protein:gRNA complexes can be made with recombinant technology using a host cell system or an *in vitro* translation-transcription system known in the art. Details of such systems and technology can be found in *e.g.*, WO2014144761 WO2014144592, WO2013176772, US20140273226, and US20140273233, the contents of which are incorporated herein by reference in their entireties.

Wild type Cas proteins

[0181] In certain embodiments, a Cas protein comprises a protein derived from a CRISPR-Cas type I, type II, or type III system, which has an RNA-guided polynucleotide binding and/or nuclease activity. *Non*-limiting examples of suitable Cas proteins include Cas3, Cas4, Cas5, Cas5e (or CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9, Cas10, Cas10d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse*1 (or C*asA), Cse2 (or CasB), Cse3 (or CasE), Cse4 (or CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, *Cmr*1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csz1, Csx15, Csf1, Csf2, Csf3, Csf4, and Cu1966. See *e.g.*, WO2014144761 WO2014144592, WO2013176772, US20140273226, and US20140273233, the contents of which are incorporated herein by reference in their entireties.

[0182] In certain embodiments, the Cas protein is derived from a type II CRISPR-Cas system. In certain embodiments, the Cas protein is or is derived from a Cas9 protein. In certain embodiments, the Cas protein is or is derived from a bacterial Cas9 protein, including those identified in WO2014144761. In certain embodiments, the Cas protein is or is derived from a *Streptococcus sp.* or *Staphylococcus sp.* Cas9 protein. In certain embodiments, the Cas protein is or is derived from the *Streptococcus thermophilus* Cas9 protein. In certain embodiments, the Cas protein is or is derived from the *Streptococcus pyogenes* Cas9 protein. In certain embodiments, the Cas protein is or is derived from the *Staphylococcus aureus* Cas9 protein. In certain embodiments, the Cas protein is or is derived from the *Streptococcus thermophilus* Cas9 protein.

[0183] In certain embodiments, the wild type Cas protein is a Cas9 protein. In certain embodiments, the wild type Cas9 protein is the Cas9 protein from *S. pyogenes* (SEQ ID NO: 115). In certain embodiments, the protein or polypeptide can comprise, consist of, or consist essentially of a fragment of SEQ ID NO: 115.

[0184] In general, a Cas protein includes at least one RNA binding domain, which interacts with the guide RNA. In certain embodiments, the Cas protein is modified to increase nucleic acid binding affinity and/or specificity, alter an enzymatic activity, and/or change another property of the protein. For example, nuclease (*i.e.*, DNase, RNase) domains of the Cas protein can be modified, mutated, deleted, or inactivated. Alternatively, the Cas protein can be truncated to remove domains that are not essential for the function of the protein. In certain embodiments, the Cas protein is truncated or modified to optimize the activity of the effector domain. In certain embodiments, the Cas protein includes a nuclear localization sequence (NLS) that effects importation of the NLS-tagged Cas protein into the nucleus of a living cell. In certain embodiments, the Cas protein includes two or more modifications.

Mutant Cas proteins

[0185] In some embodiments, the Cas protein can be a mutant of a wild type Cas protein (such as Cas9) or a fragment thereof. In other embodiments, the Cas protein can be derived from a mutant Cas protein. For example, the amino acid sequence of the Cas9 protein can be modified to alter one or more properties (*e.g.*, nuclease activity, binding affinity, stability to proteases, etc.) of the protein. Alternatively, domains of the Cas9 protein not involved in RNA-guided cleavage can be eliminated from the protein such that the modified Cas9 protein is smaller than the wild type Cas9 protein. For example, reducing the size of the Cas9 coding sequence can allow it to fit within a transfection vector that otherwise cannot accommodate the wild-type sequence, such as the AAV vector among others. In some embodiments, the present system utilizes the Cas9 protein from *S. pyogenes,* either as encoded in bacteria or codon-optimized for expression in eukaryotic cells. Shown below is the amino acid sequence of wild

type *S. pyogenes* Cas9 protein sequence (SEQ ID NO: 115, available at www.uniprot.org/uniprot/Q99ZW2).

MDKKYSIGL**D**IGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAE
ATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFG
NIVDEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSD
VDKLFIQLVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGN
LIALSLGLTPNFKSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAI
LLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYA
GYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELH
AILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSRFAWMTRKSEETITPWNFEE
VVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFL
SGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVEDRFNASLGTYHDLLKI
IKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTGWG
RLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQKAQVSGQGDSL
HEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVI**E**MARENQTTQKGQKNSRER
MKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINRLSDYDVD**H**
IVPQSFLKDDSIDNKVLTRSDK**N**RGKSDNVPSEEVVKKMKNYWRQLLNAKLITQRKFDNL
TKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKS
KLVSDFRKDFQFYKVREINNYH**H**AH**D**AYLNAVVGTALIKKYPKLESEFVYGDYKVYDVRK
MIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDF
ATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVA
YSVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPK
YSLFELENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVE
QHKHYLDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGA
PAAFKYFDTTIDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGD

[0186] A Cas9 protein generally has at least two nuclease (*e.g.,* DNase) domains. For example, a Cas9 protein can have a RuvC-like nuclease domain and an HNH-like nuclease domain. The RuvC and HNH domains work together to cut both strands in a target site to make a double-stranded break in the target polynucleotide. (Jinek et al., *Science 337*: 816–21). In certain embodiments, a mutant Cas9 protein is modified to contain only one functional nuclease domain (either a RuvC-like or an HNH-like nuclease domain). For example, in certain embodiments, the mutant Cas9 protein is modified such that one of the nuclease domains is deleted or mutated such that it is no longer functional (*i.e.*, the nuclease activity is absent). In some embodiments where one of the nuclease domains is inactive, the mutant is able to introduce a nick into a double-stranded polynucleotide (such protein is termed a "nickase") but not able to cleave the double-stranded polynucleotide. For example, an aspartate to alanine (D10A) conversion in a RuvC-like domain converts the Cas9-derived protein into a nickase. Likewise, a histidine to alanine (H840A) conversion in a HNH domain converts the Cas9-derived protein into a nickase. Likewise, an arsparagine to alanine (N863A) conversion in a HNH domain converts the Cas9-derived protein into a nickase.

[0187] In certain embodiments, both the RuvC-like nuclease domain and the HNH-like nuclease domain are modified or eliminated such that the mutant Cas9 protein is unable to nick or cleave the target polynucleotide. In certain embodiments, all nuclease domains of the Cas9-derived protein are modified or eliminated such that the Cas9-derived protein lacks all nuclease activity. In certain embodiments, a Cas9 protein that lacks some or all nuclease activity relative to a wild-type counterpart, nevertheless, maintains target recognition activity to a greater or lesser extent.

[0188] In any of the above-described embodiments, any or all of the nuclease domains can be inactivated by one or more deletion mutations, insertion mutations, and/or substitution mutations using well-known methods, such as site-directed mutagenesis, PCR-mediated mutagenesis, and total gene synthesis, as well as other methods known in the art.

[0189] In certain embodiments, the "Cas mutant" or "Cas variant" is at least 50% (*e.g.*, any number between 50% and 100%, inclusive, *e.g.*, 50%, 60%, 70%, 80%, 90%, 95%, 98%, and 99%) identical to SEQ ID NO: 115. In certain embodiments, the "Cas mutant" or "Cas variant" binds to an RNA molecule (*e.g.*, a sgRNA). In certain embodiments, the "Cas mutant" or "Cas variant" is targeted to a specific polynucleotide sequence via the RNA molecule.

Fusion Proteins

[0190] In certain embodiments, the Cas protein is fused to another protein or polypeptide heterologous to the Cas protein to create a fusion protein. In certain embodiments, the heterologous sequence includes one or more effector domains, such as a cleavage domain, a transcriptional activation domain, a transcriptional repressor domain, or an epigenetic modification domain. Additional examples of the effector domain include a nuclear localization signal, cell-penetrating or translocation domain, or a marker domain. In certain embodiments, the effector domain is located at the N-terminal, the C-terminal, or in an internal location of the fusion protein. In certain embodiments, the Cas protein of the fusion protein is or is derived from a Cas9 protein. In certain embodiments, the Cas protein of the fusion protein is or is derived from a modified or mutated Cas protein in which all the nuclease domains have been inactivated or deleted. In certain embodiments, the Cas protein of the fusion protein is or is derived from a modified or mutated Cas protein that lacks nuclease activity. In certain embodiments, the RuvC and/or HNH domains of the Cas protein are modified or mutated such that they no longer possess nuclease activity.

Cleavage Domains

[0191] In certain embodiments, the effector domain of the fusion protein is a cleavage domain. As used herein, a "cleavage domain" refers to a domain that cleaves DNA. The cleavage domain can be obtained from any endonuclease or exonuclease. Non-limiting examples of endonucleases from which a

cleavage domain can be derived include restriction endonucleases and homing endonucleases. See, for example, New England Biolabs Catalog or Belfort et al. (1997) *Nucleic Acids Res. 25*, 3379–88. Additional enzymes that cleave DNA are known (*e.g.*, 51 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease). See also Linn et al. (eds.) "Nucleases," Cold Spring Harbor Laboratory Press, 1993. One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains.

[0192] In certain embodiments, the cleavage domain can be derived from a type II-S endonuclease. Type II-S endonucleases cleave DNA specifically at sites that are typically several base pairs away from the DNA recognition site of the endonuclease and, as such, have separable recognition and cleavage domains. These enzymes generally are monomers that transiently associate to form dimers to cleave each strand of DNA at staggered locations. Non-limiting examples of suitable type II-S endonucleases include BfiI, BpmI, BsaI, BsgI, BsmBI, BsmI, BspMI, FokI, MboII, and SapI. In certain embodiments, the cleavage domain of the fusion protein is a FokI cleavage domain or a fragment or derivative thereof. See Miller et al. (2007) *Nat. Biotechnol. 25*, 778–85; Szczpek et al. (2007) *Nat. Biotechnol. 25*, 786–93; Doyon et al. (2011) *Nat. Methods*, *8*, 74–81.

Transcriptional Activation Domains

[0193] In certain embodiments, the effector domain of the fusion protein is a transcriptional activation domain. In general, a transcriptional activation domain interacts with transcriptional control elements and/or transcriptional regulatory proteins (*i.e.*, transcription factors, RNA polymerases, etc.) to increase and/or activate transcription of a gene. In certain embodiments, the transcriptional activation domain is a herpes simplex virus VP16 activation domain, VP64 (which is a tetrameric derivative of VP16), a NFκB p65 activation domain, p53 activation domains 1 and 2, a CREB (cAMP response element binding protein) activation domain, an E2A activation domain, or an NFAT (nuclear factor of activated T-cells) activation domain. In certain embodiments, the transcriptional activation domain is Gal4, Gcn4, MLL, Rtg3, Gln3, Oaf1, Pip2, Pdr1, Pdr3, Pho4, or Leu3. The transcriptional activation domain may be wild type, or it may be a modified or truncated version of the original transcriptional activation domain.

Transcriptional Repressor Domains

[0194] In certain embodiments, the effector domain of the fusion protein is a transcriptional repressor domain. In general, a transcriptional repressor domain interacts with transcriptional control elements and/or transcriptional regulatory proteins (*i.e.*, transcription factors, RNA polymerases, etc.) to decrease and/or prohibit transcription of a gene. In certain embodiments, the transcriptional repressor domains is inducible cAMP early repressor (ICER) domains, Kruppel-associated box A (KRAB-A)

repressor domains, YY1 glycine rich repressor domains, Sp1-like repressors, E(spI) repressors, IκB repressor, or MeCP2.

Epigenetic Modification Domains

[0195] In certain embodiments, the effector domain of the fusion protein is an epigenetic modification domain. In general, epigenetic modification domains alter gene expression by modifying the histone structure and/or chromosomal structure. In certain embodiments, the epigenetic modification domains is a histone acetyltransferase domain, a histone deacetylase domain, a histone methyltransferase domain, a histone demethylase domain, a DNA methyltransferase domain, or a DNA demethylase domain.

Additional Domains

[0196] In certain embodiments, the fusion protein further comprises at least one additional domain. Non-limiting examples of suitable additional domains include nuclear localization signals (NLSs), cell-penetrating or translocation domains, and marker domains. An NLS generally comprises a stretch of basic amino acids. See, *e.g.*, Lange et al. (2007) *J. Biol. Chem. 282*, 5101–5. For example, in certain embodiments, the NLS is a monopartite sequence, such as PKKKRKV (SEQ ID NO: 116) or PKKKRRV (SEQ ID NO: 117). In certain embodiments, the NLS is a bipartite sequence. In certain embodiments, the NLS is KRPAATKKAGQAKKKK (SEQ ID NO: 118).

[0197] In certain embodiments, the fusion protein comprises at least one cell-penetrating domain. In certain embodiments, the cell-penetrating domain is a cell-penetrating peptide sequence derived from the HIV-1 TAT protein. As an example, the TAT cell-penetrating sequence can be GRKKRRQRRRPPQPKKKRKV (SEQ ID NO: 119). In certain embodiments, the cell-penetrating domain is TLM (PLSSIFSRIGDPPKKKRKV; SEQ ID NO: 120), a cell-penetrating peptide sequence derived from the human hepatitis B virus. In certain embodiments, the cell-penetrating domain is MPG (GALFLGWLGAAGSTMGAPKKKRKV; SEQ ID NO: 121 or GALFLGFLGAAGSTMGAWSQPKKKRKV; SEQ ID NO: 122). In certain embodiments, the cell-penetrating domain is Pep-1 (KETWWETWWTEWSQPKKKRKV; SEQ ID NO: 123), VP22, a cell penetrating peptide from Herpes simplex virus, or a polyarginine peptide sequence.

[0198] In certain embodiments, the fusion protein comprises at least one marker domain. Non-limiting examples of marker domains include fluorescent proteins, purification tags, and epitope tags. In certain embodiments, the marker domain is a fluorescent protein. Non limiting examples of suitable fluorescent proteins include green fluorescent proteins (*e.g.*, GFP, GFP-2, tagGFP, turboGFP, EGFP, Emerald, Azami Green, Monomeric Azami Green, CopGFP, AceGFP, ZsGreen1), yellow fluorescent proteins (*e.g.* YFP, EYFP, Citrine, Venus, YPet, PhiYFP, ZsYellow1,), blue fluorescent proteins (*e.g.* EBFP, EBFP2, Azurite, mKalama1, GFPuv, Sapphire, T-sapphire,), cyan fluorescent proteins (*e.g.* ECFP,

Cerulean, CyPet, AmCyan1, Midoriishi-Cyan), red fluorescent proteins (mKate, mKate2, mPlum, DsRed monomer, mCherry, mRFP1, DsRed-Express, DsRed2, DsRed-Monomer, HcRed-Tandem, HcRed1, AsRed2, eqFP611, mRasberry, mStrawberry, Jred), orange fluorescent proteins (mOrange, mKO, Kusabira-Orange, Monomeric Kusabira-Orange, mTangerine, tdTomato) and any other suitable fluorescent protein. In certain embodiments, the marker domain is a purification tag and/or an epitope tag. Exemplary tags include, but are not limited to, glutathione-S-transferase (GST), chitin binding protein (CBP), maltose binding protein, thioredoxin (TRX), poly(NANP), tandem affinity purification (TAP) tag, myc, AcV5, AU1, AU5, E, ECS, E2, FLAG, HA, nus, Softag 1, Softag 3, Strep, SBP, Glu-Glu, HSV, KT3, S, S1, T7, V5, VSV-G, 6xHis, biotin carboxyl carrier protein (BCCP), and calmodulin.

V. Uses and Methods

[0199] In one aspect, the present invention provides a method for cleaving a target polynucleotide with a Cas protein. The method comprises contacting the target polynucleotide with (i) a guide RNA or a set of guide RNA molecules described herein, and (ii) a Cas protein. In certain embodiments, the method results in a double-strand break in the target polynucleotide. In certain embodiments, the Cas protein is a Cas protein having a single-strand nicking activity. In certain embodiments, the method results in a single-strand break in the target polynucleotide. In certain embodiments, a complex comprising a guide RNA and Cas protein having a single-strand nicking activity is used for sequence-targeted single-stranded DNA cleavage, *i.e.*, nicking.

[0200] In one aspect, the present invention provides a method for cleaving two or more target polynucleotides with a Cas protein. The method comprises contacting the target polynucleotides with (i) a set of guide RNA molecules described herein, and (ii) a Cas protein. In certain embodiments, the method results in double-strand breaks in the target polynucleotides. In certain embodiments, the Cas protein is a Cas protein having a single-strand nicking activity. In certain embodiments, the method results in single-strand breaks in the target polynucleotides. In certain embodiments, a complex comprising a guide RNA and Cas protein having a single-strand nicking activity is used for sequence-targeted single-stranded DNA cleavage, *i.e.*, nicking.

[0201] In one aspect, the present invention provides a method for binding a target polynucleotide with a Cas protein. The method comprises contacting the target polynucleotide with (i) a guide RNA or a set of guide RNA molecules described herein and (ii) a Cas protein, to result in binding of the target polynucleotide with the Cas protein. In certain embodiments, the Cas protein is a Cas variant. In certain embodiments, the Cas variant lacks some or all nuclease activity relative to a counterpart wild-type Cas protein.

[0202] In one aspect, the present invention provides a method for binding two or more target polynucleotides with a Cas protein. The method comprises contacting the target polynucleotides with (i) a set of RNA molecules described herein and (ii) a Cas protein, to result in binding of the target polynucleotides with the Cas protein. In certain embodiments, the Cas protein is a Cas variant. In certain embodiments, the Cas variant lacks some or all nuclease activity relative to a counterpart wild-type Cas protein.

[0203] In one aspect, the present invention provides a method for targeting a Cas protein to a target polynucleotide. The method comprises contacting the Cas protein with a guide RNA or a set of guide RNA molecules described herein. In certain embodiments, the method results in formation of a guide RNA:Cas protein complex. In certain embodiments, the Cas protein is a wild type Cas9 protein. In certain embodiments, the Cas protein is a mutant or variant of a Cas9 protein. In certain embodiments, the Cas protein is a Cas protein having a single-strand nicking activity. In certain embodiments, the Cas protein is a Cas protein lacking nuclease activity (e.g., a nuclease-deficient mutant of Cas protein). In certain embodiments, the Cas protein is part of a fusion protein (e.g., a fusion protein comprising (i) the Cas protein and (ii) a heterologous polypeptide).

[0204] In one aspect, the present invention provides a method for targeting a Cas protein to two or more target polynucleotides. The method comprises contacting the Cas protein with a set of guide RNA molecules described herein. In certain embodiments, the method results in formation of a guide RNA:Cas protein complex. In certain embodiments, the Cas protein is a wild type Cas9 protein. In certain embodiments, the Cas protein is a mutant or variant of a Cas9 protein. In certain embodiments, the Cas protein is a Cas protein having a single-strand nicking activity. In certain embodiments, the Cas protein is a Cas protein lacking nuclease activity (e.g., a nuclease-deficient mutant of Cas protein). In certain embodiments, the Cas protein is part of a fusion protein (e.g., a fusion protein comprising (i) the Cas protein or and (ii) a heterologous polypeptide).

[0205] In one aspect, the present invention provides a method of selecting a synthetic guide RNA. The method involves "walking" an MP modification across the guide sequence portion of a gRNA to identify which position or positions in the guide sequence enhance specificity due to the location of the MP modification. The magnitude of the specificity enhancement may be assessed for each position tested with the on-target versus off-target cleavage ratio, the cleavage percentage at the target site and the cleavage percentage at one or more off-target sites, and/or the specificity score, thus determining which modified position or positions alters the specificity of the gRNA and to what extent. The incremental walking of a single MP across the guide sequence may also identify positions for potential synergistic improvements in specificity resulting from one or more combinations of chemical modifications among the positions tested.

[0206] In an embodiment, the method comprises providing at least a first synthetic guide RNA and a second synthetic guide RNA, both comprising the same sequences of (a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and (b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence, wherein the first synthetic guide RNA comprises an MP modification at a first position of the guide sequence, and the second synthetic guide RNA comprises an MP modification at a second position of the guide sequence; forming a first gRNA:Cas protein complex comprising a Cas protein and the first synthetic guide RNA, contacting the target polynucleotide with the first gRNA:Cas protein complex, and cleaving, nicking or binding the target polynucleotide; forming a second gRNA:Cas protein complex comprising a Cas protein and the second synthetic guide RNA, contacting the target polynucleotide with the second gRNA:Cas protein complex, and cleaving, nicking or binding the target polynucleotide; determining the specificity of the first gRNA:Cas protein complex and the second gRNA:Cas protein complex in the cleaving, nicking or binding of the target polynucleotide; identifying which of the guide RNAs has greater specificity for the target polynucleotide. In certain embodiments, the first and second gRNA:Cas protein complexes are tested together in a competitive assay, such as by labeling of the first and second gRNAs with different fluorophores. In certain embodiments, the first and second gRNA:Cas protein complexes are tested individually in equivalent or split samples assayed in parallel or sequentially.

[0207] In one aspect, the present invention provides a method for analyzing the specificity of a CRISPR function performed by using a guide RNA, such as a guide RNA comprising a specificity-enhancing modification. The method comprises identifying a target sequence and one or more off-target sequences in a sample, wherein the target sequence is comprised in a target polynucleotide and the off-target sequences are comprised in one or more off-target polynucleotides, performing a CRISPR function using a guide RNA, capturing the target and off-target polynucleotides by using a library of oligonucleotide baits that are designed to hybridize to the target and off-target polynucleotides, isolating and analyzing the captured polynucleotides to assess whether the target and off-target sequences have been changed due to the CRISPR function. The relative extent of changes between the target and non-target sequences is indicative of the specificity of the CRISPR function mediated by the guide RNA. In some embodiments, the captured polynucleotides are analyzed by sequencing.

[0208] The polynucleotide baits include baits designed to hybridize to the target polynucleotide and baits designed to hybridize to the off-target polynucleotides. It is contemplated that the baits may hybridize to the target (or off-target) sequences directly, or they may hybridize to sequences near the target (or non-target) sequences, such as sequences within 2000, 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, or 20 base pairs of the target (or off-target) sequences, at

either the 5' or 3' of the target (or off-target) sequences, or both. Some baits may hybridize partially to the target (or off-target) sequences and partially outside of the target (or off-target) sequences. In some embodiments, the library of baits comprises both baits hybridizing to the target (or off-target) sequences and baits hybridizing near the target (or off-target) sequences. In some embodiments, the library only comprises baits hybridizing near the target (or off-target) sequences, but not baits hybridizing to the target (or off-target) sequences, or vice versa. In some embodiments, the baits hybridize to a region of about 1000 bp centered around the target sequence, as well as regions of about 1000 bp centered around each off-target sequences. In some embodiments, the baits are tiled.

[0209] In certain embodiments, the specificity-enhancing modification comprises 2′-O-methyl-3'-phosphonoacetate (MP), 2′-O-methyl-3'-thiophosphonoacetate (MSP), 2′-deoxy-3'-phosphonoacetate (DP), 2′-deoxy-3'-thiophosphonoacetate (DSP), or a combination thereof. In certain embodiments, the chemical modification comprises a 2′ modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification. In certain embodiments, the 2′ modification is selected from 2′-F and 2′-O-(2-methoxyethyl). In certain embodiments, the first and second synthetic guide RNAs comprise a specificity-enhancing modification at different nucleotide positions in the guide sequence portions. In certain embodiments, the specificity is determined based on ON target cleavage activity, OFF target cleavage activity, ON:OFF ratio, specificity score, or a combination thereof. In certain embodiments, the method comprises providing a first through twentieth synthetic guide RNA comprising a specificity-enhancing modification at different nucleotide positions in the guide sequence portions, forming a gRNA:Cas protein complex using each of the synthetic guide RNAs, contacting the target polynucleotide with the gRNA:Cas protein complex, cleaving, nicking or binding the target polynucleotide and measuring the specificity of each synthetic guide RNA, and identifying one or more modified positions that provide the greatest specificity enhancement. In certain embodiments, the gRNA further comprises stability-enhancing end modifications. In certain embodiments, the stability enhancing end modifications comprise 2′-O-methyl-3'-phosphonoacetate (MP), 2′-O-methyl-3'-thiophosphonoacetate (MSP), 2'-O-methyl-3'-phosphorothioate (MS), 2′-deoxy-3'-phosphonoacetate (DP), 2′-deoxy-3'-thiophosphonoacetate (DSP), 2′-fluoro-3'-phosphonoacetate (FP), 2′-fluoro-3'-thiophosphonoacetate (FSP), 2'-fluoro-3'-phosphorothioate (FS), or a combination thereof at the 5′ end and/or the 3′ end of the gRNA.

[0210] In certain embodiments, the guide RNA is introduced into a cell by transfection. Techniques for RNA transfection are known in the art and include electroporation and lipofection. Effective techniques for RNA transfection depend mostly on cell type. See, e.g., Lujambio et al. (Spanish National Cancer Centre) *Cancer Res.* Feb. 2007, which describes transfection of HTC-116 colon cancer cells and uses Oligofectamine (Invitrogen) for transfection of commercially obtained, modified miRNA or

precursor miRNA. See also, Cho et al. (Seoul National Univ.) *Nat. Biotechnol.* Mar. 2013, which describes transfection of K562 cells and uses 4D Nucleofection™ (Lonza) electroporation for transfection of transcribed sgRNAs (about 60 nts long). Techniques for transfection of RNA are also known in the art. For example, therapeutic RNA has been delivered in non-pathogenic *E. coli* coated with Invasin protein (to facilitate uptake into cells expressing β-1 integrin protein) and with the *E. coli* encoded to express lysteriolysin O pore-forming protein to permit the shRNA to pass from the *E. coli* into the cytoplasm. See also Cho *et al.* (Seoul National Univ.) *Nat. Biotechnol.* Mar. 2013.

[0211] In certain embodiments, the guide RNA is introduced or delivered into cells. Technologies that can be used for delivery of guide RNA include those that utilize encapsulation by biodegradable polymers, liposomes, or nanoparticles. Such polymers, liposomes, and nanoparticles can be delivered intravenously. In certain embodiments, for *in vivo* delivery, guide RNA can be injected into a tissue site or administered systemically. *In vivo* delivery can also be effected by a beta-glucan delivery system, such as those described in U.S. Pat. Nos. 5,032,401 and 5,607,677, and U.S. Publication No. 2005/0281781, which are hereby incorporated by reference in their entirety. In certain embodiments, guide RNA or a delivery vehicle containing guide RNA is targeted to a particular tissue or body compartment. For example, in certain embodiments, to target exogenous RNA to other tissues, synthetic carriers are decorated with cell-specific ligands or aptamers for receptor uptake, *e.g.*, RNA encased in cyclodextrin nanoparticles coated with PEG and functionalized with human transferrin protein for uptake via the transferrin receptor which is highly expressed in tumor cells. Further approaches are described herein below or known in the art.

[0212] The present invention has been tested in human cells as described in Hendel et al. (2015) *Nat. Biotechnol. 33*:9, 985–9 (which is incorporated in this application in its entirety). In the cited work, modified guide RNA was introduced into K562 cells, human primary T cells, and CD34+ hematopoietic stem and progenitor cells (HSPCs). The modified guide RNA significantly enhanced genome editing efficiencies in human cells, including human primary T cells and CD34+ HSPCs as compared to unmodified guide RNA.

[0213] Examples of other uses include genomic editing and gene expression regulation as described below.

Genomic Editing

[0214] In one aspect, the present invention provides a method for genomic editing to modify a DNA sequence *in vivo* or *in vitro* (*"in vitro"* includes, without being limited to, a cell-free system, a cell lysate, an isolated component of a cell, and a cell outside of a living organism). The DNA sequence may comprise a chromosomal sequence, an episomal sequence, a plasmid, a mitochondrial DNA sequence, or

a functional intergenic sequence, such as an enhancer sequence or a DNA sequence for a non-coding RNA. The method comprises contacting the DNA sequence with (i) a guide RNA or a set of guide RNA molecules described herein, and (ii) a Cas protein. In certain embodiments, the DNA sequence is contacted outside of a cell. In certain embodiments, the DNA sequence is located in the genome within a cell and is contacted *in vitro* or *in vivo*. In certain embodiments, the cell is within an organism or tissue. In certain embodiments, the cell is a human cell, a non-human mammalian cell, a stem cell, a non-mammalian vertebrate cell, an invertebrate cell, a plant cell, a single cell organism, or an embryo. In certain embodiments, the guide RNA aids in targeting the Cas protein to a targeted site in the DNA sequence. In certain embodiments, the Cas protein cleaves at least one strand of the DNA sequence at the targeted site. In certain embodiments, the Cas protein cleaves both strands of the DNA sequence at the targeted site.

[0215] In certain embodiments, the method further comprises introducing the Cas protein into a cell or another system. In certain embodiments, the Cas protein is introduced as a purified or non-purified protein. In certain embodiments, the Cas protein is introduced via an mRNA encoding the Cas protein. In certain embodiments, the Cas protein is introduced via a linear or circular DNA encoding the Cas protein. In certain embodiments, the cell or system comprises a Cas protein or a nucleic acid encoding a Cas protein.

[0216] In certain embodiments, a double-stranded break can be repaired via an error-prone, non-homologous end-joining ("NHEJ") repair process. In certain embodiments, a double-stranded break can be repaired by a homology-directed repair (HDR) process such that a donor sequence in a donor polynucleotide can be integrated into or exchanged with the targeted DNA sequence.

[0217] In certain embodiments, the method further comprises introducing at least one donor polynucleotide into the cell or system. In certain embodiments, the donor polynucleotide comprises at least one homologous sequence having substantial sequence identity with a sequence on either side of the targeted site in the DNA sequence. In certain embodiments, the donor polynucleotide comprises a donor sequence that can be integrated into or exchanged with the DNA sequence via homology-directed repair, such as homologous recombination.

[0218] In certain embodiments, the donor polynucleotide includes an upstream homologous sequence and a downstream homologous sequence, each of which have substantial sequence identity to sequences located upstream and downstream, respectively, of the targeted site in the DNA sequence. These sequence similarities permit, for example, homologous recombination between the donor polynucleotide and the targeted DNA sequence such that the donor sequence can be integrated into (or exchanged with) the DNA sequence targeted.

[0219] In certain embodiments, the target site(s) in the DNA sequence spans or is adjacent to a mutation, *e.g.*, point mutation, a translocation or an inversion which may cause or be associated with a disorder. In certain embodiments, the method comprises correcting the mutation by introducing into the cell or system at least one donor polynucleotide comprising (i) a wild-type counterpart of the mutation and (ii) at least one homologous sequence having substantial sequence identity with a sequence on one side of the targeted site in the DNA sequence. In certain embodiments, the donor polynucleotide comprises a homologous sequence having substantial sequence identity with a sequence on both sides of the targeted site in the DNA sequence.

[0220] In certain embodiments, the donor polynucleotide comprises an exogenous sequence that can be integrated into or exchanged with the targeted DNA sequence via a homology-directed repair process, such as homologous recombination. In certain embodiments, the exogenous sequence comprises a protein coding gene, which, optionally, is operably linked to an exogenous promoter control sequence. Thus, in certain embodiments, upon integration of the exogenous sequence, a cell can express a protein encoded by the integrated gene. In certain embodiments, the exogenous sequence is integrated into the targeted DNA sequence such that its expression in the recipient cell or system is regulated by the exogenous promoter control sequence. Integration of an exogenous gene into the targeted DNA sequence is termed a "knock in." In other embodiments, the exogenous sequence can be a transcriptional control sequence, another expression control sequence, an RNA coding sequence, and the like.

[0221] In certain embodiments, the donor polynucleotide comprises a sequence that is essentially identical to a portion of the DNA sequence at or near the targeted site, but comprises at least one nucleotide change. For example, in certain embodiments, the donor sequence comprises a modified or mutated version of the DNA sequence at or near the targeted site such that, upon integration or exchange with the targeted site, the resulting sequence at the targeted site comprises at least one nucleotide change. In certain embodiments, the at least one nucleotide change is an insertion of one or more nucleotides, a deletion of one or more nucleotides, a substitution of one or more nucleotides, or combinations thereof. As a consequence of the integration of the modified sequence, the cell may produce a modified gene product from the targeted DNA sequence.

[0222] In certain embodiments, the methods are for multiplex applications. In certain embodiments, the methods comprise introducing a library of guide RNAs into the cell or system. In certain embodiments, the library comprises at least 10 unique guide sequences. In certain embodiments, the library comprises at least 100 unique guide sequences. In certain embodiments, the library comprises at least 1,000 unique guide sequences. In certain embodiments, the library comprises at least 10,000 unique guide sequences. In certain embodiments, the library comprises at least 100,000 unique guide sequences. In certain embodiments, the library comprises at least 1,000,000 unique guide sequences. In certain

embodiments, the library targets at least 10 different polynucleotides or at least 10 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 100 different polynucleotides or at least 100 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 1,000 different polynucleotides or at least 1,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 10,000 different polynucleotides or at least 10,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 100,000 different polynucleotides or at least 100,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 1,000,000 different polynucleotides or at least 1,000,000 different sequences within one or more polynucleotides.

Genomic Editing in Human and Mammalian Cells

[0223] Embodiments of the present invention are useful in methods for genomic editing to modify a target polynucleotide, for example a DNA sequence, in a mammalian cell.

[0224] In certain embodiments, the DNA sequence is a chromosomal sequence. In certain embodiments, the DNA sequence is a protein-coding sequence. In certain embodiments, the DNA sequence is a functional intergenic sequence, such as an enhancer sequence or a non-coding sequence. In certain embodiments, the DNA is part of a human gene. In some such embodiments, the human gene is the clathrin light chain (*CLTA1*) gene, the human interleukin 2 receptor gamma (*IL2RG*) gene, the human cytotoxic T-lymphocyte–associated protein 4 (*CLTA4*) gene, the human Vascular Endothelial Growth Factor A gene (*VEGFA*), or the human hemoglobin beta (*HBB*) gene which can harbor mutations responsible for sickle cell anemia and thalassemias. Accordingly, in certain embodiments, the target polynucleotide is a *HBB* polynucleotide, a *VEGFA* polynucleotide, an *IL2RG* polynucleotide, a *CLTA1* polynucleotide, or a *CLTA4* polynucleotide.

[0225] In certain embodiments, a synthetic guide RNA comprises a guide sequence capable of hybridizing to an HBB, IL2RG, CLTA1, VEGFA, or CLTA4 polynucleotide. In certain embodiments, the guide sequence consists of nucleotides 1 through 20-N, counted from the 5' end of the guide sequence, N being an interger between -10 and 0, and the guide sequence comprises at least one specificity-enhancing modification at nucleotide 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, or 16–N. In certain embodiments, the guide sequence capable of hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 11-N. In certain embodiments, the guide sequence capable of hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 5-N. In certain embodiments, the guide sequence capable of hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 7-N. In certain embodiments, the guide sequence capable of

hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 10-N. In certain embodiments, the guide sequence capable of hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 9-N. In certain embodiments, the guide sequence capable of hybridizing to one of the above target polynucleotides has a chemical modification at nucleotide 4-N. In certain embodiments, N equals zero.

[0226] In certain embodiments, the guide sequence consists of nucleotides 1 through 19, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 3, 4, 6, 8, 9, or 10. In certain embodiments, the guide sequence consists of nucleotides 1 through 18, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 2, 3, 5, 7, 8, or 9. In certain embodiments, the guide sequence consists of nucleotides 1 through 17, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 1, 2, 4, 6, 7, or 8. In certain embodiments, the guide sequence consists of nucleotides 1 through 16, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 1, 3, 5, 6, or 7. In certain embodiments, the guide sequence consists of nucleotides 1 through 15, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 2, 4, 5, or 6. In certain embodiments, the guide sequence consists of nucleotides 1 through 14, counted from the 5' end of the guide sequence, and at least one chemical modification at one of nucleotides 1, 3, 4, or 5. In certain embodiments, the chemical modification comprises 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof. In certain embodiments, the chemical modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification. In certain embodiments, the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

[0227] In certain embodiments, the mammalian cell is a human cell. In some such embodiments, the human cell is a primary human cell. In further embodiments, the primary human cell is a human primary T cell. The human primary T cell may be stimulated or unstimulated. In certain embodiments, the human cell is a stem/progenitor cell, such as a CD34+ hematopoietic stem and progenitor cell (HSPC). In certain embodiments, the human cell is from a cultured cell line, for example such as can be obtained commercially. Exemplary cell lines include K562 cells, a human myelogenous leukemia line.

[0228] In certain embodiments, the cell is within a living organism. In certain other embodiments, the cell is outside of a living organism.

[0229] The method comprises contacting the DNA sequence with (i) a guide RNA or a set of guide RNA molecules described herein, and (ii) a Cas protein.

[0230] In certain embodiments, the method further comprises introducing or delivering the guide RNA into the cell. In some such embodiments, the guide RNA is introduced into a cell by transfection. Techniques for RNA transfection are known in the art and include electroporation and lipofection. In other embodiments, the guide RNA is introduced into a cell (and, more particularly, a cell nucleus) by nucleofection. Techniques for nucleofection are known in the art and may utilize nucleofection devices such as the Lonza Nucleofector 2b or the Lonza 4D-Nucleofector and associated reagents.

[0231] In certain embodiments, the method further comprises introducing or delivering the Cas protein into the cell. In some such embodiments, the Cas protein is introduced as a purified or non-purified protein. In other embodiments, the Cas protein is introduced via an mRNA encoding the Cas protein. In some such embodiments, the mRNA encoding the Cas protein is introduced into the cell by transfection. In other embodiments, the mRNA encoding the Cas protein is introduced into a cell (and, more particularly, a cell nucleus) by nucleofection.

[0232] In certain embodiments, the method employs ribonucleoprotein (RNP)-based delivery such that the Cas protein is introduced into the cell in a complex with the guide RNA. For example, a Cas9 protein may be complexed with a guide RNA in a Cas9:gRNA complex, which allows for co-delivery of the gRNA and Cas protein. For example, the Cas:gRNA complex may be nucleofected into cells.

[0233] In certain embodiments, the method employs an all-RNA delivery platform. For example, in some such embodiments, the guide RNA and the mRNA encoding the Cas protein are introduced into the cell simultaneously or substantially simultaneously (e.g., by co-transfection or co-nucleofection). In certain embodiments, co-delivery of Cas mRNA and modified gRNA results in higher editing frequencies as compared to co-delivery of Cas mRNA and unmodified gRNA. In particular, gRNA having 2′-O-methyl-3’-phosphorothioate (“MS”), 2′-O-methyl-3’-PACE (“MP”), or 2′-O-methyl-3’-thioPACE (“MSP”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends, provide higher editing frequencies as compared to unmodified gRNA.

[0234] In certain embodiments, the guide RNA and the mRNA encoding the Cas protein are introduced into the cell sequentially; that is, the guide RNA and the mRNA encoding the Cas protein are introduced into the cell at different times. The time period between the introduction of each agent may range from a few minutes (or less) to several hours or days. For example, in some such embodiments, gRNA is delivered first, followed by delivery of Cas mRNA 4, 8, 12 or 24 hours later. In other such embodiments, Cas mRNA is delivered first, followed by delivery of gRNA 4, 8, 12 or 24 hours later. In some particular embodiments, delivery of modified gRNA first, followed by delivery of Cas mRNA results in higher editing frequencies as compared to delivery of unmodified gRNA followed by delivery of Cas mRNA.

[0235] In certain embodiments, the gRNA is introduced into the cell together with a DNA plasmid encoding the Cas protein. In some such embodiments, the gRNA and the DNA plasmid encoding the Cas protein are introduced into the cell by nucleofection. In some particular embodiments, an RNP-based delivery platform or an all-RNA delivery platform provides lower cytotoxicity in primary cells than a DNA plasmid-based delivery system.

[0236] In certain embodiments, the method provides significantly enhanced genome editing efficiencies in human cells, including human primary T cells and CD34+ HSPCs.

[0237] In certain embodiments, modified gRNA increases the frequency of insertions or deletions (indels), which may be indicative of mutagenic NHEJ and gene disruption, relative to unmodified gRNA. In particular, modified gRNA having 2′-O-methyl-3’-phosphorothioate (“MS”), 2′-O-methyl-3’-PACE (“MP”), or 2′-O-methyl-3’-thioPACE (“MSP”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends, increases the frequency of indels relative to unmodified gRNA.

[0238] In certain embodiments, co-delivery of modified gRNA and Cas mRNA to human primary T cells increases the frequency of indels as compared to co-delivery of unmodified gRNA and Cas mRNA. In particular, modified gRNA having 2′-O-methyl-3’-phosphorothioate (“MS”), 2′-O-methyl-3’-PACE (“MP”), or 2′-O-methyl-3’-thioPACE (“MSP”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends, increases the frequency of indels in human primary T cells relative to unmodified gRNA.

[0239] In certain embodiments, modified gRNA improves gRNA stability relative to unmodified gRNA. As one example, gRNA having 2′-O-methyl (“M”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends modestly improves stability against nucleases and also improves base pairing thermostability over unmodified gRNA. As another example, gRNA having 2′-O-methyl-3’-phosphorothioate (“MS”), 2′-O-methyl-3’-PACE (“MP”), or 2′-O-methyl-3’-thioPACE (“MSP”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends, dramatically improves stability against nucleases relative to unmodified gRNA. It is contemplated that gRNA end modifications enhance intracellular stability against exonucleases, thus enabling increased efficacy of genome editing when Cas mRNA and gRNA are co-delivered or sequentially delivered into cells or cell lysates. In certain embodiments, a stability-enhancing modification at an end may also serve as a specificity-enhancing modification if a guide sequence comprises the same end. In certain embodiments, end modifications comprising 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2'-O-methyl-3'-phosphorothioate (MS), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), 2′-fluoro-3’-phosphonoacetate (FP), 2′-fluoro-3’-thiophosphonoacetate (FSP), 2'-fluoro-3'-phosphorothioate (FS), 2′-O-(2-methoxyethyl)-3’-phosphonoacetate, 2′-O-(2-methoxyethyl)-thiophosphonoacetate, 2′-O-(2-methoxyethyl)-3'-phosphorothioate, or a combination thereof increases stability and specificity of a method of the present invention. In certain embodiments, modified gRNA

stimulates gene targeting, which, in turn, allows for gene editing by, for example, homologous recombination or NHEJ. In particular, gRNA having 2′-O-methyl-3’-phosphorothioate (“MS”), 2′-O-methyl-3’-PACE (“MP”), or 2′-O-methyl-3’-thioPACE (“MSP”) incorporated at three terminal nucleotides at both the 5′ and 3′ ends, stimulates higher levels of homologous recombination than unmodified gRNA.

[0240] In certain embodiments, modified gRNA retains high specificity. In certain embodiments, the ratio of on-target to off-target indel frequencies is improved with modified gRNA as compared to unmodified gRNA. In certain embodiments, modified gRNA delivered in an RNP complex with a Cas protein provides significantly better on-target versus off-target ratios compared to a DNA plasmid-based delivery system.

Gene Expression Regulation

[0241] In certain embodiments, the guide RNA described herein is used for regulating transcription or expression of a gene of interest. For example, in certain embodiments, a fusion protein comprising a Cas protein (e.g., a nuclease-deficient Cas9) and a transcription activator polypeptide is used to increase transcription of a gene. Similarly, in certain embodiments, a fusion protein comprising a Cas protein (e.g., a nuclease-deficient Cas9) and a repressor polypeptide is used to knock-down gene expression by interfering with transcription of the gene.

[0242] In at least one aspect, the present invention provides a method for regulating the expression of a gene of interest *in vivo* or *in vitro*. The method comprises introducing into a cell or another system (i) a synthetic guide RNA described herein, and (ii) a fusion protein. In certain embodiments, the fusion protein comprises a Cas protein and an effector domain, such as a transcriptional activation domain, a transcriptional repressor domain, or an epigenetic modification domain. In certain embodiments, the fusion protein comprises a mutated Cas protein, such as a Cas9 protein that is a null nuclease. In certain embodiments, the Cas protein comprises one or more mutations, such as D10A, H840A and/or N863A.

[0243] In certain embodiments, the fusion protein is introduced into the cell or system as a purified or non-purified protein. In certain embodiments, the fusion protein is introduced into the cell or system via an mRNA encoding the fusion protein. In certain embodiments, the fusion protein is introduced into the cell or system via a linear or circular DNA encoding the fusion protein.

[0244] In certain embodiments, the guide RNA aids in directing the fusion protein to a specific target polynucleotide comprising a chromosomal sequence, an episomal sequence, a plasmid, a mitochondrial DNA sequence, or a functional intergenic sequence, such as an enhancer or the DNA sequence for a non-coding RNA. In certain embodiments, the effector domain regulates expression of a sequence in the target polynucleotide. A guide RNA for modulating gene expression can be designed to

target any desired endogenous gene or sequence encoding a functional RNA. A genomic target sequence can be selected in proximity of the transcription start site of the endogenous gene, or alternatively, in proximity of the translation initiation site of the endogenous gene. In certain embodiments, the target sequence is in a region of the DNA that is traditionally termed the "promoter proximal" region of a gene. In certain embodiments, the target sequence lies in a region from about 1,000 base pairs upstream of the transcription start site to about 1,000 base pairs downstream of the transcription start site. In certain embodiments, the target sequence is remote from the start site for transcription of the gene (e.g., on another chromosome).

[0245] In certain embodiments, the methods are for multiplex applications. In certain embodiments, the methods comprise introducing a library of guide RNAs into the cell or system. In certain embodiments, the library comprises at least 10, at least 100, at least 1,000, at least 10,000, at least 100,000, or at least 1,000,000 unique guide sequences. In certain embodiments, the library targets at least 10 different polynucleotides or at least 10 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 100 different polynucleotides or at least 100 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 1,000 different polynucleotides or at least 1,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 10,000 different polynucleotides or at least 10,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 100,000 different polynucleotides or at least 100,000 different sequences within one or more polynucleotides. In certain embodiments, the library targets at least 1,000,000 different polynucleotides or at least 1,000,000 different sequences within one or more polynucleotides.

Kits

[0246] In one aspect, the present invention provides kits containing reagents for performing the above-described methods, including producing gRNA:Cas protein complex and/or supporting its activity for binding, nicking or cleaving a target polynucleotide. In certain embodiments, one or more of the reaction components, *e.g.*, one or more guide RNAs and Cas proteins, for the methods disclosed herein, can be supplied in the form of a kit for use. In certain embodiments, the kit comprises a Cas protein or a nucleic acid encoding the Cas protein, and one or more guide RNAs described herein or a set or library of guide RNAs. In certain embodiments, the kit includes one or more other reaction components. In certain embodiments, an appropriate amount of one or more reaction components is provided in one or more containers or held on a substrate.

[0247] In certain embodiments, the present invention provides a kit for selecting a synthetic guide RNA comprising at least two synthetic guide RNAs which are identical except for different modifications

or modifications at different positions in the guide sequence. Each guide RNA comprises (a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and (b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence, wherein the guide sequence comprises nucleotides 1 through 20-N (N is an integer between -10 and 10, optionally between -10 and 6), counted from the 5′ end of the guide sequence, and at least one specificity-enhancing modification at a nucleotide in the guide sequence; wherein the at least two synthetic guide RNAs differ from each other by having at least one different specificity-enhancing modification or by having the specificity-enhancing modification at least one different position in the guide sequence. The kit also comprises a Cas protein or a polynucleotide coding for a Cas protein. In certain embodiments, each synthetic guide RNA in the kit comprises a specificity-enhancing modification at a different nucleotide. In certain embodiments, the kit comprises a series of synthetic guide RNAs, each one having a modification at a different nucleotide position in the guide sequence. In certain embodiments, the kit has the same number of different guide RNAs as the number of nucleotides in the guide sequence. In certain embodiments, the Cas protein is Cas9. In certain embodiments, the specificity-enhancing modification comprises 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), or 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof. In certain embodiments, the specificity-enhancing modifications comprise a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification. In certain embodiments, the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl). In certain embodiments, the guide RNAs are synthetic single guide RNAs.

[0248] Examples of additional components of the kits include, but are not limited to, one or more different polymerases, one or more host cells, one or more reagents for introducing foreign nucleic acid into host cells, one or more reagents (*e.g.*, probes or PCR primers) for detecting expression of the guide RNA and/or the Cas mRNA or protein or for verifying the status of the target nucleic acid, and buffers, transfection reagents or culture media for the reactions (in 1X or more concentrated forms). In certain embodiments, the kit includes one or more of the following components: biochemical and physical supports; terminating, modifying and/or digesting reagents; osmolytes; and apparati for reaction, transfection and/or detection.

[0249] The reaction components used can be provided in a variety of forms. For example, the components (*e.g.*, enzymes, RNAs, probes and/or primers) can be suspended in an aqueous solution or bound to a bead or as a freeze-dried or lyophilized powder or pellet. In the latter case, the components, when reconstituted, form a complete mixture of components for use in an assay. The kits of the invention can be provided at any suitable temperature. For example, for storage of kits containing protein

components or complexes thereof in a liquid, it is preferred that they are provided and maintained below 0°C, preferably at about -20°C, possibly in a freeze-resistant solution containing glycerol or other suitable antifreeze.

[0250] A kit or system may contain, in an amount sufficient for at least one assay, any combination of the components described herein. In some applications, one or more reaction components may be provided in pre-measured single use amounts in individual, typically disposable, tubes or equivalent containers. With such an arrangement, a RNA-guided nuclease reaction can be performed by adding a target nucleic acid, or a sample or cell containing the target nucleic acid, to the individual tubes directly. The amount of a component supplied in the kit can be any appropriate amount and may depend on the market to which the product is directed. The container(s) in which the components are supplied can be any conventional container that is capable of holding the supplied form, for instance, microfuge tubes, microtiter plates, ampoules, bottles, or integral testing devices, such as fluidic devices, cartridges, lateral flow, or other similar devices.

[0251] The kits can also include packaging materials for holding the container or combination of containers. Typical packaging materials for such kits and systems include solid matrices (*e.g.*, glass, plastic, paper, foil, micro-particles and the like) that hold the reaction components or detection probes in any of a variety of configurations (*e.g.*, in a vial, microtiter plate well, microarray, and the like). The kits may further include instructions recorded in a tangible form for use of the components.

EXAMPLES

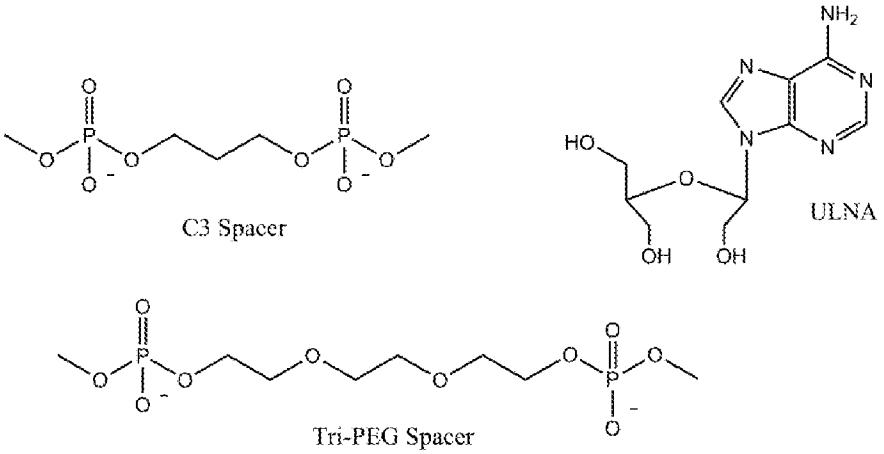
Example 1

[0252] To evaluate the effect of chemical modification on the binding energy at physiological salt conditions, a 43-nucleotide crRNA with a 20-nucleotide guide sequence was made. A duplex was formed by mixing the crRNA with a complementary 40-nucleotide DNA oligonucleotide that comprised a 10 nucleotides overhanging on each end of the DNA oligonucleotide. The melting temperature ("Tm") of the duplex was measured. Seven additional 43-nt crRNAs were made, with several sequential modifications in the sampling and locking region and an intervening or "spacer" nucleotide at position 10 in the 20-nt guide sequence. Fig. 6A shows the type and placement of the modifications. The exonuclease resistance modification was 2′-O-methyl-3'-PACE ("MP"), and the spacer was an unmodified RNA nucleotide. Duplexes were formed individually by mixing each modified crRNA 601 with the complementary 40-nt DNA oligonucleotide 603, and the melting temperature of each RNA/DNA duplex 605 containing modifications in the RNA strand was measured to quantify the effect that the various modifications had on the binding energy under physiological salt conditions.

[0253] The effect that modifications have on the cooperativity of the duplex melting can be evaluated qualitatively by measuring the slope of the melting curve: a larger slope indicates greater cooperativity in the unbinding (and therefore in the binding during a reverse process as well, according to well-established principles of equilibrium reversibility). FIG. 6B shows the melting curve for unmodified gRNA, and FIGs. 6C through 6G show melting curves for various types of modifications at nucleotides 6 through 9 in the 20-nt guide sequence portions. The addition of 2′-O-methyl ("M") and 2′-O-methyl phosphorothioate ("MS") increased the binding energy by about 0.2°C per modification, whereas 2′-O-methyl-3'-PACE ("MP") decreased the Tm or binding energy by 1°C per modification and 2′-O-methyl-3'-thioPACE ("MSP") decreased the Tm by 1.4°C per modification. Fig. 7 is a graph showing change in melting temperature of a 20–base pair gRNA/DNA duplex as the gRNA was truncated to 17, 18 or 19 nucleotides or extended to 21 or 22 nucleotides in length. The measurements were performed in physiological salt concentrations. The measurements indicated a change in melting temperature of about 2°C per base pair for the extension or truncation of the 20-nucleotide guide sequence.

Example 2

[0254] Further melting temperature measurements were performed using experimental crRNAs containing a linker or linker-like modification at nucleotide 9 in the guide sequences of crRNAs. The linker or linker-like modifications comprised a ULNA (unlocked nucleic acid), an abasic spacer, an alkylene spacer comprising $-PO_4Y-(CR^3_2)_m-PO_4Y-$, or an ethylene glycol spacer comprising $(-PO_4Y-(CR^3_2CR^3_2O)_n-PO_3Y-)$, where m is 2, 3 or 4, n is 1, 2 or 3, each R^3 is independently selected from the group consisting of H, an alkyl and substituted alkyl, and each Y is H or a negative charge. For example, in certain embodiments, the alkylene spacer is a "C3 spacer" in which m is 3. In other embodiments, the ethylene glycol spacer is a "Tri-PEG spacer" in which n is 3. These modifications also decrease the binding energy by giving more flexibility to the strand which decreases the cooperativity of hybridization.



[0255] The results of these melting temperature measurements are shown in Table 1:

Table 1

Entry	crRNA Name	T_m
1	Control_1XMP	**50.2**
2	rA-ULNA_9_1XMP	**44.8**
3	C3-Spacer_9_1XMP	**40.9**
4	Tri-PEG-Spacer_9_1XMP	**38.3**

Examples 3 – 7

[0256] To evaluate the ability of the chemically synthesized guide RNAs to bind and cleave a DNA target sequence and to evaluate the effect of various modifications on Cas9-mediated cleavage, an *in vitro* cleavage assay was used. Briefly, PAM-addressable DNA constructs comprising target polynucleotide sequences (ON) or off-target polynucleotide sequences (OFF) set forth in Tables 2 and 7 were prepared by preparative PCR amplification of plasmid-borne human sequences of various target genes. As exemplary genes to demonstrate the ability of the present compositions and methods to selectively edit genes, the human clathrin light chain *CLTA* gene, the human hemoglobin beta (*HBB*) gene, the human interleukin 2 receptor subunit gamma (*IL2RG*) gene, and the human cytotoxic T-lymphocyte-associated protein 4 (*CLTA4*) gene were used as target genes. These are representative of the general approach disclosed herein for evaluating and editing target genes.

[0257] Tables 3 to 6 set forth synthetic guide RNAs. In most of the guide RNAs, the first 20 nucleotides at the 5′ end are complementary to the target sequence in target DNA – these complementary nucleotides make up the guide sequence. In some guide RNAs, an overhang or extension was present at the 5′ end of the guide sequence, which is not complementary to the target sequence. In some guide RNAs, the 5′ end of the guide sequence was truncated such that nucleotides 1, or 1 and 2, or 1 and 2 and 3, are not present. On-target constructs ("ON") comprise the 20-nt target sequence. Off-target constructs ("OFF") comprise most of the same 20 nucleotides as the target DNA, with 1, 2 or 3 nucleotide differences. Accordingly, the gRNA was mostly, but not completely, complementary to the sequence of the OFF target constructs. The OFF target constructs are based on gene sequences known to occur in the human genome.

[0258] The gRNAs were synthesized on an ABI 394 Synthesizer (Life Technologies, Carlsbad, CA, USA) using 2′-O-thionocarbamate-protected nucleoside phosphoramidites according to procedures described in Dellinger et al. (2011) *J. Am. Chem. Soc.*, *133*, 11540–56. 2′-O-methyl phosphoramidites were incorporated into RNA oligomers under the same conditions as the 2′-O-thionocarbamate protected

phosphoramidites. The 2′-O-methyl-3’-O-(di-*iso*-propylamino)phosphinoacetic acid-1,1-dimethylcyano-ethyl ester-5′-O-dimethoxytrityl nucleosides used for synthesis of thiophosphonoacetate (thioPACE)-modified RNAs were synthesized essentially according to published methods. See Dellinger et al. (2003) *J. Am. Chem. Soc. 125*, 940–50; and Threlfall et al. (2012) *Org. Biomol. Chem. 10*, 746–54.

[0259] All the oligonucleotides were purified using reversed-phase high-performance liquid chromatography (HPLC) and analyzed by liquid chromatography–mass spectrometry (LC-MS) using an Agilent 1290 Infinity series LC system coupled to an Agilent 6520 Q-TOF (time-of-flight) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The yields for the synthesis and purification of the sgRNAs were estimated using deconvolution of mass spectra obtained from LC-MS–derived total ion chromatograms. The chemical synthesis of the 100-mer sgRNAs typically yielded 25–35% full-length product from a nominal 1 micromole scale synthesis. Reversed-phase HPLC purification using ion pairing buffer conditions typically gave 20% yield from the crude product with an estimated purity of the final sgRNA in the range of 90% to 95%.

[0260] The DNA target constructs comprised the target sequences (also known as on-target sequences or identified as “ON”) and off-target sequences (“OFF”) set forth in Table 2, with differences in the off-target sequences from the target in bold italics, and the PAM sequences (when shown) are underlined:

Table 2

On-target or Off-target Site	DNA Sequence	SEQ ID NO.
CLTA1 ON	AGTCCTCATCTCCCTCAAGCAG<u>G</u>	1
CLTA1 OFF1	AGTCCTCA***A***CTCCCTCAAGCAG<u>G</u>	2
CLTA1 OFF3	A***C***TCCTCATC***C***CCCTCAAGC<u>CGG</u>	3
CLTA4 ON	GCAGATGTAGTGTTTCCACA<u>GG</u> <u>G</u>	4
CLTA4 OFF1	GCAGATGTAGT***A***TTTCCACA<u>GG</u> <u>G</u>	5
CLTA4 OFF2	***C***CAGATGTAG***C***GTTTCCACA<u>GG</u> <u>G</u>	6
CLTA4 OFF3	GCAGATGT***T***GTGTTTCCACA<u>GG</u> <u>G</u>	7
HBB ON	CTTGCCCCACAGGGCAGTAA<u>CG</u> <u>G</u>	8
HBB OFF1	***TCA***GCCCCACAGGGCAGTAA<u>GG</u> <u>G</u>	9
IL2RG ON	TGGTAATGATGGCTTCAACA<u>TG</u> <u>G</u>	10
IL2RG OFF2	TGGT***GA******G***GATGGCTTCAACA<u>CG</u>	191

	G	
IL2RG OFF3	TGGTAATGATG*A*CTTCAACATA G	11
VEGFA ON	GGTGAGTGAGTGTGTGCGTGTG G	192
VEGFA OFF2	*T*GTG*G*GTGAGTGTGTGCGTGAG G	193

[0261] Note that *HBB* OFF1 differs from *HBB* ON in the first 3 nucleotides of the potential target sequence. Therefore, a gRNA truncated at the 5′ end of its 20-nt guide sequence by 3 nucleotides to provide a 17-nt guide sequence cannot distinguish between *HBB*-ON and *HBB*-OFF1 target sequences. The full sequence of the DNA target constructs used in Examples 3 – 7 is set forth in Table 8 below.

[0262] Included for comparative purposes are guide RNAs having truncation of the guide sequence of a gRNA from 20 nucleotides to 18 or 17 nucleotides, with an evaluation of the ratio of cleavage of target gene sites to known off-target sites. It was seen that, in contrast to the teachings and conclusion by Yanfang et al. (2014), truncation had an effect on cleavage of specific off-target sites but did not have an effect on cleavage at other off-target sites. In spite of the teachings of Yanfang et al. (2014), the present inventors have sought and identified novel compounds and methods for CRISPR-Cas cleaving, nicking or binding a target polynucleotide with enhanced specificity and without truncation of the guide sequence.

[0263] In a 20-uL reaction volume, 2.5 nM of linearized DNA target in the presence of 50 nM sgRNA, 40 nM recombinant purified Cas9 protein (*S. pyogenes*; Agilent) and 0.8 mM $MgCl_2$ at pH 7.6 was incubated at 37 °C for 1 hr. Upon completion, 0.5 uL of RNace It (Agilent) was added, and incubation was continued at 37 °C for 5 min and then at 70 °C for 15 min. Crude products were loaded into a DNA 1000 or DNA 7500 LabChip for analysis on an Agilent Bioanalyzer 2200 or were loaded onto a Genomic DNA ScreenTape or a D5000 ScreenTape for analysis on an Agilent TapeStation 2200 or 4200. The workup steps serve to release Cas9 from binding of target DNA, which was assayed for cleavage.

[0264] Cleavage yields were calculated by the formula: a/(a+b) × 100 where a is the sum of the band intensities of the two cleavage products and b is the remaining uncleaved DNA if present. A cleavage percentage of 100% means that all of the target DNA construct was cleaved, within the limits of detection.

Example 3

[0265] A series of 32 sgRNAs were made for targeting the "*CLTA1*" locus in the human *CLTA* gene. Briefly, individual RNA strands were synthesized and HPLC purified. All oligonucleotides were quality control approved on the basis of full-length strand purity by HPLC analysis and chemical

composition by mass spectrometry analysis. Table 3 sets forth the sequences of the various *CLTA1* sgRNAs. Table 3 shows the sequences of sgRNAs as Entries 1 through 31, and the table discloses certain embodiments of the present gRNAs containing one or more specificity-enhancing modifications. Entry 1 was unmodified and serves as a comparative example. Entry 2 contains MS modifications in the guide sequence at nucleotides 1, 2 and 3. Entry 3 contains MSP modifications in the guide sequence at nucleotides 1, 2 and 3. Entry 4 contains an MSP modification at nucleotide 1 of the guide sequence. Entries 5 and 6 are comparable examples having a gRNA truncated at the 5′ end of its 20-nt guide sequence to an 18-nucleotide guide sequence or a 17-nucleotide guide sequence, respectively. Entries 7 and 8 are comparable examples having unmodified gRNA with one- or two-nucleotide overhangs, respectively, at the 5′ end of the 20-nt guide sequence. Entries 9, 10, 11, 12, and 13 contain MP modifications in the guide sequence at nucleotide 1, nucleotides 1–2, nucleotides 1–3, nucleotides 1–4, and nucleotides 1–5, respectively. Entries 14 and 15 contain MP modifications in the tracrRNA region of the gRNA at nucleotides 2–5 counted from the 3′ end of the sgRNA or nucleotides 2–6 counted from the 3′ end of the sgRNA, respectively, noting that nucleotides are generally counted from 5′ ends of polynucleotides. Therefore, the counting described for entries 14 and 15 is an exception to the general rule. Entries 16 and 17 contain MP modifications in the 20-nt guide sequence at nucleotides 1–2, with an MP-modified C or G nucleotide overhang, respectively. Entries 18 and 19 contain MP modifications in the 20-nt guide sequence at nucleotides 1–3, with an MP-modified UC or AG dinucleotide overhang, respectively. Entries 20 and 21 contain MP modifications in the 20-nt guide sequence at nucleotides 1–4, with an MP-modified CUC or GAG trinucleotide overhang, respectively, plus MP modifications in the tracrRNA region of the gRNA at the 3′-end of the sgRNA. Entries 22– 25 contain MP modification in the guide sequence at nucleotide 20, 19, 18 or 17, respectively. Entry 26 contains MP modifications in the guide sequence at nucleotides 18 and 17. Entries 27–29 contain an M modification in the guide sequence at nucleotide 19, 18 or 17, respectively. Entry 30 contains M modifications in the guide sequence at nucleotides 18 and 17. Entry 31 contains M modifications in the guide sequence at nucleotides 1–20. Entry 32 contains M modifications in the 20-nt guide sequence at nucleotides 1–7, 9–11, 13–14 and 20, plus M modifications at several select positions across the remainder of the sgRNA sequence, specifically at nucleotides 30–31, 33, 35-36, 39, 42, 45, 47, 50, 60, 65–66, 70, 71, 76–77, 80–82, 90, 93, 95–96, 100–101, 104, and 106–112.

Table 3

Entry	sgRNA Name	5' → 3'	SEQ. ID. NO.
1	CLTA1_unmodif	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	12
2	CLTA1_3xMS	AsGsUsCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUsUsUsU	13
3	CLTA1_3xMSP	A*sG*sU*sCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*sU*sU*sU	14
4	CLTA1_1xMSP	A*sGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*sU	15
5	CLTA1_Truncated_18mer	UCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUUU	16
6	CLTA1_Truncated_17mer	CCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUUU	17
7	CLTA1_1xExtraG	G.AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUUU	18
8	CLTA1_2xExtraG	G.G.AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUUU	19
9	CLTA1_1xMP{5'}	A*GUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	20
10	CLTA1_2xMP{5'}	A*G*UCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	21
11	CLTA1_3xMP{5'}	A*G*U*CCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	22
12	CLTA1_4xMP{5'}	A*G*U*C*CUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	23
13	CLTA1_5xMP{5'}	A*G*U*C*C*UCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	24
14	CLTA1_4xMP{3'}	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U*U*U*U	25
15	CLTA1_5xMP{3'}	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCU*U*U*U*U*U	26
16	CLTA1+1over_3xMP	C.*A*G*UCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	27
17	CLTA1+1overNC_3xMP	G.*A*G*UCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	28
18	CLTA1+2over_5xMP	U.*C.*A*G*U*CCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	29
19	CLTA1+2overNC_5xMP	A.*C.*A*G*U*CCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	30
20	CLTA1+3over_7xMP	C.*U.*C.*A*G*U*C*CUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U*U*U*U	31
21	CLTA1+3overNC_7xMP	G.*A.*G.*A*G*U*C*CUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U*U*U*U	32
22	CLTA1_20MP	AGUCCUCAUCUCCCUCAAGC*GUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	33
23	CLTA1_19MP	AGUCCUCAUCUCCCUCAAG*CGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	34
24	CLTA1_18MP	AGUCCUCAUCUCCCUCAA*GCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	35
25	CLTA1_17MP	AGUCCUCAUCUCCCUCA*AGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	36
26	CLTA1_17.18MP	AGUCCUCAUCUCCCUCA*A*GCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	37
27	CLTA1_19M	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	38
28	CLTA1_18M	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	39
29	CLTA1_17M	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	40
30	CLTA1_17.18M	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	41
31	CLTA1_20xM	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	42
32	CLTA1_47xM	AGUCCUCAUCUCCCUCAAGCGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	124

LEGEND

Ns = 2'-O-methyl-3'-phosphorothioate modification of nucleotide N

N* = 2'-O-methyl-3'-PACE modification of nucleotide N

N*s = 2'-O-methyl-3'-thioPACE modification of nucleotide N

N.* = 2'-O-methyl-3'-PACE modification of an overhanging nucleotide N (where N is located in a 5' overhang sequence covalently linked to the guide sequence of a sgRNA)

N = 2'-O-methyl modification of nucleotide N

[0266] FIG. 8A shows the impact of chemical modifications in the gRNAs from Table 3 (SEQ ID NO: 12-42 and 124) with regard to Cas9-mediated target polynucleotide cleavage versus off-target polynucleotide cleavage. More particularly, the cleavage percentages of a *CLTA1* target polynucleotide sequence (the on-target sequence, or "ON") and comparable off-target polynucleotide sequences (OFF1 and OFF3) are shown numerically and in bar graph form. FIG. 8B is derived from the results in FIG. 8A, with a ratio calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA assayed (SEQ ID NO: 12-42 and 124). Also calculated is a Specificity Score obtained by multiplying each ratio by the respective ON-target cleavage percentage per sgRNA assayed. The shaded values in entries 6, 12, 13, 20, 21, and 31 of FIG. 8B result from slightly to substantially reduced cleavage yields for the target polynucleotide sequence (ON1). Importantly, the reduction was even greater for one or both of the off-target polynucleotide sequences, yielding ON:OFF cleavage ratios greater than 2.0. The shaded values indicate that at least two-fold improvements in specificity can be obtained. Among the various chemical modifications and combinations tested in this experiment, the 2′-O-methyl-3'-PACE ("MP") modification gave the largest desired effect of decreasing off-target cleavage while retaining high levels of on-target cleavage, especially when incorporated at an optimal number of positions in the guide sequence. These examples serve as embodiments of the present teaching.

Example 4

[0267] For the example represented in FIG. 9A, single or triple MP modifications were "walked" across the 20-nt guide sequence to see which modified positions could yield improvements in specificity as judged by on-target versus off-target cleavage activities. As listed in Table 4, a series of 28 sgRNAs (SEQ ID NO: 43-70) were made for targeting the "*CLTA4*" locus in the human *CLTA* gene, in which experimental sgRNAs contained a 2′-O-methyl-3'-PACE ("MP") modification at one or more positions in the guide sequence, in addition to having an MP modification at nucleotide 1 and also at the penultimate nucleotide in the tracrRNA region at the 3′ end of the sgRNA which includes the last (i.e., most 3′) internucleotide linkage. Thus, the modifications in the terminal internucleotide linkages were designed to protect such modified sgRNAs against degradation by exonucleases. Individual RNA strands were synthesized and HPLC purified. All oligonucleotides were quality control approved on the basis of full-length strand purity by HPLC analysis and chemical composition by mass spectrometry analysis. The cleavage percentages of a *CLTA4* target polynucleotide sequence (ON) and comparable off-target polynucleotide sequences (OFF1, OFF2 and OFF3) are shown numerically and in bar graph form in FIG 9A. FIG. 9B is derived from the results in FIG. 9A, with a ratio calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA (SEQ ID NO: 43-70) assayed. Ratios recorded as "large" indicate that no cleavage of off-target DNA polynucleotide was

detected in those particular assays. As stated above in the description for FIG. 8B, a Specificity Score is calculated by multiplying a ratio by its respective on-target cleavage percentage. In the example represented by FIG. 9B, specificity scores ≥ 2.0 are shaded to indicate an improvement in specificity relative to unshaded scores. Shading indicates which MP positions in the guide sequence provided at least two-fold improvements in specificity. The results of the MP walk in entries 1–18 indicate that placement of the walked MP modification has an effect on specificity, and a trend is apparent for each set of specificity scores per off-target site assayed which shows that an MP modification near the 5' end of the guide sequence enhances specificity more so than an MP at other positions in the guide sequence, as seen in entries 1 and 2 relative to entries 3–18. This trend is consistent with a specificity enhancement trend observed for MP modifications in gRNAs targeted to the *CLTA1* target sequence, in which MP modifications added to the 5' end of the *CLTA1* sgRNA enhanced specificity, as indicated by the shaded scores in entries 12–13 and 20–21 in FIG. 8B. Although perhaps not effective in every case, a general strategy for improving specificity is to incorporate 1, 2, 3, 4 or 5 MP modifications at consecutive phosphodiester internucleotide linkages at the 5' end of a guide sequence in a gRNA.

Table 4

Entry	sgRNA Name	5' → 3'	SEQ. ID NO.
1	CLTA4_5-7MP_1xMP	G*CAGA*U*G*UAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	43
2	CLTA4_6-8MP_1xMP	G*CAGAU*G*U*AGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	44
3	CLTA4_7-9MP_1xMP	G*CAGAUG*U*A*GUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	45
4	CLTA4_8-10MP_1xMP	G*CAGAUGU*A*G*UGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	46
5	CLTA4_9-11MP_1xMP	G*CAGAUGUA*G*U*GUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	47
6	CLTA4_10-12MP_1xMP	G*CAGAUGUAG*U*G*UUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	48
7	CLTA4_11-13MP_1xMP	G*CAGAUGUAGU*G*U*UUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	49
8	CLTA4_12-14MP_1xMP	G*CAGAUGUAGUG*U*U*UCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	50
9	CLTA4_13-15MP_1xMP	G*CAGAUGUAGUGU*U*U*CCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	51
10	CLTA4_14-16MP_1xMP	G*CAGAUGUAGUGUU*U*C*CACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	52
11	CLTA4_4MP_1xMP	G*CAG*AUGUAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	53
12	CLTA4_5MP_1xMP	G*CAGA*UGUAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	54
13	CLTA4_6MP_1xMP	G*CAGAU*GUAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	55
14	CLTA4_7MP_1xMP	G*CAGAUG*UAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	56
15	CLTA4_8MP_1xMP	G*CAGAUGU*AGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	57
16	CLTA4_9MP_1xMP	G*CAGAUGUA*GUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	58
17	CLTA4_10MP_1xMP	G*CAGAUGUAG*UGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	59
18	CLTA4_11MP_1xMP	G*CAGAUGUAGU*GUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	60
19	CLTA4_12MP_1xMP	G*CAGAUGUAGUG*UUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	61
20	CLTA4_13MP_1xMP	G*CAGAUGUAGUGU*UCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	62
21	CLTA4_14MP_1xMP	G*CAGAUGUAGUGUU*CCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	63
22	CLTA4_15MP_1xMP	G*CAGAUGUAGUGUUU*CCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	64
23	CLTA4_16MP_1xMP	G*CAGAUGUAGUGUUUC*CACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	65
24	CLTA4_17MP_1xMP	G*CAGAUGUAGUGUUUCC*ACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	66
25	CLTA4_18MP_1xMP	G*CAGAUGUAGUGUUUCCA*CAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	67
26	CLTA4_19MP_1xMP	G*CAGAUGUAGUGUUUCCAC*AGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	68
27	CLTA4_20MP_1xMP	G*CAGAUGUAGUGUUUCCACA*GUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU*U	69
28	CLTA4_unmodif	GCAGAUGUAGUGUUUCCACAGUUUAAGAGCUAUGCUGGUAACAGCAUAGCAAGUUUAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	70

LEGEND

Ns = 2'-O-methyl-3'-phosphorothioate modification of nucleotide N

N* = 2'-O-methyl-3'-PACE modification of nucleotide N

N*s = 2'-O-methyl-3'-thioPACE modification of nucleotide N

N_n* = 2'-O-methyl-3'-PACE modification of an overhanging nucleotide N (where N is located in a 5' overhang sequence covalently linked to the guide sequence of a sgRNA)

N = 2'-O-methyl modification of nucleotide N

Example 5

[0268] In the example represented by FIG. 10, single or triple MP modifications were "walked" across the 20-nt guide sequence to see which modified positions could yield improvements in specificity as judged by on-target versus off-target cleavage activities. As listed in Table 5, a series of thirty sgRNAs (SEQ ID NO: 71-86 and 173-186) were made for targeting a locus in the human *IL2RG* gene. The experimental sgRNAs contained 2′-O-methyl-3'-PACE ("MP") modification at one or more positions in the guide sequence, in addition to having an MP modification at nucleotide 1 and also at the penultimate nucleotide in the tracrRNA region at the 3′ end of the sgRNA which includes the last (i.e., most 3′) internucleotide linkage. Thus, the modifications in the terminal internucleotide linkages were designed to protect the sgRNAs against exonucleases. Individual RNA strands were synthesized and HPLC purified. All oligonucleotides were quality control approved on the basis of full-length strand purity by HPLC analysis and chemical composition by mass spectrometry analysis. The cleavage percentages of an *IL2RG* target polynucleotide sequence (ON) and a comparable off-target polynucleotide sequence (OFF3) are shown numerically in FIG. 10. The figure also shows a ratio calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA (SEQ ID NO: 71-86 and 173-186) assayed. A Specificity Score is calculated by multiplying a ratio by its respective on-target cleavage percentage. Specificity scores $\geq$ 2.0 are shaded to indicate an improvement in specificity relative to unshaded scores, therefore the shading indicates which MP positions in the guide sequence provided at least two-fold improvements in specificity. In FIG. 10, the positions in the guide sequence which yielded the greatest improvements in specificity are indicated by darker shading. The results from MP modification of position 7, 14 or 16 are shown in entries 6, 13 and 15, respectively. Guide RNAs possessing such compositions can enhance specificity performance relative to other commonly used compositions such as gRNAs lacking MP modifications, particularly for various CRISPR-Cas applications of gRNAs targeting the clinically important *IL2RG* locus. The example represented by FIG. 10 also instantiates the present novel method of "walking" an MP modification across the guide sequence portion of a gRNA to identify which position or positions yield specificity enhancement due to the location of the walked MP modification. The magnitude of the specificity enhancement is assessed by the on-target versus off-target cleavage ratio for each position tested, and a practitioner can consider such values alongside the percentage of on-target cleavage measured for each design when deciding which MP-modified position or positions is likely to benefit the overall performance of the gRNA. The incremental walking of a single MP across the guide sequence may also identify positions in the sequence for potential synergistic improvements in specificity resulting from one or more combinations of MP modifications at the positions tested by the walk.

Table 5

Entry	sgRNA Name	5' → 3'	SEQ ID NO
1	IL2RG_2MP_1xMP	U*G*GUAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	176
2	IL2RG_3MP_1xMP	U*GG*UAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	177
3	IL2RG_4MP_1xMP	U*GGU*AAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	178
4	IL2RG_5MP_1xMP	U*GGUA*AUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	179
5	IL2RG_6MP_1xMP	U*GGUAA*UGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	180
6	IL2RG_7MP_1xMP	U*GGUAAU*GAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	181
7	IL2RG_8MP_1xMP	U*GGUAAUG*AUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	182
8	IL2RG_9MP_1xMP	U*GGUAAUGA*UGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	183
9	IL2RG_10MP_1xMP	U*GGUAAUGAU*GGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	184
10	IL2RG_11MP_1xMP	U*GGUAAUGAUG*GCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	185
11	IL2RG_12MP_1xMP	U*GGUAAUGAUGG*CUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	186
12	IL2RG_13MP_1xMP	U*GGUAAUGAUGGC*UUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	78
13	IL2RG_14MP_1xMP	U*GGUAAUGAUGGCU*UCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	79
14	IL2RG_15MP_1xMP	U*GGUAAUGAUGGCUU*CAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	80
15	IL2RG_16MP_1xMP	U*GGUAAUGAUGGCUUC*AACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	81
16	IL2RG_17MP_1xMP	U*GGUAAUGAUGGCUUCA*ACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	82
17	IL2RG_18MP_1xMP	U*GGUAAUGAUGGCUUCAA*CAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	83
18	IL2RG_19MP_1xMP	U*GGUAAUGAUGGCUUCAAC*AGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	84
19	IL2RG_20MP_1xMP	U*GGUAAUGAUGGCUUCAACA*GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	85
20	IL2RG_1xMP (control)	U*GGUAAUGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	86
21	IL2RG_4-6MP_1xMP	U*GGU*A*A*UGAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	173
22	IL2RG_5-7MP_1xMP	U*GGUA*A*U*GAUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	174
23	IL2RG_6-8MP_1xMP	U*GGUAA*U*G*AUGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	175
24	IL2RG_7-9MP_1xMP	U*GGUAAU*G*A*UGGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	71
25	IL2RG_8-10MP_1xMP	U*GGUAAUG*A*U*GGCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	72
26	IL2RG_9-11MP_1xMP	U*GGUAAUGA*U*G*GCUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	73
27	IL2RG_10-12MP_1xMP	U*GGUAAUGAU*G*G*CUUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	74
28	IL2RG_11-13MP_1xMP	U*GGUAAUGAUG*G*C*UUCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	75
29	IL2RG_12-14MP_1xMP	U*GGUAAUGAUGG*C*U*UCAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	76
30	IL2RG_13-15MP_1xMP	U*GGUAAUGAUGGC*U*U*CAACAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	77

LEGEND

N* = 2'-O-methyl-3'-PACE modification of nucleotide N

Example 6

[0269] A modification "walk" was done with an MP modification installed at incremental positions across a guide sequence targeted to the human *HBB* gene as shown in FIG. 11A to see if various sites in the 20-nt guide sequence may give substantial cleavage of the on-target site and decreased cleavage of the off-target site. A series of 65 sgRNAs were made for targeting the *HBB* gene as listed in Table 6, in which experimental sgRNAs contained a 2′-O-methyl-3'-PACE ("MP") modification at one or more internal nucleotide positions in the guide sequence, in addition to having an MP modification at nucleotide 1 and also at the penultimate nucleotide in the tracrRNA region at the 3′ end of the sgRNA which includes the last (i.e., most 3′) internucleotide linkage. Thus, the modifications in the terminal internucleotide linkages were designed to protect the sgRNAs against degradation by exonucleases. Individual RNA strands were synthesized and HPLC purified. All oligonucleotides were quality control approved on the basis of full-length strand purity by HPLC analysis and chemical composition by mass spectrometry analysis. The cleavage percentages of an *HBB* target polynucleotide sequence (ON) and a comparable off-target polynucleotide sequence (OFF1) are shown numerically in FIG 11A. The figure also shows a ratio calculated for cleaved target polynucleotide versus cleaved off-target polynucleotide for each synthetic sgRNA (SEQ ID NO: 87-103) assayed. A Specificity Score is calculated by multiplying a ratio by its respective on-target cleavage percentage. Specificity scores ≥ 2.0 are shaded to indicate an improvement in specificity relative to unshaded scores, therefore the shading indicates which MP positions in the guide sequence provided at least two-fold improvements in specificity. In FIG. 11A the positions in the guide sequence that yielded the greatest improvements in specificity are indicated by darker shading, resulting from MP modification of positions 5, 9 or 11 as shown in entries 2, 6 and 8, respectively. Guide RNAs possessing such compositions can enhance specificity relative to other commonly used compositions, such as gRNAs lacking MP modifications, particularly for various CRISPR-Cas applications of gRNAs targeting the clinically important *HBB* locus. The example represented by FIG. 11A also instantiates our method of "walking" an MP modification across the guide sequence portion of a gRNA to identify which position or positions yield specificity enhancement due to the location of the walked MP modification. The magnitude of the specificity enhancement is assessed by the on-target versus off-target cleavage ratio for each position tested, and a practitioner can consider such values alongside the percentage of on-target cleavage measured for each design when deciding which MP-modified position or positions is likely to benefit the overall performance of the gRNA. The incremental walking of a single MP across the guide sequence may also identify positions in the sequence for potential synergistic improvements in specificity resulting from one or more combinations of MP modifications at the positions tested by the walk.

[0270] In a related experiment using *HBB* sgRNAs having the same 20-nt guide sequence, specificity was evaluated for editing of the genomic *HBB* target locus in K562 cells in which the 20–base

pair target sequence *in vivo* was the same as was tested in polynucleotide constructs *in vitro* for FIG. 11A. FIG. 11B shows the impact of various types of modifications in sgRNAs targeting the human *HBB* gene in K562 cells by co-transfecting each synthetic sgRNA (SEQ ID NO: 187-190) with Cas9 mRNA and measuring cleavage of the target locus and three off-target loci including the same off-target sequence OFF1 as evaluated in FIG. 11A. A ratio is calculated for cleaved target (ON) versus cleaved off-target polynucleotide (OFF1) for each synthetic sgRNA assayed. A Specificity Score is calculated by multiplying a ratio by its respective on-target cleavage percentage. The results show that the PACE modifications in the guide sequence yielded a substantial improvement in specificity as evaluated by the Specificity Scores for the various types of modifications tested, especially with respect to the primary off-target activity (at OFF1).

Table 6

Entry	sgRNA Name	5' → 3'	SEQ. ID. NO.
1	HBB_4MP_1xMP	C*UUGU*CCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	87
2	HBB_5MP_1xMP	C*UUGC*CCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	88
3	HBB_6MP_1xMP	C*UUGCC*CCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	89
4	HBB_7MP_1xMP	C*UUGCCC*CACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	90
5	HBB_8MP_1xMP	C*UUGCCCC*ACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	91
6	HBB_9MP_1xMP	C*UUGCCCC*CAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	92
7	HBB_10MP_1xMP	C*UUGCCCCAC*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	93
8	HBB_11MP_1xMP	C*UUGCCCCACA*GGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	94
9	HBB_12MP_1xMP	C*UUGCCCCACAG*GGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	95
10	HBB_13MP_1xMP	C*UUGCCCCACAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	96
11	HBB_14MP_1xMP	C*UUGCCCCACAGGC*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	97
12	HBB_15MP_1xMP	C*UUGCCCCACAGGGC*AGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	98
13	HBB_16MP_1xMP	C*UUGCCCCACAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	99
14	HBB_17MP_1xMP	C*UUGCCCCACAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	100
15	HBB_18MP_1xMP	C*UUGCCCCACAGGGCAGU*AAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	101
16	HBB_19MP_1xMP	C*UUGCCCCACAGGGCAGUA*AGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	102
17	HBB_1xMP (control)	C*UUGCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	103
18	HBB_16,17MP_1xMP	C*UUGCCCCACAGGGCA*G*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	125
19	HBB_14,17MP_1xMP	C*UUGCCCCACAGGC*CAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	126
20	HBB_13,17MP_1xMP	C*UUGCCCCACAGG*GCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	127
21	HBB_10,17MP_1xMP	C*UUGCCCCAC*AGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	128
22	HBB_9,17MP_1xMP	C*UUGCCCCA*CAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	129
23	HBB_8,17MP_1xMP	C*UUGCCCC*ACAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	130
24	HBB_7,17MP_1xMP	C*UUGCCC*CACAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	131
25	HBB_6,17MP_1xMP	C*UUGCC*CCACAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	132
26	HBB_5,17MP_1xMP	C*UUGC*CCCACAGGGCAG*UAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	133
27	HBB_14,16MP_1xMP	C*UUGCCCCACAGGC*CA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	134
28	HBB_13,16MP_1xMP	C*UUGCCCCACAGG*GCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	135
29	HBB_10,16MP_1xMP	C*UUGCCCCAC*AGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	136
30	HBB_9,16MP_1xMP	C*UUGCCCCA*CAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	137
31	HBB_8,16MP_1xMP	C*UUGCCC*CACAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	138
32	HBB_7,16MP_1xMP	C*UUGCCC*CACAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	139
33	HBB_6,16MP_1xMP	C*UUGCC*CCACAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	140
34	HBB_5,16MP_1xMP	C*UUGC*CCCACAGGGCA*GUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	141
35	HBB_13,14MP_1xMP	C*UUGCCCCACAGG*G*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	142
36	HBB_10,14MP_1xMP	C*UUGCCCCAC*AGG*G*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	143
37	HBB_9,14MP_1xMP	C*UUGCCCCA*CAGGG*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	144
38	HBB_8,14MP_1xMP	C*UUGCCCC*ACAGGG*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	145
39	HBB_6,14MP_1xMP	C*UUGCC*CCACAGGG*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	146
40	HBB_7,14MP_1xMP	C*UUGCCC*CACAGGG*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	147
41	HBB_5,14MP_1xMP	C*UUGC*CCCACAGGG*CAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	148
42	HBB_10,13MP_1xMP	C*UUGCCCCAC*AGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	149
43	HBB_9,13MP_1xMP	C*UUGCCCCA*CAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	150
44	HBB_8,13MP_1xMP	C*UUGCCC*ACAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	151
45	HBB_7,13MP_1xMP	C*UUGCCC*CACAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	152
46	HBB_6,13MP_1xMP	C*UUGCC*CCACAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	153
47	HBB_5,13MP_1xMP	C*UUGC*CCCACAGG*GCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	154
48	HBB_6,11MP_1xMP	C*UUGC*CCACA*GGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	155
49	HBB_5,11MP_1xMP	C*UUGC*CCCACA*GGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	156
50	HBB_9,10MP_1xMP	C*UUGCCCC*A*C*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	157
51	HBB_8,10MP_1xMP	C*UUGCCC*C*C*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	158
52	HBB_7,10MP_1xMP	C*UUGCCC*CAC*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	159
53	HBB_6,10MP_1xMP	C*UUGCC*CCAC*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	160
54	HBB_5,10MP_1xMP	C*UUGC*CCCA*C*AGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	161
55	HBB_8,9MP_1xMP	C*UUGCCCC*A*CAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	162
56	HBB_7,9MP_1xMP	C*UUGCCC*CA*CAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	163
57	HBB_6,9MP_1xMP	C*UUGCC*CCA*CAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	164
58	HBB_5,9MP_1xMP	C*UUGC*CCCA*CAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	165
59	HBB_6,8MP_1xMP	C*UUGCC*C*CACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	166
60	HBB_5,8MP_1xMP	C*UUGC*CCC*ACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	167
61	HBB_6,7MP_1xMP	C*UUGCC*C*CACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	168
62	HBB_5,7MP_1xMP	C*UUGC*C*CACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	169
63	HBB_5,6MP_1xMP	C*UUGC*C*CCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUU*U	170
64	HBB_onmodif	CUUGCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	171
65	HBB_3xMS	C*sU*sUsGCCCCACAGGGCAGUAAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUsUsUsU	172

LEGEND

N* = 2'-O-methyl-3'-PACE modification of nucleotide N

Ns = 2'-O-methyl-3'-phosphorothioate modification of nucleotide N

Example 7

[0271] Further experiments for determining on-target versus off-target specificity enhancements due to MP modifications were performed either in solution using an *in vitro* cleavage assay or in cultured human cells of two types. The two cell types were K562 cells and induced pluripotent stem cells (also known as iPS cells or iPSCs). Cultured cells were transfected in individual wells with an MP-modified gRNA pre-complexed with recombinant Cas9 protein. Specificity was evaluated for editing of a genomic *HBB* target locus in which the 20–base pair target sequence in cultured cells was the same as was assayed in polynucleotide constructs *in vitro* as assessed in FIGs. 11A and 12A. Entries 1–17 (SEQ ID NO: 87-103) of FIG. 12A show the same results as FIG. 11A. The data in FIG. 12A are ranked according to Specificity Score from highest to lowest. Entries 18–64 (SEQ ID NO: 125-171) show the impact of additional MP modifications at various locations in gRNAs targeted to a sequence in the human *HBB* gene with regard to *in vitro* cleavage of target polynucleotide called "ON" and separately assayed cleavage of an off-target polynucleotide called "OFF1," representing *HBB* ON-target and *HBB* OFF1-target, respectively. For all entries, a ratio was calculated for cleaved target polynucleotide (ON) versus cleaved off-target polynucleotide (OFF1) for each synthetic sgRNA assayed. A Specificity Score was calculated by multiplying a ratio by its respective on-target cleavage percentage. Entries 18–64 are ranked according to Specificity Score from highest to lowest. Entries with the highest scores are shaded.

[0272] FIG. 12B shows the results of editing a genomic *HBB* target which has the same 20–base pair sequence as was tested in polynucleotide constructs *in vitro* for FIG. 12A. For FIG. 12B, the genomic *HBB* target site is endogenous in the human K562 cells and iPS cells tested. The results are grouped according to the number of MP modifications incorporated in the 20-nt guide sequence. Entries 1–17 in FIG. 12A and entries 1–12 and 22–33 in FIG. 12B represent testing of a single internal MP at various positions in the 20-nt guide sequence, in addition to having an MP modification at the very first internucleotide linkage at the 5' terminus and another MP at the very last internucleotide linkage at the 3′ terminus of each sgRNA to protect it against exonucleolytic degradation. For entries 1–12 and 22–33 of FIG. 12B, the sgRNA tested were SEQ ID NO: 88, 90, 92-94, 96, 97, 99, 100, 103, 171 and 172. For entries 13–19 and 34–40 of FIG. 12B, the sgRNA tested were 128, 129, 133, 141, 160, 165, and 168. Included in these groupings are the results of controls lacking an internal MP modification, such as shown in entry 14 in FIG. 12A and entries 7–9 and 29–31 in FIG. 12B. Other groupings of results were made for sgRNAs having two internal MP modifications at various positions in the 20-nt guide sequence, in addition to having a single MP modification on each terminus of the sgRNAs to inhibit exonucleases, as shown for entries 18–64 in FIG. 12A and entries 13–19 and 34–40 in FIG. 12B. The various groupings are separated in the figures by heavy black lines. These figures show that the internal MP positions which give the largest specificity enhancements *in vitro* are the same as those which give the largest

enhancements in both cell types, as shown in entries 1–2 in FIG. 12A and entries 1–2 and 22–23 in FIG. 12B. The next lower tier of specificity enhancements due to an internal MP modification is indicated by lighter shading, as shown for entries 3–5 in FIG. 12A and entries 3–5 and 24–26 in FIG. 12B. The relative ranking of specificity enhancements resulting from the position of internal MP modification in *HBB* sgRNAs is strikingly consistent across *in vitro* and *in vivo* assays as shown in entries 1–17 of FIG. 12A and entries 1–12 and 22–33 of FIG. 12B.

[0273] Results for a pair of internal MP modifications are grouped separately in entries 18–64 in FIG. 12A and in entries 13–19 and 34–40 in FIG. 12B, and the relative performance of these designs *in vitro* and *in vivo* as ranked by Specificity Score is remarkably consistent across the various assays and cell types. A slightly different way of evaluating specificity enhancement is simply to consider the on-target versus off-target ratio, which is ON:OFF1 for the *HBB* examples in FIGs. 12A and 12B. Using this alternative metric, a re-ranking of the groupings in FIG. 12B according to measured ratio, sorted from highest to lowest ratio per grouping, is shown in FIG. 12C. The various groupings in the figure are separated by heavy black lines. Comparable to our observations for Specificity Scores in FIG. 12B, the simpler assessment of specificity by ON:OFF1 ratio as presented in FIG. 12C shows similar outcomes across both cell types. Examples 6 and 7 demonstrate that specificity enhancements are dependent on the positions of MP modifications in the 20-nt guide sequence targeting the *HBB* gene.

[0274] In an experiment using some of the IL2RG sgRNAs of Table 5, specificity was evaluated for editing of the genomic IL2RG target locus in K562 cells. Also, using the VEGFA sgRNAs of Table 7 (shown below), specificity was evaluated for editing of the genomic VEGFA target locus in K562 cells.

Table 7

Entry	sgRNA Name	5' → 3'	SEQ. ID. NO.
1	**VEGFA_11MP_1xMP**	**G***GUGAGUGAG**U***GUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**194**
2	**VEGFA_10MP_1xMP**	**G***GUGAGUGA**G***UGUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**195**
3	**VEGFA_9MP_1xMP**	**G***GUGAGUG**A***GUGUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**196**
4	**VEGFA_7MP_1xMP**	**G***GUGAG**U***GAGUGUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**197**
5	**VEGFA_5MP_1xMP**	**G***GUG**A***GUGAGUGUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**198**
6	**VEGFA_1xMP (control)**	**G***GUGAGUGAGUGUGUGCGUGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUU**U***U	**199**

[0275] In this way, the 20-nt guide sequences were tested in vivo. FIG. 14 shows the impact of various types of modifications in IL2RG sgRNAs and VEGFA sgRNAs in K562 cells. Each synthetic sgRNA was co-transfected with Cas9 mRNA, and cleavage of the target locus and of an off-target locus OFF2 were measured. A ratio is calculated for cleaved target (ON) versus cleaved off-target polynucleotide (OFF2) for each synthetic sgRNA assayed. A Specificity Score is calculated by multiplying a ratio by its respective on-target cleavage percentage.

[0276] The results in FIG. 14 show that modifications in the guide sequence of synthetic gRNAs targeting IL2RG and VEGFA yielded substantial improvements in specificity as evaluated by the Specificity Scores for the various types of modifications tested, especially with respect to the off-target activity (at OFF2). In contrast with entry 1, entry 2 shows a significant specificity enhancement resulted from MP modification at both ends of the IL2RG sgRNA, in which the 5' and 3' terminal internucleotide linkages are modified. In contrast with entries 1 & 2, entries 3 & 4 show impressive specificity enhancements for IL2RG sgRNAs having an MP modification at an internal position in the IL2RG guide sequence, namely at position 5 or 11, respectively. Furthermore, MP modification of position 5 gave the largest enhancement of specificity among entries 1 thru 4 in Fig. 14.

[0277] In contrast with entry 5, entries 6 thru 10 show significant specificity enhancements for VEGFA sgRNA having an MP modification at an internal position in the VEGFA guide sequence, namely at position 5, 7, 9, 10, or 11, respectively. When comparing the enhancements across entries 5 thru 10, it is notable for entry 6 that an MP modification at position 5 of the 20-nt guide sequence gave the largest enhancement of specificity for VEGFA.

[0278] By conducting MP walks across guide sequences in gRNAs targeting different DNA sequences and assaying cleavage of on-target and off-target polynucleotides *in vitro* as presented in Examples 4 through 7, a composite map of MP-modified positions in various guide sequences which yielded specificity enhancements is shown in FIG. 13. In particular, FIG. 13 shows a comparison of *in vitro* cleavage results from FIGs. 9A, 9B, 10 and 11A. FIG. 13 shows several important trends. First, as shown in Entry 12 in contrast to all other entries in FIG. 13, gRNAs having an MP modification at position 15 in their 20-nt guide sequences suffered a substantial loss of Cas9-mediated cleavage activity. This suggests that position 15 was particularly intolerant of an MP modification for Cas9-mediated cleavage. Second, there is a trend for specificity enhancements throughout entries 1–2, as indicated by shaded specificity scores. This trend suggests that incorporating MP modifications at or near the 5' end of various 20-nt guide sequences can be a generally useful design strategy for enhancing the target specificity of gRNAs. Another example of the utility of this approach was discussed above in Example 3 for results presented in FIGs. 8A and 8B regarding MP modifications at the 5' end of gRNAs targeting the *CLTA1* locus *in vitro*. A further example of the utility of the general approach was discussed above in

Example 6 regarding MSP modifications at the 5' end of a gRNA targeting the *HBB* gene in transfected human K562 cells as shown in FIG. 11B. A third example regarding specificity effects of MP modifications is apparent in entry 3 of FIG. 13, where specificity scores resulting from MP modification of position 6 in differently targeted 20-nt guide sequences constitute the lowest scores per MP walk, disregarding the anomalous effects for position 15 mentioned above. For each series of MP walks involving *CLTA4* OFF3, *IL2RG* OFF3 and *HBB* OFF1, a nadir of specificity enhancement due to MP modification of position 6 is especially noticeable in contrast to specificity scores resulting from MP modification of an adjacent position in the same guide sequence, namely position 4, 5 or 7 (shown as shaded scores throughout entries 1, 2 or 4, respectively).

[0279] Examples 3 through 7 demonstrate that gRNAs containing modifications at specific positions in guide sequences are tolerated by active Cas protein and gRNA:Cas protein complexes, as modifications at many sequence positions in gRNAs did not prevent target-specific cleavage of on-target polynucleotides. Examples 3 through 7 also demonstrate that the present gRNAs, complexes and methods can achieve on-target versus off-target ratios of at least 1.2, alternatively at least 1.5, alternatively at least 2, alternatively at least 2.5, alternatively at least 3, alternatively at least 3.5, alternatively at least 4, alternatively at least 4.5, alternatively at least 5, and/or the ratio is up to 10, 12, 15 or 20. In many instances, the 2′-O-methyl-3'-PACE ("MP") modification had a positive effect on ratios by decreasing off-target cleavage levels while retaining high levels of on-target cleavage as desired for specificity enhancements.

[0280] As a result of these experiments it is calculated that incorporation of multiple modifications that decrease binding energy at specific sites across a guide sequence will lower known and unknown off-target cleavage events.

[0281] As mentioned above, the full sequences of the DNA constructs used in Examples 3 – 7 are set forth in Table 8. The target polynucleotide or off-target sequence, along with PAM sequence, is shown in bold, and the PAM sequence is also underlined.

Table 8

CLTA1 ON1-target (SEQ ID NO: 104):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
GCAGGCCAAAGATGTCTCCCGCATGCGCTC**AGTCCTCATCTCCCTCAAGC<u>AGG</u>**CCCTGCTGGTGCAC
TGAAGAGCCACCCTGTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA1 OFF1-target (SEQ ID NO: 105):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA

ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
GCAGGGCAAAGAGGTCTCCTGTATGCACTC**AGTCCTCAACTCCCTCAAGC<u>AGG</u>**CGACCCTTGGTGCA
CTGACAAACCGCTCCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA1 OFF3-target (SEQ ID NO: 106):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC

ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
AGGAGAGGGAGCCATGCTCATCTCCAGCCC**ACTCCTCATCCCCCTCAAGCCGG**TCCCAGGCTGAGA
GGCTAAAGCTTGTCTTTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCG
CGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC
ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC
GTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC
GCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAG
GTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC
CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTC
ATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA
GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCT
GAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCG
GTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC
TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATC
AAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATG
AGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTC
GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCC
CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCA
GCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG
CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCA
TCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA
CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAA
GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTT
GCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA4 ON-target (SEQ ID NO: 107):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
AAGAGCTTCACTGAGTAGGATTAAGATATT**GCAGATGTAGTGTTTCCACAGGG**TGGCTCTTCAGTGC

ACCAGCGGAACCTGCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA4 OFF1-target (SEQ ID NO: 108):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
AAGAGCTTCACTGAGTAGGATTAAGATATT**GCAGATGTAGTATTTCCACAGGG**TGGCTCTTCAGTGC
ACCAGCGGAACCTGCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC

GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA4 OFF2-target (SEQ ID NO: 109):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
AAGAGCTTCACTGAGTAGGATTAAGATATT**CCAGATGTAGCGTTTCCACAGGG**TGGCTCTTCAGTGC
ACCAGCGGAACCTGCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC

CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

CLTA4 OFF3-target (SEQ ID NO: 110):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
AAGAGCTTCACTGAGTAGGATTAAGATATT**GCAGATGTTGTGTTTCCACAGGG**TGGCTCTTCAGTGC
ACCAGCGGAACCTGCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC

GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

HBB ON-target (SEQ ID NO: 111):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
GGCCTCACCACCAACTTCATCCACGTTCAC**CTTGCCCCACAGGGCAGTAA**<u>**CGG**</u>CAGACTTCTCCTCA
GGAGTCAGATGCACCAGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC

TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

HBB OFF1-target (SEQ ID NO: 112):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
GGATAGGAAAGGTGAAGTCAGAGCAGTGCT**TCAGCCCCACAGGGCAGTAAGGG**CAGCCTTCCTCTA
AATACCAGATTCCCAAAGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCG
CGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC
ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC
GTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC
GCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAG
GTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC
CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTC
ATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA
GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCT
GAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCG
GTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC
TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATC
AAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATG
AGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTC
GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCC
CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCA
GCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG
CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCA
TCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA
CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAA
GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTT
GCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

IL2RG ON-target (SEQ ID NO: 113):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA

AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA
GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
GGGCAGCTGCAGGAATAAGAGGGATGTGAA**TGGTAATGATGGCTTCAACATGG**CGCTTGCTCTTCA
TTCCCTGGGTGTAGTCTGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCG
CGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAAC
ATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGC
GTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC
GCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAG
GTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC
CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTC
ATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACG
ACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA
GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCT
GAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCG
GTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC
TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATC
AAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATG
AGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTC
GTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCC
CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCA
GCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG
CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCA
TCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTA
CATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAA
GATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTT
GCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

IL2RG OFF3-target (SEQ ID NO: 114):

GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGA
AATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTT
TTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGA

GTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAA
ACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTG
CCGTAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCG
AACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG
GTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCCATTCGCC
ATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGA
AAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAA
ACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATCGATGCGGCCTC
CAATATTGAGAGTGAATGAAAAGTGTCAGC**TGGTAATGATGACTTCAACATAG**TCAGAACTCTTTGG
GCTGTTCCAAACATCAGCGCGTGATATGCAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGC
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCG
TTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTT
GCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGT
GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAT
AGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCC
CCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAG
AGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTG
AAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG
TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAA
AAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTT
CATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCC
AGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGC
CGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC
GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGA
TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGC
TCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGC

Example 8

[0282] This experiment demonstrates a homology-directed repair (HDR) process stimulated by Cas9 cleavage using synthetic gRNAs according to the present disclosure. K562 cells were provided having a normal *HBB* gene on both pairs of chromosomes (Chr11). Also provided was DNA donor template for HDR carrying the Sickle Cell Disease (SCD) mutation. The experiment sought to edit the *HBB* gene in the K562 cells to add, or knock-in, the SCD mutation to create a heterozygous cell line. Such a cell line would be useful to model the SCD disease.

[0283] sgRNAs with and without chemical modifications were used. More particularly, experiments were run with the following 20-nt synthetic guide RNAs: unmodified gRNA (Unmodif), gRNA having MP modifications at the 5′ and 3′ ends (1xMP), gRNA having MP modifications at position 5 and at the 5′ and 3′ ends (5MP_1xMP), gRNA having MP modifications at position 11 and at the 5′ and 3′ ends (11MP_1xMP), and mock-transfected cells treated with buffer instead of sgRNA:Cas9 complex (Mock). Each sgRNA was pre-complexed separately with Cas9 protein for transfection into K562 cells (via electroporation) along with a ssDNA oligo to serve as a repair template for HDR at the *HBB* target site. In each group, experiments were run with donor DNA template (Donor + in Figure 15) and without donor DNA template (Donor – in Figure 15). The target site was cleaved by sgRNA:Cas9 complex to form a double-stranded break. The break was repaired by an HDR process such that the sequence of the donor DNA template was used by the endogenous DNA repair enzymes in the transfected cell to direct repair of the cleaved DNA target sequence.

[0284] The sequence of the single-stranded donor DNA template transfected into the K562 cells to serve as a repair template was:

TCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTC AAACAGACACCATGGTGCACCTGACTCCTgtaGAGAAGTCTGCGGTTACTGCCCTGTGGGGCA AGGTGAACGTGGATGAAGTTGGTGGTGAGGCC (SEQ ID NO:200).

[0285] The oligonucleotide is 158 nucleotides long, and the lower case “gta” is the codon for the SCD mutation being knocked-in by HDR. Transfected cells were cultured and subsequently harvested at 48 hours post-transfection. Genomic DNA was isolated, and the *HBB* locus and its major off-target locus were both amplified by PCR for deep sequencing to quantify editing outcomes which comprised (i) formation of Cas9-induced indels (ON target Indel and OFF target Indel in Figure 15), (ii) partial HDR (partial ON target HDR), and (iii) full HDR which installs the SCD codon (ON target HDR and OFF target HDR). All three types of editing were measured at the on-target site (Locus ON in Figure 15), and two of the three types of editing were detected at a major off-target site in the genome on Chr9 (Locus OFF in Figure 15).

[0286] Figure 15 summarizes the results from these experiments of homology-directed repair (HDR) of the *HBB* gene in human K562 cells. For "Locus ON", the results reported (from left to right) are ON target HDR%, partial ON target HDR%, and ON target Indel%. For "Locus OFF", the results reported (from left to right) are OFF target HDR% and OFF target Indel%.

[0287] This experiment demonstrated that chemical modifications for enhancing specificity, particularly MP modifications at position 5 and position 11 in a 20-nt guide sequence, not only reduced the formation of off-target indels quite substantially, but also reduced the off-target HDR activity

significantly. It was noted that, in this experiment, the off-target site had sufficient sequence similarity to the ssDNA repair template to undergo HDR editing as well.

Example 9

[0288] In this experiment, off-target activity by synthetic guide RNAs was evaluated in two different but complementary ways. Several chemically synthesized guide RNAs were used to bind and cleave the *HBB* target site and off-target sites using the same *in vivo* assay described above with respect to Example 7 and FIG. 12B. Thus, for the first evaluation, practitioner-specified polynucleotide sequences or loci in genomic DNA from human K562 cells were amplified by PCR, and for the second evaluation, a library of about 1,000 polynucleotide sequences or loci (specified by the practitioner) in fragments of the genomic DNA were enriched by using a commercially available loci enrichment kit. More particularly, in the first evaluation, off-target activity was evaluated by performing PCR on the genomic DNA to amplify 16 off-target sites that constitute the sequences in the human genome that possess 3 or fewer mismatches with respect to the 20-bp target sequence in *HBB* and which also have either an NGG or an NAG PAM sequence at positions 21-23 relative to the target sequence; such PAM sequences are required for Cas9 recognition. In the second evaluation, off-target activity was evaluated by using biotinylated oligonucleotide baits (SureSelect Target Enrichment Kit from Agilent Technologies) on the genomic DNA from the K562 cells to capture target and off-target sites. The baits were designed to selectively capture the sequences in the human genome (approximately 960 sequences in total) that possess 5 or fewer mismatches with respect to the 20-bp target sequence in *HBB* and which also have either an NGG or an NAG PAM sequence at positions 21-23. The on-target sequence and off-target sequences were:

Table 9

***HBB* on-target locus & similar off-target loci**	**Target Site Sequence in Human Genome** PAM sequences underscored; mismatches in italic; bulges in lower case	**Genomic Coordinates** in Human Assembly GRCh38/hg38	**SEQ ID NO.**
HBB ON	**CTTGCCCCACAGGGCAGTAACGG**	Chr11: 5,226,968-5,226,990	201
OFF1	***TCA*GCCCCACAGGGCAGTAAGGG**	Chr9:101,833,584-101,833,606	202
OFF2	**CTTGCCCCAga CAGGGCAGTAAGGG**	Chr17:78,243,545-78,243,567	203
OFF3	**C*CTCT*CCCACAGGGCAGTAAAGG**	Chr17:68,628,098-68,628,120	204
OFF4	**CTTGCCCC*TAGA*GGGCA*C*TAAAGG**	Chr11:76,420,075-76,420,097	205
OFF5	***GC*TGCCCCACAGGGCAG*C*AAAGG**	Chr12:124,319,282-124,319,304	206
OFF6	**C*C*TG*T*CCCACAGGGCAG*G*AAGGG**	Chr19:33,890,015-33,890,037	207
OFF7	***G*TG*G*CCCCACAGGGCAG*G*AATGG**	ChrX:75,786,405-75,786,427	208
OFF8	**CT*C*GCCCC*T*CAGGGCAGTA*G*TGG**	Chr2:11,637,669-11,637,691	209
OFF9	***A*TTGCCCCAC*G*GGGCAGT*G*ACGG**	Chr12:93,155,409-93,155,431	210
OFF10	**CTTGC*A*CCACAG*A*GCA*C*TAAGGG**	Chr19:37,048,134-37,048,156	211
OFF11	**CTTGCCCCA*G*AGGGC*T*GT*T*AAGG**	Chr2:79,966,568-79,966,590	212
OFF12	**CTTG*G*CCCACAGGGCA*C*T*G*AGGG**	Chr19:48,638,948-48,638,970	213
OFF13	**C*C*TGCCCCACAGGGCAG*CC*AAGG**	Chr15:87,945,584-87,945,606	214
OFF14	**CTcTGCCCCACAGG*C*CAG*G*AAGGG**	Chr5:132,087,686-132,087,708	215
OFF15	**CTTGtCCCCACAGGGCAGT*GG*CGG**	Chr16:535,479-535,501	216
OFF16	**CTTGCCCCACAGG*T*CA*T*TAAT*AG***	Chr18:45,734,262-45,734,284	217

[0289] For both evaluations, the following 20-nt synthetic guide RNAs were used: unmodified gRNA (Unmodif), gRNA having MP modifications at the 5′ and 3′ ends (1xMP), gRNA having MP modifications at position 5 and at the 5′ and 3′ ends (5MP_1xMP), and mock-transfected cells treated with buffer instead of sgRNA:Cas9 complex (Mock). The first evaluation also included gRNA having MP modifications at position 11 and at the 5′ and 3′ ends (11MP_1xMP).

[0290] Human K562 cells were transfected with gRNA and Cas9 using the same procedures used in the preceding Examples. More particularly, human K562 cells were obtained from ATCC and were cultured in RPMI 1640 media supplemented with 10% bovine growth serum (Thermo Fisher). K562 cells (within passage number 3 to 9) were nucleofected using a Lonza 4D-Nucleofector (96-well Shuttle device, program FF-120) per manufacturer's instructions. Nucleofection conditions utilized a Lonza SF Cell Line kit (V4SC-2960) with 0.2 million cells in 20 uL of media, 125 pmoles of chemically modified sgRNA, and 50 pmoles of recombinant Cas9 protein (Thermo Fisher). Cells were cultured at 37 °C in ambient oxygen and 5% carbon dioxide. Cultured cells were harvested at 48 hours post-transfection.

[0291] For the second evaluation, a library comprising target enrichment baits was designed (using the SureSelect enrichment platform from Agilent) to have overlapping sequence coverage (also referred to as "tiling") to capture a 1-Kbp segment of genomic DNA approximately centered on the *HBB* target site. The library also comprised similarly-tiled baits to capture 1-Kbp segments centered on 960 discrete off-target sites in the human genome. The 960 off-target sites are believed to constitute all sequences in the human genome which possess 5 or fewer mismatches with respect to the 20-bp target sequence for CRISPR-Cas9 targeting in *HBB* and which also possess either an NGG or an NAG PAM sequence adjoining the 20-bp off-target sequence as would be required to allow Cas9 recognition and binding, etc. Genomic DNA was isolated per triplicate sample per treatment, and each isolate was processed according to the Agilent SureSelect HiSeq protocol.

[0292] For both evaluations, the amplified or captured DNA was sequenced on an Illumina HiSeq 4000 sequencer using Illumina reagents for paired-end 2x150-bp sequencing reads. HiSeq raw data was preprocessed by discarding reads that did not overlap the ON- or OFF-target sites by at least 30 bp on either side of the Cas9 cleavage site per on- or off-target sequence. Mapping of reads to the human genome was performed using BWA-MEM (bwa-0.7.10) software set to default parameters. Reads yielding inconsistent paired-end mappings, low quality mappings, or secondary mappings were discarded from the analysis. Each retained mapped read was scored as possessing an indel or not according to whether it had an insertion or deletion of sequence (i.e., indel) within 10 bp of the mapped cut site for Cas9 cleavage. Mapped reads per ON- or OFF-target site were binned according to whether they possessed an indel or not, and the tally of reads per bin was used to calculate %indels formed at the ON-

target site and likewise at each of the 960 OFF-target sites. The %indels results were used to calculate ON:OFF target cleavage ratios and Specificity Scores (not shown).

[0293] Figure 16 summarizes the results from the first evaluation, which measured indel formation at the *HBB* on-target site and 16 similar off-target sites for the modified or unmodified sgRNA. Deep sequencing of the PCR amplicons generated sequence reads for the 17 sites (the on-target site and 16 off-target sites) using isolated genomic DNA per treatment. All of the modified or unmodified gRNAs resulted in high percentages of Indels at the ON-target site. At the OFF1 site, the unmodified gRNA and the 1xMP gRNA also produced high percentages of Indels; in contrast, the 5MP_1xMP gRNA and the 11MP_1xMP gRNA gave much lower percentages of Indels. At the OFF5 site, the 5MP_1xMP gRNA and the 11MP_1xMP gRNA produced much lower percentages of Indels than the unmodified gRNA and the 1xMP gRNA. At the other OFF sites, all of the gRNAs produced very low percentages of Indels. This evaluation shows that chemical modifications for enhancing specificity, particularly MP modifications at position 5 and position 11 in a 20-nt guide sequence, reduced the formation of off-target indels quite substantially in the sequences in the human genome which possess 3 or fewer mismatches with respect to the 20-bp on-target sequence in *HBB* and which also have either an NGG or an NAG PAM sequence at positions 21-23.

[0294] Figure 17 summarizes results from the deep sequencing analysis of amplified DNA loci (PCR) or captured DNA loci (SureSelect) from both evaluations. FIG. 17 shows the measured percentage of Indel formation at the *HBB* target site (HBB ON) and three OFF-target sites (OFF1, OFF5 and OFF9) from experiments with the following 20-nt synthetic guide RNAs: unmodified gRNA (HBB_unmodif (PCR) and HBB_unmodif (SureSelect)), gRNA having MP modifications at the 5′ and 3′ ends (HBB_1xMP (PCR) and HBB_1xMP (SureSelect)), gRNA having MP modifications at position 5 and at the 5′ and 3′ ends (HBB_5xMP (PCR) and HBB_5xMP (SureSelect)), and mock-transfected cells treated with buffer instead of sgRNA:Cas9 complex (Mock (PCR) and Mock (SureSelect)). The percentages of Indels detected were consistent between the two evaluations, with analysis of captured DNA by SureSelect enrichment finding similar percentages of Indels as found in analysis of discrete DNA loci amplified by PCR. All of the modified or unmodified gRNAs resulted in high percentages of Indels at the HBB ON target site. At the OFF1 site, both HBB_unmodif (PCR) and HBB_unmodif (SureSelect) experiments and the HBB_1xMP (PCR) and HBB_1xMP (SureSelect) experiments showed high percentages of Indels. In contrast, the 5MP_1xMP gRNA and the 11MP_1xMP gRNA showed much lower percentages of Indels by the PCR and SureSelect evaluations. At the OFF5 site, both evaluations using the 5MP_1xMP gRNA (*i.e.*, HBB_5xMP gRNA (PCR) and HBB_5xMP gRNA (SureSelect)) produced much lower percentages of Indels than the unmodified gRNA and HBB_1xMP gRNA. At the OFF9 site, all of the gRNAs tested had very low percentages of Indels. Of the remaining 957 off-target

loci evaluated by SureSelect enrichment, only 32 produced indels at a level greater than the detection limit of 0.1% (results not shown), and the highest Indel% among these was less than 0.5%. Thus, chemical modifications for enhancing specificity, particularly an MP modification at position 5 in a 20-nt guide sequence, reduced the formation of off-target indels quite substantially. Such specificity enhancement can be demonstrated by evaluating genomic loci amplified by PCR or genomic loci enriched by using a targeted library enrichment kit such as SureSelect.

EXEMPLARY EMBODIMENTS

Exemplary embodiments provided in accordance with the presently disclosed subject matter include, but are not limited to, the claims and the following embodiments:

A1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence comprises at least one specificity-enhancing modification, wherein the synthetic guide RNA has gRNA functionality.

A2. The synthetic guide RNA of embodiment A1, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

A3. The synthetic guide RNA of embodiment A1, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

A4. The synthetic guide RNA of embodiment A1, wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

A5. The synthetic guide RNA of any of the preceding embodiments, further comprising at least one stability-enhancing modification on the 5′ end or the 3′ end or both ends of the guide RNA.

A6. The synthetic guide RNA of any of the preceding embodiments, wherein the guide sequence comprises a locking region, a sampling region, and a seed region, and the at least one specificity-enhancing modification is present in the sampling region and/or in the seed region.

A7. The synthetic guide RNA of embodiment A6, wherein the at least one specificity-enhancing modification is present in the seed region and/or in the sampling region and/or in the locking region.

A8. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification comprises an internucleotide linkage modification.

A9. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification comprises a chemically modified nucleobase.

A10. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification is located in at least one nucleotide sugar moiety.

A11. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification comprises a 3'-phosphonoacetate internucleotide linkage, a 3'-phosphonoacetate methyl ester internucleotide linkage, a 3'-methylphosphonate internucleotide linkage, a 3'-thiophosphonoacetate internucleotide linkage, a 3'-methylthiophosphonate internucleotide linkage, a 3'-boranophosphonate internucleotide linkage, or combinations thereof.

A12. The synthetic guide RNA of any of the preceding embodiments, comprising a 2′ modification that confers a C3′-endo sugar pucker.

A13. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification comprises:

(a) 2′-deoxyribose, 2′-deoxy-2′-fluoroarabinofuranosyl, 2′-deoxy-2′-fluororibofuranosyl, sugars such as ribose having 2′-O-phenyl, 2′-thiophenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino, or 2′-O-substituted phenyl, or combinations thereof;

(b) phosphonoacetates, thiophosphonoacetates, phosphonopropionates, thiophosphonopropionates, methylphosphonates, methylthiophosphonates, or boranophosphonates; or combinations thereof; or

(c) combinations of (a) and (b).

A14. The synthetic guide RNA any of embodiments A8, A9, A11 or A13, further comprising a 2′-O-methyl modification.

A15. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification is an unstructured nucleic acid ("UNA"), an unlocked nucleic acid ('ULNA'), an abasic nucleotide, or an alkylene spacer comprising -PO_4Y-(CR^3_2)m-PO_4Y-, or an ethylene glycol spacer comprising (-PO4Y-$(CR^3_2CR^3_2O)$n-PO_3Y-), where m is 2, 3 or 4, n is 1, 2 or 3, each R^3 is independently selected from the group consisting of H, an alkyl and a substituted alkyl, and each Y is H or a negative charge.

A16. The synthetic guide RNA of any of embodiments A1 to A9, A11 to A13, or A15, wherein the at least one specificity-enhancing modification does not comprise a nucleobase modification.

A17. The synthetic guide RNA of any of embodiments A1 to A15, wherein the at least one specificity-enhancing modification is a nucleobase selected from the group consisting of 2-thioU, 2-thioC,

4-thioU, 6-thioG, 2-aminopurine, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylC, 5-methylU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-ethynylcytosine, 5-aminoallylU, 5-aminoallylC, an abasic nucleotide, a UNA base, isoC, isoG, 5-methyl-pyrimidine, x(A,G,C,T,U), y(A,G,C,T,U), and combinations thereof.

A18. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification is a nucleotide or nucleotide analog selected from the group consisting of 5-nitroindole, nebularine, inosine, diaminopurine, an abasic linkage, and an abasic fluorophore linkage such as 3-O-yl-2-(4-butylamidofluorescein)propyl-1-O-yl-phosphodiester.

A19. The synthetic guide RNA of any of the preceding embodiments, wherein the at least one specificity-enhancing modification comprises a modification that lowers a melting temperature (Tm) of a first DNA/RNA duplex formed by the synthetic guide RNA and the target polynucleotide, relative to the Tm of a duplex without the specificity-enhancing modification.

A20. The synthetic guide RNA of embodiment A19, wherein the at least one specificity-enhancing modification lowers the Tm by about 0.5°C per modification, alternatively by about 0.5–1.0°C per modification, alternatively by about 1.0 – 2.0°C per modification, alternatively by 2–8°C per modification.

A21. The synthetic guide RNA of any of the preceding embodiments, wherein the guide sequence comprises 20 nucleotides and optionally comprises a 5′-overhang sequence.

A22. The synthetic guide RNA of embodiment A21, wherein the guide sequence comprises or consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence, and at least one specificity-enhancing modification at nucleotide 1, alternatively at nucleotides 1 and 2, alternatively at nucleotides 1, 2, and 3, alternatively at nucleotides 1, 2, 3 and 4; alternatively at nucleotides 1, 2, 3, 4 and 5.

A23. The synthetic guide RNA of embodiment A21, wherein the guide sequence consists of nucleotides 1 through 20-N, counted from the 5′ end of the guide sequence, where N is an integer between -10 and 10 (optionally between -10 and 6), and the at least one specificity-enhancing modification is within nucleotides 4-N to 20-N, alternatively within nucleotides 5-N to 20-N, alternatively within nucleotides 10-N to 20-N, alternatively within nucleotides 13-N to 20-N, alternatively within nucleotides 13-N through 14-N or 16-N through 19-N, alternatively within nucleotides 13-N through 14-N or 16-N through 18-N.

A24. The synthetic guide RNA of embodiment A21, wherein the guide sequence consists of nucleotides 1 through 20-N, wherein N is a positive or negative integer between -10 and 10 (optionally

between -10 and 6), counted from the 5′ end of the guide sequence, and the guide sequence comprises one specificity-enhancing modification located at nucleotide 11-N, 12-N, 13-N, 14-N, 16-N, 17-N, 18-N, 19-N or 20-N, alternatively located at nucleotide 13-N, 14-N, 16-N, 17-N, 18-N, 19-N or 20-N, alternatively located at nucleotide 13-N, 14-N, 16-N, 17-N, or 18-N.

A25. The synthetic guide RNA of any of the preceding embodiments, further comprising at least one stability-enhancing modification starting at nucleotide 1 on the 5′ end and at least one stability-enhancing modification within the five terminal nucleotides on the 3′ end of the guide RNA.

B1. A gRNA:Cas protein complex comprising a synthetic guide RNA of any of the preceding embodiments and a Cas protein, capable of cleaving, nicking or binding a target polynucleotide, or having cleaving activity, nicking activity and/or binding activity.

C1. A method for cleaving, nicking or binding a target polynucleotide comprising:

contacting the target polynucleotide with the gRNA:Cas protein complex of embodiment B1, and

cleaving, nicking or binding the target polynucleotide.

C2. The method of embodiment C1, wherein the synthetic guide RNA comprising the at least one specificity-enhancing modification decreases the cleaving, nicking or binding of an off-target polynucleotide in comparison to an unmodified gRNA.

C3. The method of embodiment C2, wherein the at least one off-target polynucleotide is cleaved, nicked or bound by the CRISPR-associated protein, and wherein a ratio of cleaved, nicked or bound target polynucleotide to cleaved, nicked or bound off-target polynucleotide is at least 1.2, alternatively at least 1.5, alternatively at least 2, alternatively at least 2.5, alternatively at least 3, alternatively at least 3.5, alternatively at least 4, alternatively at least 4.5, alternatively at least 5 or at least 10, 12, 15 or 20.

D1. A method of preparing a synthetic guide RNA comprising:

selecting a target polynucleotide in a genome wherein the target polynucleotide comprises a respective nucleotide sequence;

identifying at least one off-target polynucleotide in the genome wherein the off-target polynucleotide comprises a respective nucleotide seqeuence;

aligning the sequence of the target polynucleotide with the sequence of the off-target polynucleotide to identify one or more identical portions of both sequences;

designing a synthetic guide RNA comprising;

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence includes a specificity-enhancing modification within a portion of its sequence complementary to one of the identified identical portions.

D2. The method of embodiment D1, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

D3. The method of embodiment D1, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

D4. The method of embodiment D1, wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

D5. The method of any of the preceding embodiments, further comprising synthesizing the designed guide RNA.

D6. The method of any of the preceding embodiments, wherein the off-target polynucleotide is identified by an algorithm to predict off-target sites such as those found at http://www.rgenome.net/Cas-OFFinder; https://cm.jefferson.edu/Off-Spotter; or http://crispr.mit.edu, or other technique for identifying and quantifying the activation of off-target sites in actual cases, such as disclosed in Tsai et al. (2015) Nat. Biotechnol. 33, 187–97; Ran et al. (2015) Nature 520, 186–91; and/or Frock et al. (2015) Nat. Biotechnol. 33, 179–86.

D7. The method of any of the preceding embodiments, further comprising:

identifying at least one distinguishing nucleotide position between the target polynucleotide and the off-target polynucleotide, wherein the target polynucleotide and the off-target polynucleotide have a different nucleotide at the at least one distinguishing position, and

including in the synthetic guide RNA a nucleotide matching the nucleotide at the at least one distinguishing position in the target polynucleotide.

D8. The method of any of the preceding embodiments, wherein the specificity-enhancing modification lowers the melting temperature ("Tm") of a first DNA/RNA duplex formed by the guide

sequence of the synthetic guide RNA and the target polynucleotide, relative to the Tm of a duplex without the specificity-enhancing modification.

D9. The method of embodiment D8, wherein the specificity-enhancing modification lowers the Tm of the first DNA/RNA duplex by about 0.5°C per modification, alternatively by about 0.5–1°C per modification, alternatively by about 1–2°C per modification, alternatively by about 2–8°C per modification.

D10. The method of embodiment D8, wherein the specificity-enhancing modification lowers the Tm of the first DNA/RNA duplex by at least about 1°C, at least about 2°C, at least about 3°C, at least about 4°C, at least about 5°C, and/or up to about 6°C, alternatively up to about 8°C, alternatively up to about 10°C, alternatively up to about 13°C, for example by lowering the Tm from about 1°C to about 13°C, alternatively from about 1°C to about 6°C.

D11. The method of embodiments D8, wherein the specificity-enhancing modification lowers the Tm of a second DNA/RNA duplex formed by the guide sequence of the synthetic guide RNA and the at least one off-target polynucleotide.

D12. The method of embodiments D8, wherein the Tm of the first DNA/RNA duplex is higher than the Tm of the second DNA/RNA duplex, for example at least at least about 0.5°C higher, alternatively at least about 1°C higher.

E1. A method for cleaving, nicking or binding a target polynucleotide comprising:

selecting a target polynucleotide in a genome;

providing and/or designing a synthetic guide RNA comprising;

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence includes a specificity-enhancing modification;

calculating melting temperature (Tm) of a DNA/RNA duplex of the guide sequence and the target polynucleotide; and

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA;

contacting the target polynucleotide with a gRNA:Cas protein complex at a temperature within 10°C of the Tm, alternatively within 5°C of the Tm, alternatively at approximately the Tm; and

cleaving, nicking or binding the target polynucleotide by the contacting.

F1. A method for cleaving, nicking or binding a target polynucleotide comprising:

selecting a target polynucleotide in a genome;

providing and/or designing a synthetic guide RNA comprising;

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence includes at least one specificity-enhancing modification; selecting said modification or combination of modifications so that melting temperature (Tm) of the DNA/RNA duplex of the guide sequence and the target polynucleotide is between 25°C and 49°C;

forming a gRNA:Cas protein complex comprising a Cas protein and the designed synthetic guide RNA;

contacting the target polynucleotide with a gRNA:Cas protein complex at a temperature within 12°C of the Tm, alternatively within 8°C of the Tm, alternatively within 5°C of the Tm, alternatively at approximately the Tm; and

cleaving, nicking or binding the target polynucleotide.

G1. A method for cleaving, nicking or binding a target polynucleotide comprising:

selecting a target polynucleotide in a genome;

providing and/or designing a synthetic guide RNA comprising;

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence includes at least one specificity-enhancing modification;

selecting or including in the guide sequence said modification or combination of modifications so that the melting temperature (Tm) of the DNA/RNA duplex of the guide sequence and the target polynucleotide is at least 0.5–1°C lower than the Tm of the unmodified gRNA/target duplex, or at least 1–3°C lower or at least 1–12°C lower; and

forming a gRNA:Cas protein complex comprising a Cas protein and the designed synthetic guide RNA;

contacting the target polynucleotide with a gRNA:Cas protein complex at a temperature within 12°C of the Tm, alternatively within 8°C of the Tm, alternatively within 5°C of the Tm, alternatively at approximately the Tm; and

cleaving, nicking or binding the target polynucleotide.

EFG2. The method of any of the preceding embodiments E1, F1 or G1, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

EFG3. The synthetic guide RNA of any of embodiments E1, F1 or G1,wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

EFG4. The synthetic guide RNA of any of embodiments E1, F1 or G1,wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

H1. The method of any of the preceding embodiments, wherein the cleaving, nicking or binding takes place *in vitro*.

H2. The method of any of the preceding embodiments, wherein the cleaving, nicking or binding takes place in a cell.

H3. The method of embodiment H2, wherein the cell is isolated from a multicellular source prior to contacting the target polynucleotide with the gRNA:Cas protein complex.

H4. The method of embodiment H3, wherein the source is a plant, an animal, a multicellular protist, or a fungus.

H5. The method of any one of embodiments H2 to H4, wherein the cell, or a cell derived therefrom, is returned to the source after contacting the target polynucleotide with the gRNA:Cas protein complex.

H6. The method of any of the preceding embodiments, wherein the cleaving, nicking or binding takes place *in vivo*.

H7. The method of any one of embodiments any of of the preceding embodiments, wherein the Cas protein is Cas9.

H8. The method of any of the preceding embodiments, wherein the cleaving or nicking results in gene editing.

H9. The method of any of the preceding embodiments, wherein the cleaving, nicking or binding results in alteration of gene expression.

H10. The method of any of the preceding embodiments, wherein the cleavage, nicking or binding results in a functional knockout of a target gene.

H11. The method of any of the preceding embodiments, further comprising repairing the cleaved target polynucleotide by homology-directed repair with an exogenous or endogenous template polynucleotide.

H12. The method of embodiment H11, wherein the exogenous or endogenous template polynucleotide comprises at least one sequence having substantial sequence identity with a sequence on either side of the cleavage site.

H13. The method of any of the preceding embodiments, further comprising repairing the cleaved target polynucleotide by non-homologous end joining.

H14. The method of any of the preceding embodiments, wherein the repairing step produces an insertion, deletion, or substitution of one or more nucleotides of the target polynucleotide.

H15. The method of embodiment H14, wherein the insertion, deletion, or substitution results in one or more amino acid changes in a protein expressed from a gene comprising the target polynucleotide.

I1. A set or library of synthetic guide RNA molecules comprising two or more synthetic guide RNAs of any of the preceding embodiments.

J1. A kit comprising a synthetic guide RNA of any of the preceding embodiments, and one or more other components.

K1. An array of RNA molecules comprising two or more synthetic guide RNAs of any of the preceding embodiments.

L1. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, comprising a single RNA strand that comprises both the crRNA segment and the tracrRNA segment.

L2. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, comprising two RNA strands, and the crRNA segment and the tracrRNA segment are in different RNA strands.

L3. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA is a single-guide RNA, wherein the crRNA segment and the tracrRNA segment are linked through a loop L.

L4. The synthetic guide RNA, method, set or library, kit or array of embodiment L3, wherein the loop L comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides.

L5. The synthetic guide RNA, method, set or library, kit or array of embodiments L3 or L4, wherein the loop L comprises a nucleotide sequence of GNRA, wherein N represents A, C, G, or U and R represents A or G.

L6. The synthetic guide RNA, method, set or library, kit or array of any of embodiments L3 to L5, wherein the loop L comprises a nucleotide sequence of GAAA.

L7. The synthetic guide RNA, method, set or library, kit or array of any of embodiments L3 to L6, wherein the loop L comprises one or more modified nucleotides.

L8. The synthetic guide RNA, method, set or library, kit or array of any of embodiments L3 to L7, wherein the loop L comprises a fluorescent dye.

L9. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, comprising one or more isotopic labels.

L10. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, comprising one or more fluorescent labels.

L11. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-methyl-3’-phosphonoacetate (2′-O-methyl-3’-PACE) nucleotide.

L12. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2-thioU.

L13. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 6-thioG.

L14. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2-thioC.

L15. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-deoxy-2′-fluoroarabinofuranosyl modification.

L16. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-deoxy-2′-fluororibofuranosyl modification.

L17. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-phenyl ribose.

L18. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-thiophenyl ribose.

L19. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-S-thiophenyl ribose.

L20. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-methyl ribose.

L21. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-ethyl ribose.

L22. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-propyl ribose.

L23. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-allyl ribose.

L24. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-allylphenyl ribose.

L25. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-methylhydroxy ribose.

L26. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-methyloxymethyl ribose.

L27. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-carbamate ribose.

L28. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-ethylamino ribiose.

L29. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-allylamino ribose.

L30. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-propylamino ribose.

L31. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the synthetic guide RNA comprises at least one 2′-O-substituted phenyl ribose.

L32. The synthetic guide RNA, method, set or library, kit or array of any of the preceding embodiments, wherein the guide sequence of the synthetic guide RNA consists of nucleotides 1 through 20-N, counted from the 5′ end of the guide sequence, where N is an integer between -10 and 10 (optionally between -10 and 6), and the region of nucleotides 6-N through 14-N comprises the specificity-enhancing modification(s).

M1. A method for cleaving, nicking or binding a target *HBB* polynucleotide comprising:

selecting a target sequence in an *HBB* locus within a target polynucleotide;

providing a synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target *HBB* polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, and the guide sequence comprises at least one specificity-enhancing modification at a nucleotide selected from positions 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, and 16–N; and

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA;

contacting the target *HBB* polynucleotide with the gRNA:Cas protein complex; and

cleaving, nicking or binding the target polynucleotide.

M2. The method of embodiment M1, wherein the at least one specificity-enhancing modification is at nucleotide 11-N.

M3. The method of any one of embodiments M1 or M2, wherein the at least one specificity-enhancing modification is at nucleotide 5-N.

M4. The method of any one of embodiments M1 to M3, wherein the at least one specificity-enhancing modification is at nucleotide 7-N.

M5. The method of any one of embodiments M1 to M4, wherein the at least one specificity-enhancing modification is at nucleotide 10-N.

M6. The method of any one of embodiments M1 to M5, wherein the at least one specificity-enhancing modification is at nucleotide 9-N.

M7. The method of any one of embodiments M1 to M6, wherein the at least one specificity-enhancing modification is at nucleotide 4-N.

M8. The method of any one of embodiments M1 to M7, wherein the at least one specificity-enhancing modification comprises modification with 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

M9. The method of any one of embodiments M1 to M7, wherein the at least one specificity-enhancing modification is selected from a 2′-modification that confers a C3′-endo sugar pucker, a phosphonoacetate or thiophosphonoacetate linkage modification, and a combination thereof.

M10. The method of embodiment M9, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

M11. The method of any one of embodiments M1 to M10, wherein the cleaving, nicking or binding takes place *in vitro*.

M12. The method of any one of embodiments M1 to M10, wherein the cleaving, nicking or binding takes place in a cell.

M13. The method of any one of embodiments M1 to M12, wherein the guide sequence further comprises a modification at its 5’end, 3’end, or both, optionally one or more stability-enhancing modifications selected from 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2'-O-methyl-3'-phosphorothioate (MS), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), 2′-fluoro-3’-phosphonoacetate (FP), 2′-fluoro-3’-thiophosphonoacetate (FSP), 2'-fluoro-3'-phosphorothioate (FS), or a combination thereof.

M14. The method of any one of embodiments M1 to M13, wherein the Cas protein is Cas9 or Cpf1.

M15. The method of any one of embodiments M1 to M14, wherein the guide RNA is a synthetic single guide RNA.

M16. The method of any one of embodiments M1 to M15, wherein the method involves cleaving the target *HBB* polynucleotide.

M17. The method of any one of embodiments M1 to M15, wherein the method involves nicking the target *HBB* polynucleotide.

M18. The method of any one of embodiments M1 to M15, wherein the method involves binding the target *HBB* polynucleotide.

M19. The method of any of embodiments M1 to M18, wherein the at least one specificity-enhancing modification increases specificity of the method.

N1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target *HBB* polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, and the guide sequence comprises at least one specificity-enhancing modification at a nucleotide selected from positions 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, and 16–N, and wherein the synthetic guide RNA has gRNA functionality.

N2. The synthetic guide RNA of embodiment N1, wherein the at least one specificity-enhancing modification is at nucleotide 11-N.

N3. The synthetic guide RNA of embodiment N1 or N2, wherein the at least one specificity-enhancing modification is at nucleotide 5-N.

N4. The synthetic guide RNA of any one of embodiments N1 to N3, wherein the at least one specificity-enhancing modification is at nucleotide 7-N.

N5. The synthetic guide RNA of any one of embodiments N1 to N4, wherein the at least one specificity-enhancing modification is at nucleotide 10-N.

N6. The synthetic guide RNA of any one of embodiments N1 to N5, wherein the at least one specificity-enhancing modification is at nucleotide 9-N.

N7. The synthetic guide RNA of any one of embodiments N1 to N6, wherein the at least one specificity-enhancing modification is at nucleotide 4-N.

N8. The synthetic guide RNA of any one of embodiments N1 to N7, wherein the at least one specificity-enhancing modification comprises 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-

thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

N9. The synthetic guide RNA of any one of embodiments N1 to N8, wherein the at least one specificity-enhancing modification is selected from a 2’-modification that confers a C3′-endo sugar pucker, a phosphonoacetate or thiophosphonoacetate linkage modification, and a combination thereof.

N10. The synthetic guide RNA of embodiment N9, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

N11. The synthetic guide RNA of any one of embodiments N1 to N10, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target *HBB* polynucleotide.

N12. The synthetic guide RNA of any one of embodiments N1 to N10, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

N13. The synthetic guide RNA of any one of embodiments N1 to N10, wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target *HBB* polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

N14. The synthetic guide RNA of any one of embodiments N1 to N13, wherein the guide RNA is a synthetic single guide RNA.

N15. The method of any one of embodiments N1 to N14, wherein the guide sequence consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence.

O1. A method for cleaving, nicking or binding a target polynucleotide comprising:

selecting a target polynucleotide;

providing a synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, and the guide sequence comprises at least one specificity-enhancing modification at a nucleotide selected from positions 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, and 16–N; and

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA;

contacting the target polynucleotide with the gRNA:Cas protein complex ; and

cleaving, nicking or binding the target polynucleotide.

O2. The method of embodiment O1, wherein the target polynucleotide is selected from the group consisting of *VEGFA* polynucleotide, *IL2RG* polynucleotide, *CLTA1* polynucleotide, and a *CLTA4* polynucleotide.

O3. The method of embodiment O1 or O2, wherein the at least one specificity-enhancing modification at nucleotide 11-N.

O4. The method of any one of embodiments O1 to O3, wherein the at least one specificity-enhancing modification at nucleotide 5-N.

O5. The method of any one of embodiments O1 to O4, wherein the at least one specificity-enhancing modification is at nucleotide 7-N.

O6. The method of any one of embodiments O1 to O5, wherein the at least one specificity-enhancing modification is at nucleotide 10-N.

O7. The method of any one of embodiments O1 to O6, wherein the at least one specificity-enhancing modification is at nucleotide 9-N.

O8. The method of any one of embodiments O1 to O7, wherein the at least one specificity-enhancing modification is at nucleotide 4-N.

O9. The method of any of embodiments O1 to O8, wherein the at least one specificity-enhancing modification comprises modification with 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

O10. The method of any of embodiments O1 to O8, wherein the at least one specificity-enhancing modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification.

O11. The method of embodiment O10, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

O12. The method of any of embodiments O1 to O11, wherein the at least one specificity-enhancing modification increases specificity of the method.

O13. The method of any of embodiments O1 to O12, wherein the cleaving, nicking or binding takes place *in vitro*.

O14. The method of any of embodiments O1 to O12, wherein the cleaving, nicking or binding takes place in a cell.

O15. The method of any of embodiments O1 to O14, wherein the Cas protein is Cas9 or Cpf1.

O16. The method of any of embodiments O1 to O15, wherein the guide RNA is a synthetic single guide RNA.

O17. The method of any of embodiments O1 to O16, wherein the method involves cleaving the target *HBB* polynucleotide.

O18. The method of any of embodiments O1 to O16, wherein the method involves nicking the target *HBB* polynucleotide.

O19. The method of any of embodiments O1 to O16, wherein the method involves binding the target *HBB* polynucleotide.

O20. The method of any of embodiments O1 to O19, wherein the guide sequence consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence.

P1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, and the guide sequence comprises at least one specificity-enhancing modification at a nucleotide selected from positions 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, and 16–N, and wherein the synthetic guide RNA has gRNA functionality.

P2. The synthetic guide of embodiment P1, wherein the target polynucleotide is selected from the group consisting of VEGFA polynucleotide, IL2RG polynucleotide, CLTA1 polynucleotide, and a CLTA4 polynucleotide.

P3. The synthetic guide RNA of embodiment P1 or P2, wherein the at least one specificity-enhancing modification is at nucleotide 11-N.

P4. The synthetic guide RNA of any one of embodiments P1 to P3, wherein the at least one specificity-enhancing modification is at nucleotide 5-N.

P5. The synthetic guide RNA of any one of embodiments P1 to P4, wherein the at least one specificity-enhancing modification is at nucleotide 7-N.

P6. The synthetic guide RNA of any one of embodiments P1 to P5, wherein the at least one specificity-enhancing modification is at nucleotide 10-N.

P7. The synthetic guide RNA of any one of embodiments P1 to P6, wherein the at least one specificity-enhancing modification is at nucleotide 9-N.

P8. The synthetic guide RNA of any one of embodiments P1 to P7, wherein the at least one specificity-enhancing modification is at nucleotide 4-N.

P9. The synthetic guide RNA of any one of embodiments P1 to P8, wherein the specificity-enhancing modifications comprise 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

P10. The synthetic guide RNA of any one of embodiments P1 to P9, wherein the at least one specificity-enhancing modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification.

P11. The synthetic guide RNA of embodiment P10, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

P12. The synthetic guide RNA of any one of embodiments P1 to P11, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

P13. The synthetic guide RNA of any one of embodiments P1 to P12, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

P14. The synthetic guide RNA of any one of embodiments P1 to P13, wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

P15. The synthetic guide RNA of any one of embodiments, P1 to P14, wherein the guide RNA is a synthetic single guide RNA.

P16. The synthetic guide RNA of any one of embodiments P1 to P15, wherein the guide sequence consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence.

Q1. A method for cleaving, nicking or binding a target polynucleotide comprising:

selecting a target polynucleotide in a genome;

providing a synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence comprises at least two consecutive specificity-enhancing modifications; and

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA;

contacting the target polynucleotide with a gRNA:Cas protein complex; and

cleaving, nicking or binding the target polynucleotide,

wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

Q2. The method of embodiment Q1, wherein the guide sequence comprises modifications at nucleotide 1 and 2 counted from a 5'-end of the guide sequence.

Q3. The method of embodiment Q1, wherein the guide sequence comprises modifications at nucleotide 1, 2, and 3 counted from a 5'-end of the guide sequence.

Q4. The method of embodiment Q1, wherein the guide sequence comprises modifications at nucleotides 1, 2, 3, and 4 counted from a 5'-end of the guide sequence.

Q5. The method of embodiment Q1, wherein the guide sequence comprises modifications at nucleotides 1, 2, 3, 4, and 5 counted from a 5'-end of the guide sequence.

Q6. The method of embodiment Q1, wherein the guide sequence comprises consecutive specificity-enhancing modifications at a 5′ end.

Q7. The method of embodiment Q1, wherein the consecutive specificity-enhancing modifications begin at nucleotide 1, 2, 3, or 4 counted from a 5'-end of the guide sequence.

Q8. The method of any one of embodiments Q1 to Q7, wherein the target polynucleotide is selected from the group consisting of a *HBB* polynucleotide, a *VEGFA* polynucleotide, an *IL2RG* polynucleotide, a *CLTA1* polynucleotide, and a *CLTA4* polynucleotide.

Q9. The method of any one of embodiments Q1 to Q8, wherein the specificity-enhancing modifications comprise 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

Q10. The method of any one of embodiments Q1 to Q8, wherein the at least one specificity-enhancing modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification.

Q11. The method of embodiment Q10, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

Q12. The method of any one of embodiments Q1 to Q11, wherein the cleaving, nicking or binding takes place *in vitro*.

Q13. The method of any one of embodiments Q1 to Q11, wherein the cleaving, nicking or binding takes place in a cell.

Q14. The method of any one of embodiments Q1 to Q13, wherein the method involves cleaving the target *HBB* polynucleotide.

Q15. The method of any one of embodiments Q1 to Q13, wherein the method involves nicking the target *HBB* polynucleotide.

Q16. The method of any one of embodiments Q1 to Q13, wherein the method involves binding the target *HBB* polynucleotide.

Q17. The method of any one of embodiments Q1 to Q16, wherein the guide RNA is a synthetic single guide RNA.

Q18. The method of any one of embodiments Q1 to Q17, wherein the guide sequence comprises or consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence.

R1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence comprises at least two consecutive specificity-enhancing modifications, and wherein the synthetic guide RNA has gRNA functionality, and

wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

R2. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises specificity-enhancing modifications at nucleotide 1 and 2 counted from a 5'-end of the guide sequence.

R3. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises specificity-enhancing modifications at nucleotide 1, 2, and 3 counted from a 5'-end of the guide sequence.

R4. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises specificity-enhancing modifications at nucleotides 1, 2, 3, and 4 counted from a 5'-end of the guide sequence.

R5. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises specificity-enhancing modifications at nucleotides 1, 2, 3, 4, and 5 counted from a 5'-end of the guide sequence.

R6. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises consecutive specificity-enhancing modifications at the 5′ end counted from a 5'-end of the guide sequence.

R7. The synthetic guide RNA of embodiment R1, wherein the consecutive specificity-enhancing modifications begin at nucleotide 1, 2, 3, or 4 counted from a 5'-end of the guide sequence.

R8. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises three consecutive specificity-enhancing modifications.

R9. The synthetic guide RNA of embodiment R1, wherein the guide sequence comprises four consecutive specificity-enhancing modifications.

R10. The synthetic guide RNA of any one of embodiments R1 to R9, wherein the target polynucleotide is selected from the group consisting of a *HBB* polynucleotide, a *VEGFA* polynucleotide, an *IL2RG* polynucleotide, a *CLTA1* polynucleotide, and a *CLTA4* polynucleotide.

R11. The synthetic guide RNA of any one of embodiments R1 to R10, wherein the specificity-enhancing modifications comprise 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

R12. The synthetic guide RNA of any one of embodiments R1 to R10, wherein the at least one specificity-enhancing modification is selected from a 2’-modification that confers a C3′-endo sugar pucker, a phosphonoacetate or thiophosphonoacetate linkage modification, and a combination thereof.

R13. The synthetic guide RNA of embodiment R12, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

R14. The synthetic guide RNA of any one of embodiments R1 to R13, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and the target polynucleotide.

R15. The synthetic guide RNA of any one of embodiments R1 to R13, wherein the at least one specificity-enhancing modification weakens hybridization between the guide sequence and an off-target polynucleotide.

R16. The synthetic guide RNA of any one of embodiments R1 to R13, wherein the at least one specificity-enhancing modification strengthens hybridization between the guide sequence and the target polynucleotide and weakens hybridization between the guide sequence and an off-target polynucleotide.

R17. The synthetic guide RNA of any one of embodiments R1 to R16, wherein the guide RNA is a synthetic single guide RNA.

R18. The method of any one of embodiments R1 to R17, wherein the guide sequence comprises or consists of nucleotides 1 through 20, counted from the 5′ end of the guide sequence.

S1. A method of selecting a synthetic guide RNA comprising:

providing at least a first synthetic guide RNA and a second synthetic guide RNA, each comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein each of the guide sequences consists of 20-N nucleotides, where N is an integer between -10 and 10, counted from the 5′ end of the guide sequence,

wherein the first synthetic guide RNA comprises a specificity-enhancing modification at a first position within the guide sequence, and the second synthetic guide RNA comprises a specificity-enhancing modification at a second position within the guide sequence;

forming a first gRNA:Cas protein complex comprising a Cas protein and the first synthetic guide RNA, contacting the target polynucleotide with the first gRNA:Cas protein complex, and cleaving, nicking or binding the target polynucleotide;

forming a second gRNA:Cas protein complex comprising a Cas protein and the second synthetic guide RNA, contacting the target polynucleotide with the second gRNA:Cas protein complex, and cleaving, nicking or binding the target polynucleotide;

determining the specificity of the first gRNA:Cas protein complex and the second gRNA:Cas protein complex in the cleaving, nicking or binding of the target polynucleotide;

identifying which of the first gRNA:Cas protein complex and the second gRNA:Cas protein complex has greater specificity for the target polynucleotide.

S2. The method of embodiment S1, wherein the specificity-enhancing modification comprises 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), or a combination thereof.

S3. The method of embodiment S2, wherein the specificity-enhancing modification comprises 2′-O-methyl-3’-phosphonoacetate (MP) or 2′-O-methyl-3’-thiophosphonoacetate (MSP).

S4. The method of embodiment S1, wherein the at least one specificity-enhancing modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification.

S5. The method of embodiment S4, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

S6. The method of any one of embodiments S1 to S5, wherein the first and second synthetic guide RNA comprise a specificity-enhancing modification at different nucleotides.

S7. The method of any one of embodiments S1 to S6, wherein the specificity is selected from ON and/or OFF target cleaving, binding, or nicking percentages, ON:OFF ratio, specificity score, or a combination thereof.

S8. The method of any one of embodiments S1 to S7, wherein the method comprises providing a first through twentieth synthetic guide RNA comprising a specificity-enhancing modification at different nucleotide positions in the guide sequence portions, forming a gRNA:Cas protein complex using each of the synthetic guide RNAs, contacting the target polynucleotide with the gRNA:Cas protein complex, cleaving, nicking or binding the target polynucleotide and measuring the specificity of each synthetic guide RNA, and identifying one or more modified positions that provide the greatest specificity enhancement.

S9. The method of any one of embodiments S1 to S8, wherein the guide sequence further comprises modification with 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2'-O-methyl-3'-phosphorothioate (MS), 2′-deoxy-3’-phosphonoacetate (DP), 2′-deoxy-3’-thiophosphonoacetate (DSP), 2′-fluoro-3’-phosphonoacetate (FP), 2′-fluoro-3’-thiophosphonoacetate (FSP), 2'-fluoro-3'-phosphorothioate (FS), or a combination thereof at the 5′ end and the 3′ end.

S10. The method of any one of embodiments S1 to S9, wherein the method involves cleaving a target *HBB* polynucleotide.

S11 The method of any one of embodiments S1 to S9, wherein the method involves nicking a target *HBB* polynucleotide.

S12. The method of any one of embodiments S1 to S9, wherein the method involves binding a target *HBB* polynucleotide.

S13. The method of any one of embodiments S1 to S12, wherein the first and second guide RNAs are a synthetic single guide RNA.

T1. A kit for selecting a synthetic guide RNA comprising:

at least two synthetic guide RNAs comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, counted from the 5′ end of the guide sequence, and comprises at least one specificity-enhancing modification at a nucleotide in the guide sequence; and wherein the at least two synthetic guide

RNAs differ from each other by having at least one different specificity-enhancing modification or by having the specificity-enhancing modification at least one different position in the guide sequence; and

a Cas protein or a polynucleotide encoding said Cas protein.

T2. The kit of embodiment T1, wherein the kit comprises at least twenty synthetic guide RNAs.

T3. The kit of embodiment T1 or T2, wherein the Cas protein is Cas9 or Cpf1.

T4. The kit of any one of embodiments T1 to T3, wherein the specificity-enhancing modification comprises 2′-O-methyl-3’-phosphonoacetate (MP), 2′-O-methyl-3’-thiophosphonoacetate (MSP), 2′-deoxy-3’-phosphonoacetate (DP), or 2′-deoxy-3’-thiophosphonoacetate (DSP).

T5. The kit of any one of embodiments T1 to T3, wherein the specificity-enhancing modification comprises a 2’-modification that confers a C3′-endo sugar pucker and a phosphonoacetate or thiophosphonoacetate linkage modification.

T6. The kit of embodiment T5, wherein the 2′-modification is selected from 2′-F and 2′-O-(2-methoxyethyl).

T7. The kit of any one of embodiments T1 to T6, wherein the guide RNAs are synthetic single guide RNAs.

U1. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has a specificity score of greater than 1, preferably at least 1.1, more preferably at least 1.5, even more preferably at least 2, even more preferably at least 5, even more preferably at least 10, or optimally at least 20.

U2. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has a specificity score of from about 2 to about 60 or preferably about 10 to about 60.

U3. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has an ON target cleavage of at least 30%, preferably at least 50%, more preferably at least 70%, or optimally at least 90%.

U4. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has an ON target cleavage of from about 25% to 99.9% or preferably from about 50% to about 99.9%.

U5. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has a ON:OFF ratio of greater than 1, preferably at least 1.1:1, more preferably at least 1.5:1, even more preferably at least 3:1, even more preferably at least 10:1, even more preferably at least 20:1, or optimally at least 40:1.

U6. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the gRNA:Cas protein complex comprises a specificity-enhancing modification and has a ON:OFF ratio of from about 1.5:1 to about 99.9:1 or preferably from about 10:1 to about 99.9:1.

W1. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 19, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at one of nucleotides selected from positions 3, 4, 6, 8, 9, and 10.

W2. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 18, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at one of nucleotides selected from positions 2, 3, 5, 7, 8, and 9.

W3. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 17, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at one of nucleotides selected from positions 1, 2, 4, 6, 7, and 8.

W4. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 16, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at one of selected from positions nucleotides 1, 3, 5, 6, and 7.

W5. The synthetic guide RNA, method, or kit any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 15, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at one of nucleotides selected from positions 2, 4, 5, and 6.

W6. The synthetic guide RNA, method, or kit of any of the preceding embodiments, wherein the guide sequence consists of nucleotides 1 through 14, counted from the 5' end of the guide sequence, and comprises at least one chemical modification at a nucleotide selected from positions 1, 3, 4, or 5.

In the exemplary embodiments below, "X embodiments" means all the embodiments of which the numbers start with an X. Likewise, in the exemplary embodiments below, "Xn embodiments" means all the embodiments of which the numbers start with Xn. By way of example, "X9 embodiments" as recited in any claim means "X9", and "X9a" up to "X9z" if existing.

X. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, wherein the target polynucleotide comprises a target sequence adjacent to a PAM site, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10;

wherein the guide sequence comprises at least one modification.

X1. A synthetic crRNA comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, wherein the target polynucleotide comprises a target sequence adjacent to a PAM site, and (ii) a stem sequence;

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10;

wherein the guide sequence comprises at least one modification.

X1a. The synthetic guide RNA or crRNA of embodiment X or X1, wherein the at least one modification comprises a modification at position 1-N of the guide sequence.

X1b. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 2-N of the guide sequence.

X1c. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 3-N of the guide sequence

X2. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 4-N of the guide sequence.

X3. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 5-N of the guide sequence.

X3a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 6-N of the guide sequence.

X4. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 7-N of the guide sequence.

X4a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 8-N of the guide sequence.

X5. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 9-N of the guide sequence.

X6. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 10-N of the guide sequence.

X7. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 11-N of the guide sequence.

X7a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 12-N of the guide sequence.

X7b. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 13-N of the guide sequence.

X7c. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 14-N of the guide sequence.

X7d. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 15-N of the guide sequence.

X7e. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 16-N of the guide sequence.

X7f. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 17-N of the guide sequence.

X7g. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 18-N of the guide sequence.

X7h. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 19-N of the guide sequence.

X7i. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modification at position 20-N of the guide sequence.

X8. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a phosphonocarboxylate internucleotide linkage, optionally a phosphonoacetate internucleotide linkage (P).

X8a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a phosphonocarboxylate ester internucleotide linkage, optionally a phosphonoacetate ester internucleotide linkage.

X8b. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a phosphonopropionate internucleotide linkage.

X9. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a thiophosphonocarboxylate internucleotide linkage, optionally a thiophosphonoacetate internucleotide linkage (SP).

X9a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a thiophosphonopropionate internucleotide linkage.

X9b. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a thiophosphonocarboxylate ester internucleotide linkage, optionally a thiophosphonoacetate ester internucleotide linkage.

X9c. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a phosphorothioate internucleotide linkage.

X9d. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a chiral phosphorothioate internucleotide linkage.

X9e. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a phosphorodithioate internucleotide linkage.

X9f. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a boranophosphonate intenucleotide linkage.

X9g. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a C_{1-4} alkyl phosphonate internucleotide linkage.

X9h. The synthetic guide RNA or crRNA of the X9g embodiment, wherein the at least one modification comprises a methylphosphonate internucleotide linkage.

X9i. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a methylthiophosphonate internucleotide linkage.

X10. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modified sugar.

X10a. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-deoxyribose (2’-deoxy).

X10b. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-NH_2.

X10c. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-arabinofuranosyl (2’-arabino).

X10d. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-deoxy-2′-fluoroarabinofuranosyl (2’-F-arabino).

X10e. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-LNA.

X10f. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-ULNA.

X10g. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 4'-thioribosyl.

X10h. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-O-C_{1-4}alkyl.

X10i. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl.

X10j. The synthetic guide RNA or crRNA of embodiment X10, wherein said at least one modification is selected from 2′-O-phenyl, 2′-thiophenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino, and 2′-O-substituted phenyl.

X10k. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a 2'-modification that confers a C3'-endo sugar pucker configuration.

X11. The synthetic guide RNA or crRNA of embodiment X10k, wherein said 2'-modification is selected from 2'-O-methyl, 2'-fluoro, and 2'-O-(2-methoxyethyl).

X11a. The synthetic guide RNA or crRNA of embodiment X11, wherein said 2'-modification is 2'-O-methyl.

X11b. The synthetic guide RNA or crRNA of embodiment X11, wherein said 2'-modification is 2′-deoxy-2′-fluororibofuranosyl (2'-fluoro).

X11c. The synthetic guide RNA or crRNA of embodiment X11, wherein said 2'-modification is 2'-O-(2-methoxyethyl).

X12. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a 2'-O-methyl-3'-phosphonoacetate (MP).

X13. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a 2'-O-methyl-3'-thiophosphonoacetate (MSP).

X14. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a 2'-O-deoxy-3'-phosphonoacetate (DP).

X15. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a 2'-O-deoxy-3'-thiophosphonoacetate (DSP).

X16. The synthetic guide RNA or crRNA of embodiment X or X1, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at any position from position 4-N to 20-N of the guide sequence, and wherein said modification is not at position 15-N of the guide sequence.

X16a. The synthetic guide RNA or crRNA of embodiment X or X1, wherein the at least one modification comprises an internucleotide linkage modification and wherein position 15–N does not comprise 2′-O-methyl-3′-phosphonoacetate (MP) or 2′-O-methyl-3′-thiophosphonoacetate (MSP).

X17. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X16a, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 4-N of the guide sequence.

X18. The synthetic guide RNA or crRNA of any one of embodiments X, X1, and X16 to X17, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 5-N of the guide sequence.

X19. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X18, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 6-N of the guide sequence.

X20. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X19, wherein the at least one modification comprises a phosphonocarboxylate or

thiophosphonocarboxylate internucleotide linkage modification at position 7-N of the guide sequence.

X21. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X20, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 8-N of the guide sequence.

X22. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X21, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 9-N of the guide sequence.

X23. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X22, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 10-N of the guide sequence.

X24. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X23, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 11-N of the guide sequence.

X25. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X24, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 12-N of the guide sequence.

X26. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X25, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 13-N of the guide sequence.

X27. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X26, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 14-N of the guide sequence.

X27a. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16a-X26, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 15-N of the guide sequence.

X28. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X27, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 16-N of the guide sequence.

X29. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X28, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 17-N of the guide sequence.

X30. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X29, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 18-N of the guide sequence.

X31. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X30, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 19-N of the guide sequence.

X32. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X31, wherein the at least one modification comprises a phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at position 20-N of the guide sequence.

X33. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X32, wherein the at least one modification comprises a phosphonocarboxylate internucleotide linkage.

X34. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X32, wherein the at least one modification comprises a thiophosphonocarboxylate internucleotide linkage.

X35. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X33, wherein said phosphonocarboxylate internucleotide linkage is a phosphonoacetate linkage (P).

X36. The synthetic guide RNA or crRNA of any one of embodiments X, X1 and X16-X33, wherein said phosphonocarboxylate internucleotide linkage is a thiophosphonoacetate linkage (SP).

X37. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, further comprising at least one modification at the 5'-end, 3'-end, or both ends of said guide RNA.

X38. The synthetic guide RNA or crRNA of embodiment X37, wherein said at least one modification at the 5'-end, the 3'-end, or both ends is independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphorothioate (MS), a 2'-O-methyl-3'-phosphosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphosphonoacetate (MSP) or a combination thereof.

X38a. The synthetic guide RNA or crRNA of embodiment X37, wherein said at least one modification at the 5'-end, the 3'-end, or both ends is independently selected from a a 2′-deoxy-3′-phosphonoacetate (DP), a 2′-O-deoxy-3′-thiophosphonoacetate (DSP), or a combination thereof.

X39. The synthetic guide RNA or crRNA of any one of embodiments X-X38a, wherein said target polynucleotide is located within an *HBB* polynucleotide.

X40. The synthetic guide RNA or crRNA of any one of embodiments X-X38a, wherein said target polynucleotide is located within an *IL2RG* polynucleotide.

X41. The synthetic guide RNA or crRNA of any one of embodiments X-X38a, wherein said target polynucleotide is located within a *VEGFA* polynucleotide.

X42. The synthetic guide RNA or crRNA of any one of embodiments X-X38a, wherein said target polynucleotide is located within a *CLTA1* polynucleotide.

X43. The synthetic guide RNA or crRNA of any one of embodiments X-X38a, wherein said target polynucleotide is located within a *CLTA4* polynucleotide.

X44. The synthetic guide RNA or crRNA of embodiment X39, wherein the target polynucleotide comprises GCCCCACAGGGCAGTAA.

X45. The synthetic guide RNA or crRNA of embodiment X40, wherein the target polynucleotide comprises TAATGATGGCTTCAACA.

X46. The synthetic guide RNA or crRNA of embodiment X41, wherein the target polynucleotide comprises GAGTGAGTGTGTGCGTG.

X47. The synthetic guide RNA or crRNA of embodiment X42, wherein the target polynucleotide comprises CCTCATCTCCCTCAAGC.

X48. The synthetic guide RNA or crRNA of embodiment X43, wherein the target polynucleotide comprises GATGTAGTGTTTCCACA.

X48a. The synthetic guide RNA or crRNA of any one of the preceding X embodiments, wherein the at least one modification comprises a modified base.

X48b. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 2-thioU, 2-thioC, 4-thioU, 6-thioG, 2-aminoA, 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylC, 5-methylU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-

allylU, 5-allylC, 5-aminoallylU, 5-aminoallylC, an abasic nucleotide, a UNA base, isoC, isoG, 5-methyl-pyrimidine, x(A,G,C,T,U), y(A,G,C,T,U), and combinations thereof.

X48c. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 2-thioU, 2-thioC, 4-thioU and 6-thioG.

X48d. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 2-aminoA and 2-aminopurine.

X48e. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from pseudouracil and hypoxanthine.

X48f. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is inosine.

X48g. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, and 7-deaza-8-azaadenine.

X48h. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 5-methyl-cytosine, 5-methyl-uracil and 5-methyl-pyrimidine.

X48i. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine and 5-ethynyluracil.

X48j. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from 5-allyluracil, 5-allylcytosine, 5-aminoallyluracil and 5-aminoallylcytosine.

X48k. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is an abasic nucleotide.

X48l. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is selected from isoC and isoG.

X48m. The synthetic guide RNA or crRNA of embodiment X48a, wherein the modified base is a UNA base.

X49. A method for enhancing the specificity of a CRISPR function, comprising:

selecting a target polynucleotide;

providing at least one synthetic guide RNA or crRNA of any one of the preceding X embodiments;

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA or crRNA; and

contacting the target polynucleotide with the gRNA:Cas protein complex under conditions to result in hybridization between the target polynucleotide and the guide RNA or crRNA, as well as performance of the CRISPR function.

X49a. A method for enhancing the specificity of a CRISPR function, comprising:

selecting a target polynucleotide;

providing at least one synthetic crRNA of any one of the preceding X embodiments;

forming a crRNA:Cas protein complex comprising a Cas protein and the synthetic crRNA; and

contacting the target polynucleotide with the crRNA:Cas protein complex.

X49b. A method for analyzing the specificity of a CRISPR function, the method comprising:

providing a sample comprising (1) a target polynucleotide comprising a target sequence; and (2) one or more off-target polynucleotides comprising off-target sequences;

providing at least one synthetic guide RNA or crRNA of any of the preceding X embodiments for the target sequence;

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA or crRNA, wherein said Cas protein is provided as a protein or a polynucleotide encoding said Cas protein;

contacting the sample with the gRNA:Cas protein complex under conditions to result in hybridization of said gRNA or crRNA to said target polynucleotide and performance of a CRISPR function;

adding a library of oligonucleotide baits, the baits including baits designed to hybridize to the target polynucleotide and baits designed to hybridize to the one or more off-target polynucleotides, to capture the target polynucleotide and said one or more off-target polynucleotide;

isolating and analyzing said captured target polynucleotides and said captured off-target polynucleotides to identify changes caused by the CRISPR function, and determining the relative changes in the target polynucleotide and the off-target polynucleotides, thereby assessing the specificity of said CRISPR function.

X49c. The method of embodiment X49b, wherein said sample comprises two or more target polynucleotides comprising each a target sequence.

X49d. The method of embodiment X49b, wherein said guide RNA is a synthetic crRNA of any preceding X embodiments and said gRNA:Cas protein complex is crRNA:Cpf1 complex.

X50. The method of any one of the X49 embodiments, wherein the contacting is performed in a cell.

X51. The method of embodiment X50, wherein the cell is a primary cell.

X52. The method of any one of embodiments X49-51, wherein the Cas protein is provided as a protein or a polynucleotide expressing the Cas protein.

X52a. The method of any one of embodiments X49-52, wherein the Cas protein is Cas9.

X52b. The method of any one of embodiments X49-52, wherein the Cas protein is Cpf1.

X53. The method of any one of the X52 embodiments, wherein the polynucleotide is an mRNA.

X54. The method of any one of the preceding X embodiments, wherein said forming is performed outside of a cell.

X55. The method of any one of the preceding X embodiments, wherein the CRISPR function is gene editing.

X55a. The method of any one of the preceding X embodiments, wherein the CRISPR function is gene editing and further comprises providing a single-stranded or double-stranded polynucleotide as a donor template for homology-directed repair.

X56. The method of any one of the preceding X embodiments, wherein the CRISPR function is CRISPRa.

X57. The method of any one of the preceding X embodiments, wherein the CRISPR function is CRISPRi.

X57a. The method of any one of embodiments X55 to X57 that comprises analyzing captured polynucleotides, wherein the analyzing is performed by sequencing.

X57b. The method of embodiment X56 or X57 further comprising analyzing said CRISPR function by measuring gene expression levels, optionally by qRT-PCR.

X57c. The method of embodiment X57b wherein said measuring gene expression levels is performed on a microarray.

X58. The method of any one of the preceding X embodiments, wherein the CRISPR function is regulation of gene expression.

X59. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 0, and the guide sequence consists of 20 nucleotides.

X60. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 1, and the guide sequence consists of 19 nucleotides.

X61. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 2, and the guide sequence consists of 18 nucleotides.

X62. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 3, and the guide sequence consists of 17 nucleotides.

X63. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 4, and the guide sequence consists of 16 nucleotides.

X64. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals 5, and the guide sequence consists of 15 nucleotides.

X65. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals -1, and the guide sequence consists of 21 nucleotides.

X66. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals -2, and the guide sequence consists of 22 nucleotides.

X67. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals -3, and the guide sequence consists of 23 nucleotides.

X68. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals -4, and the guide sequence consists of 24 nucleotides.

X69. The synthetic guide RNA or crRNA or method of any one of embodiments X-X58, wherein N equals -5, and the guide sequence consists of 25 nucleotides.

X70. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 10 nucleotides in the guide sequence comprise modifications.

X71. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 9 nucleotides in the guide sequence comprise modifications.

X72. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 8 nucleotides in the guide sequence comprise modifications.

X73. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 7 nucleotides in the guide sequence comprise modifications.

X74. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 6 nucleotides in the guide sequence comprise modifications.

X75. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 5 nucleotides in the guide sequence comprise modifications.

X76. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 4 nucleotides in the guide sequence comprise modifications.

X77. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 3 nucleotides in the guide sequence comprise modifications.

X78. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein no more than 2 nucleotides in the guide sequence comprise modifications.

X79. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein, if the first 4 nucleotides of the guide sequence are all modified, they are not modified in an identical manner.

X80. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein, if the first 5 nucleotides of the guide sequence are all modified, they are not modified in an identical manner.

X81. The synthetic guide RNA or method of any one of the preceding X embodiments, wherein the guide RNA is a single guide RNA.

X82. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein the guide RNA has higher specificity for the target polynucleotide or higher gRNA functionality than a corresponding guide RNA without the modification.

X83. The method of any one of the preceding X embodiments, where at least two different synthetic guide RNAs are provided for two different target polynucleotides.

X84. The method of embodiment X83, wherein the two different target polynucleotides are located in the same gene.

X85. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein N is between -10 and 6, and the guide sequence consists of 14 to 30 nucleotides.

X86. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein N is between -5 and 6, and the guide sequence consists of 14 to 25 nucleotides.

X87. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein N is between -4 and 3, and the guide sequence consists of 17 to 24 nucleotides.

X88. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein the at least one modification comprises 2'-deoxy-3'-phosphonoacetate (DP).

X89. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, wherein the at least one modification comprises 2'-O-deoxy-3'-thiophosphonoacetate (DSP).

X90. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, comprising at least one modification at the 5'-end, the 3'-end, or both ends, which comprises 2'-deoxy-3'-phosphonoacetate (DP).

X91. The synthetic guide RNA or crRNA or method of any of the preceding X embodiments, comprising at least one modification at the 5'-end, the 3'-end, or both ends, which comprises 2'-O-deoxy-3'-thiophosphonoacetate (DSP).

Y1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, wherein the target polynucleotide comprises a target sequence adjacent to a PAM site, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, optionally between -10 and 6; and wherein the guide sequence further comprises at least one modification located at position 4-N, 5-N, 7-N, 9-N, 10-N, 11-N, 14–N, or 16–N of the guide sequence, or a combination thereof.

Y2. The synthetic guide RNA of embodiment Y1 wherein said guide RNA is a single guide RNA.

Y3. The synthetic guide RNA of embodiment Y1 wherein said at least one modification is selected from a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), and a 2'-modification that confers a C3'-endo sugar pucker configuration or a combination thereof.

Y4. The synthetic guide RNA of embodiment Y3 wherein said 2'-modification is selected from 2'-O-methyl, 2'-fluoro, and 2'-O-(2-methoxyethyl).

Y5. The synthetic guide RNA of embodiment Y1 wherein said at least one modification is selected from a 2'-O-methyl-3'-phosphonoacetate (MP) and 2'-O-methyl-3'-thiophosphonoacetate (MSP).

Y6. The synthetic guide RNA of embodiment Y5 wherein said at least one modification is located at position 5-N or 11-N of the guide sequence, or a combination thereof.

Y7. The synthetic guide RNA of embodiment Y1 further comprising at least one modification at the 5'-end, 3'-end, or both ends of said guide RNA.

Y8. The synthetic guide RNA of embodiment Y7 wherein said at least one modification at the 5'-end, the 3'-end, or both ends is independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage

(P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphorothioate (MS), a 2'-O-methyl-3'-phosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphonoacetate (MSP), or a combination thereof.

Y9. The synthetic guide RNA of embodiment Y5 further comprising at least one modification at the 5'-end, the 3'-end, or both ends independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphoroatioate (MS), a 2'-O-methyl-3'-phosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphonoacetate (MSP), or a combination thereof.

Y10. The synthetic guide RNA of embodiment Y5 wherein said target polynucleotide is located within the *HBB* gene, the *IL2RG* gene, or the *VEGFA* gene.

Y11. The synthetic guide RNA of embodiment Y10, wherein the target polynucleotide comprises GCCCCACAGGGCAGTAA of the *HBB* gene, TAATGATGGCTTCAACA of the *IL2RG* gene, or GAGTGAGTGTGTGCGTG of the *VEGFA* gene.

Y12. The synthetic guide RNA of embodiment Y10, wherein said guide RNA is a single guide RNA and wherein said at least one modification at the 5'-end, 3'-end or both ends of said guide RNA is independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphoroatioate (MS), a 2'-O-methyl-3'-phosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphonoacetate (MSP), or a combination thereof.

Y13. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, wherein the target polynucleotide comprises a target sequence adjacent to a PAM sequence, (ii) a stem sequence; and
(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20-N nucleotides, where N is an integer between -10 and 10, optionally between -10 and 6;
wherein the guide sequence further comprises at least one phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at any position from position 4-N to 20-N of the guide sequence, and wherein said modification is not at position 15-N of the guide sequence.

Y14. The synthetic guide RNA of embodiment Y13 wherein said phosphonocarboxylate internucleotide linkage is selected from a phosphonoacetate linkage (P) and a thiophosphonoacetate linkage (SP).

Y15. The synthetic guide RNA of embodiment Y13 further comprising at least one modification at the 5'-end, 3'-end or both ends of said guide RNA.

Y16. The synthetic guide RNA of embodiment Y13 wherein said guide RNA is a single guide RNA.

Y17. The synthetic guide RNA of embodiment Y15 wherein said at least one modification at the 5'-end, the 3'-end, or both ends is independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphoroatioate (MS), a 2'-O-methyl-3'-phosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphonoacetate (MSP), or a combination thereof.

Y18. A synthetic crRNA comprising a guide sequence capable of hybridizing to a target polynucleotide comprising a target sequence adjacent to a PAM site, wherein said guide sequence consists of 20-N nucleotides, wherein N is an integer between -10 and 10, optionally between -10 and 6; wherein the guide sequence further comprises at least one phosphonocarboxylate or thiophosphonocarboxylate internucleotide linkage modification at any position from position 4-N to 20-N of the guide sequence, and wherein said modification is not at position 15-N of the guide sequence.

Y19. The synthetic crRNA of embodiment Y18 further comprising at least one modification at the 5'-end, 3'-end or both ends of said crRNA.

Y20. A method for enhancing the specificity of a CRISPR function, comprising:

selecting a target polynucleotide;

providing at least one synthetic guide RNA of embodiment Y13;

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA; and

contacting the target polynucleotide with the gRNA:Cas protein complex;

wherein said Cas protein is provided as a protein or as a polynucleotide encoding said Cas protein.

Y21. The method of embodiment Y20, wherein said guide RNA is a single guide RNA.

Y22. The method of embodiment Y20, wherein said guide RNA further comprises at least one modification at the 5'-end, the 3'-end, or both ends of said guide RNA.

Y23. The method of embodiment Y22 wherein said at least one modification at the 5'-end, the 3'-end, or both ends is independently selected from a 2'-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2'-O-methyl-3'-phosphoroatioate (MS), a 2'-O-methyl-3'-phosphonoacetate (MP) and a 2'-O-methyl-3'-thiophosphonoacetate (MSP), or a combination thereof.

Y24. The method of embodiment Y20 wherein said guide RNA comprises at least one modification selected from a phosphonoacetate internucleotide linkage and a thiophosphonoacetate internucleotide linkage or a combination thereof.

Y25. The method of embodiment Y20 wherein said guide RNA comprises at least one modification selected from a 2'-O-methyl-3'-phosphonoacetate (MP) and 2'-O-methyl-3'-thiophosphonoacetate (MSP).

Y26. The method of embodiment Y20 wherein said contacting of said polynucleotide target with said gRNA:Cas protein complex is performed in a cell.

Y27. The method of embodiment Y26 wherein said Cas protein is a Cas9 protein.

Y28. The method of embodiment Y27, wherein said target polynucleotide is located within the *HBB* gene, the *IL2RG* gene, or the *VEGFA* gene.

Y29. The method of embodiment Y28, wherein the target polynucleotide comprises GCCCCACAGGGCAGTAA of the *HBB* gene, TAATGATGGCTTCAACA of the *IL2RG* gene, or GAGTGAGTGTGTGCGTG of the *VEGFA* gene.

Y30. The method of embodiment Y20 wherein said forming is performed outside of a cell.

The foregoing description of exemplary or preferred embodiments should be taken as illustrating, rather than as limiting, the present invention as defined by the claims. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended to be included within the scope of the following claims. All references cited herein are incorporated by reference in their entireties.

We claim:

1. A synthetic guide RNA comprising:

(a) a crRNA segment comprising (i) a guide sequence capable of hybridizing to a target polynucleotide, wherein the target polynucleotide comprises a target sequence adjacent to a PAM site, (ii) a stem sequence; and

(b) a tracrRNA segment comprising a nucleotide sequence that is partially or completely complementary to the stem sequence,

wherein the guide sequence consists of 20–N nucleotides, where N is an integer between –10 and 6; and wherein the guide sequence comprises at least one modification located at any position from 4–N to 20–N of the guide sequence.

2. The synthetic guide RNA of claim 1, wherein said guide RNA is a single guide RNA (sgRNA).

3. A synthetic crRNA comprising a guide sequence capable of hybridizing to a target polynucleotide comprising a target sequence adjacent to a PAM site, wherein said guide sequence consists of 20–N nucleotides, wherein N is an integer between –10 and 6; wherein the guide sequence comprises at least one modification located at any position from 4–N to 20–N of the guide sequence.

4. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein the at least one modification is located at position 4–N, 5–N, 7–N, 9–N, 10–N,11–N, 14–N, or 16–N.

5. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is located at position 5–N or 11–N of the guide sequence, or a combination thereof.

6. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is an internucleotide linkage modification, and wherein position 15–N does not comprise 2′-O-methyl-3′-phosphonoacetate (MP) or 2′-O-methyl-3′-thiophosphonoacetate (MSP).

7. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is selected from a phosphorothioate internucleotide linkage, a chiral phosphorothioate internucleotide linkage, a phosphorodithioate internucleotide linkage, a boranophosphonate intenucleotide linkage, a C_{1-4} alkyl phosphonate internucleotide linkage, a phosphonocarboxylate internucleotide linkage, a phosphonocarboxylate ester internucleotide linkage, a thiophosphonocarboxylate internucleotide linkage, and a thiophosphonocarboxylate ester internucleotide linkage.

8. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is selected from a phosphonocarboxylate internucleotide linkage, a thiophosphonocarboxylate internucleotide linkage, a modified base, and a 2′-modification that confers a C3′-endo sugar pucker configuration, or a combination thereof.

9. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is a phosphonoacetate internucleotide linkage (P) or thiophosphonoacetate internucleotide linkage (SP).

10. The synthetic guide RNA or crRNA of claim 8, wherein said 2′-modification is selected from 2′-O-methyl, 2′-fluoro, and 2′-O-(2-methoxyethyl).

11. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification is selected from a 2′-deoxy-3′-phosphonoacetate (DP), 2′-O-deoxy-3′-thiophosphonoacetate (DSP), 2′-O-methyl-3′-phosphonoacetate (MP) and 2′-O-methyl-3′-thiophosphonoacetate (MSP).

12. The synthetic guide RNA or crRNA of any one of the preceding claims, further comprising one or more modifications at the 5′-end, the 3′-end, or both ends of said guide RNA or crRNA.

13. The synthetic guide RNA or crRNA of claim 12, wherein said at one or more modifications at the 5′-end, the 3′-end, or both ends are independently selected from a 2′-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2′-O-methyl-3′-phosphorothioate (MS), a 2′-O-methyl-3′-phosphonoacetate (MP), a 2′-O-methyl-3′-

thiophosphonoacetate (MSP), a 2′-deoxy-3′-phosphonoacetate (DP), a 2′-O-deoxy-3′-thiophosphonoacetate (DSP), or a combination thereof.

14. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification comprises a modified base.

15. The synthetic guide RNA or crRNA of claim 14, wherein the modified base is selected from 2-thioU, 2-thioC, 4-thioU, 6-thioG, 2-aminoA, 2-aminopurine, pseudouracil, hypoxanthine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deazaadenine, 7-deaza-8-azaadenine, 5-methylC, 5-methylU, 5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5,6-dehydrouracil, 5-propynylcytosine, 5-propynyluracil, 5-ethynylcytosine, 5-ethynyluracil, 5-allylU, 5-allylC, 5-aminoallylU, 5-aminoallylC, an abasic nucleotide, a UNA base, isoC, isoG, 5-methyl-pyrimidine, x(A,G,C,T,U), y(A,G,C,T,U), and combinations thereof.

16. The synthetic guide RNA or crRNA of claim 15 wherein said at least one modification is selected from 2-thioU and 2-aminoA.

17. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said at least one modification comprises a modified sugar.

18. The synthetic guide RNA or crRNA of claim 17 wherein said at least one modification is selected from 2′-O-C_{1-4}alkyl, 2′-O-C_{1-3}alkyl-O-C_{1-3}alkyl, 2′-deoxy, 2′-F, 2′-NH_2, 2′-arabino, 2′-F-arabino, 2′-LNA, 2′-ULNA and 4′-thioribosyl.

19. The synthetic guide RNA or crRNA of claim 17, wherein the at least one modification comprises:

(a) 2′-deoxyribose, 2′-deoxy-2′-fluoroarabinofuranosyl, 2′-deoxy-2′-fluororibofuranosyl, 2′-O-phenyl, 2′-thiophenyl, 2′-S-thiophenyl, 2′-methyl, 2′-ethyl, 2′-propyl, 2′-allyl, 2′-allylphenyl, 2′-methylhydroxy, 2′-methyloxymethyl, 2′-O-carbamate, 2′-O-ethylamino, 2′-O-allylamino, 2′-O-propylamino, or 2′-O-substituted phenyl;

(b) phosphonoacetate, thiophosphonoacetate, phosphonopropionate, thiophosphonopropionate, methylphosphonate, methylthiophosphonate, or boranophosphonate; or

(c) combinations of (a) and (b).

20. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein said target polynucleotide is located within the *HBB* gene, the *IL2RG* gene, or the *VEGFA* gene.

21. The synthetic guide RNA or crRNA of any one of the preceding claims, wherein the target polynucleotide comprises GCCCCACAGGGCAGTAA of the *HBB* gene, TAATGATGGCTTCAACA of the *IL2RG* gene, or GAGTGAGTGTGTGCGTG of the *VEGFA* gene.

22. The synthetic guide RNA or crRNA of claim 20 or 21, wherein said guide RNA is a single guide RNA (sgRNA) and comprises one or more modifications at the 5′-end, the 3′-end or both ends independently selected from a 2′-O-methyl (M), a phosphorothioate internucleotide linkage (S), a phosphonoacetate internucleotide linkage (P), a thiophosphonoacetate internucleotide linkage (SP), a 2′-O-methyl-3′-phosphoroatioate (MS), a 2′-O-methyl-3′-phosphonoacetate (MP), a 2′-O-methyl-3′-thiophosphonoacetate (MSP), a 2′-deoxy-3′-phosphonoacetate (DP), a 2′-O-deoxy-3′-thiophosphonoacetate (DSP), and combinations thereof.

23. A method for enhancing the specificity of a CRISPR function, comprising:

selecting a target polynucleotide;

providing at least one synthetic guide RNA of any of the preceding claims;

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA; and

contacting the target polynucleotide with the gRNA:Cas protein complex under conditions to result in hybridization of said gRNA to said target polynucleotide and performance of a CRISPR function;

wherein said Cas protein is provided as a protein or as a polynucleotide encoding said Cas protein.

24. The method of claim 23, wherein said contacting of said polynucleotide target with said gRNA:Cas protein complex is performed in a cell and wherein said forming said complex is performed outside or inside the cell.

25. The method of claim 23 or 24, wherein said Cas protein is a Cas9 protein.

26. The method of any of claims 23 to 25, wherein said forming is performed outside a cell.

27. The method of any one of claims 23-26 wherein said CRISPR function is genome editing and further comprises providing at least one single-stranded or double-stranded DNA polynucleotide as a donor template for homology-directed repair.

28. A set or a library of guide RNA comprising at least two different synthetic guide RNAs of any one of the preceding claims.

29. A set or a library of crRNA comprising at least two different synthetic crRNAs of any one of the preceding claims.

30. A kit comprising a synthetic guide RNA or crRNA of any one of the preceding claims.

31. A microarray comprising at least one synthetic guide RNA or crRNA of any one of the preceding claims.

32. A method for analyzing the specificity of a CRISPR function, the method comprising:

providing a sample comprising (1) a target polynucleotide comprising a target sequence; and (2) one or more off-target polynucleotides comprising off-target sequences;

providing at least one synthetic guide RNA of any of the preceding claims for the target sequence;

forming a gRNA:Cas protein complex comprising a Cas protein and the synthetic guide RNA wherein said Cas protein is provided as a protein or a polynucleotide encoding said Cas protein;

contacting the sample with the gRNA:Cas protein complex under conditions to result in hybridization of said gRNA to said target polynucleotide and performance of a CRISPR function;

adding a library of oligonucleotide baits, the baits including baits designed to hybridize to the target polynucleotide and baits designed to hybridize to the one or more off-target polynucleotides, to capture the target polynucleotide and said one or more off-target polynucleotide;

isolating and analyzing said captured target polynucleotides and said captured off-target polynucleotides to identify changes caused by the CRISPR function, and determining the relative changes in the target polynucleotide and the off-target polynucleotides, thereby assessing the specificity of said CRISPR function.

33. The method of claim 32, where the captured target polynucleotide and off-target polynucleotides are analyzed by sequencing.

34. The method of claim 32 or 33, wherein the library of oligonucleotide baits is provided in solution, and hybridization of the baits to the target and off-target polynucleotides occur in solution.

35. The method of claim 32 or 33, wherein the library of oligonucleotide baits is provided on a solid support, and hybridization of the baits to the target and off-target polynucleotides occur on the solid support.

36. The method of any one of claim 32-35, wherein the oligonucleotide baits comprise baits that are capable of hybridizing to sequences in the target polynucleotide within one kilobase (kb) of the target sequence, or sequences in the off-target polynucleotides within one kb of the off-target sequences, rather than to the target sequence or the off-target sequences.

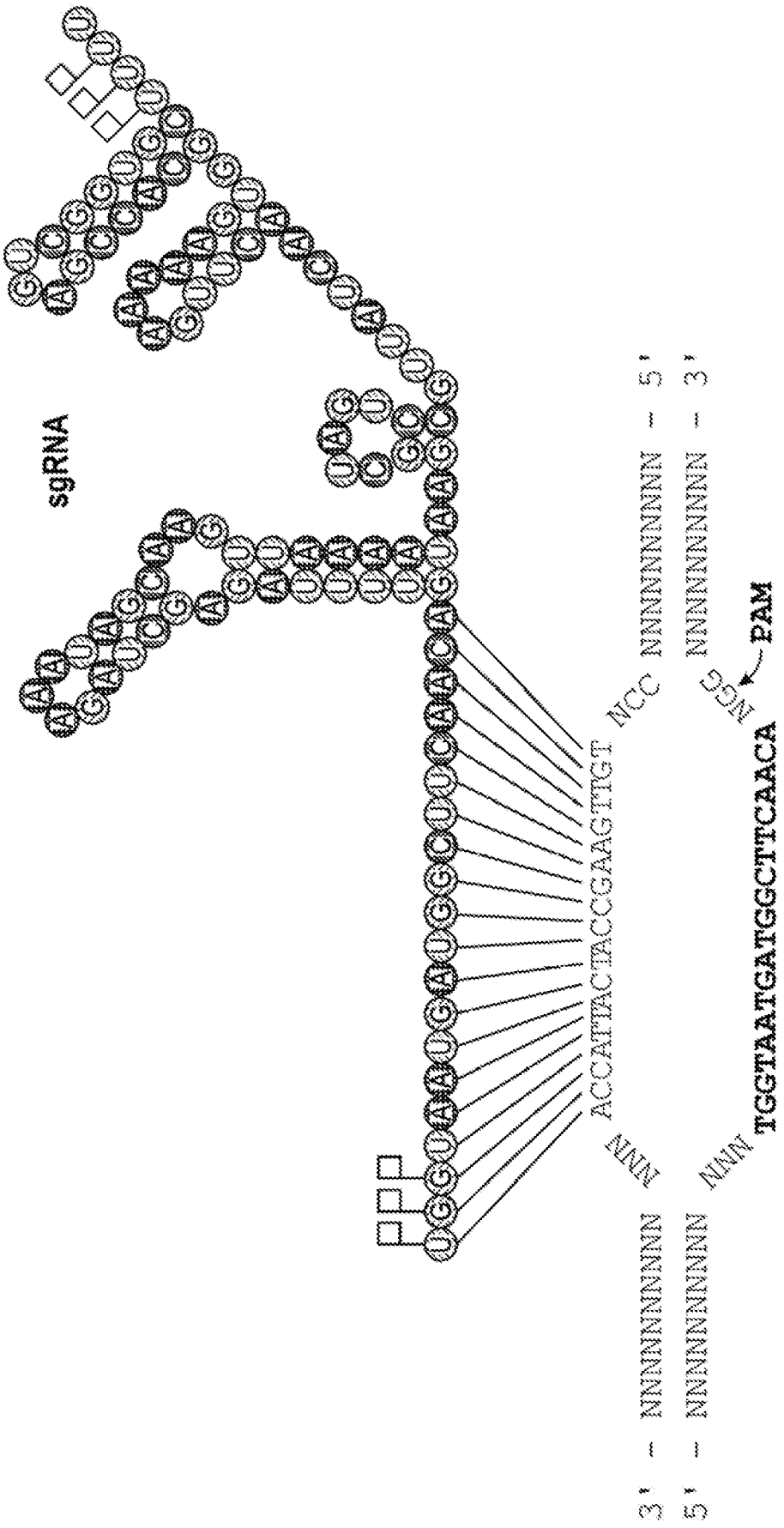

sgRNA
3' – NNNNNNNNNNNNNNNNNNNN – 5'
5' – NNNNNNNNNNNNNNNNNNNN – 3'
ACCATTACTACCGAAGTTGT
TGGTAATGATGGCTTCAACA
NCC
NGG
PAM
Target Site

FIG. 1

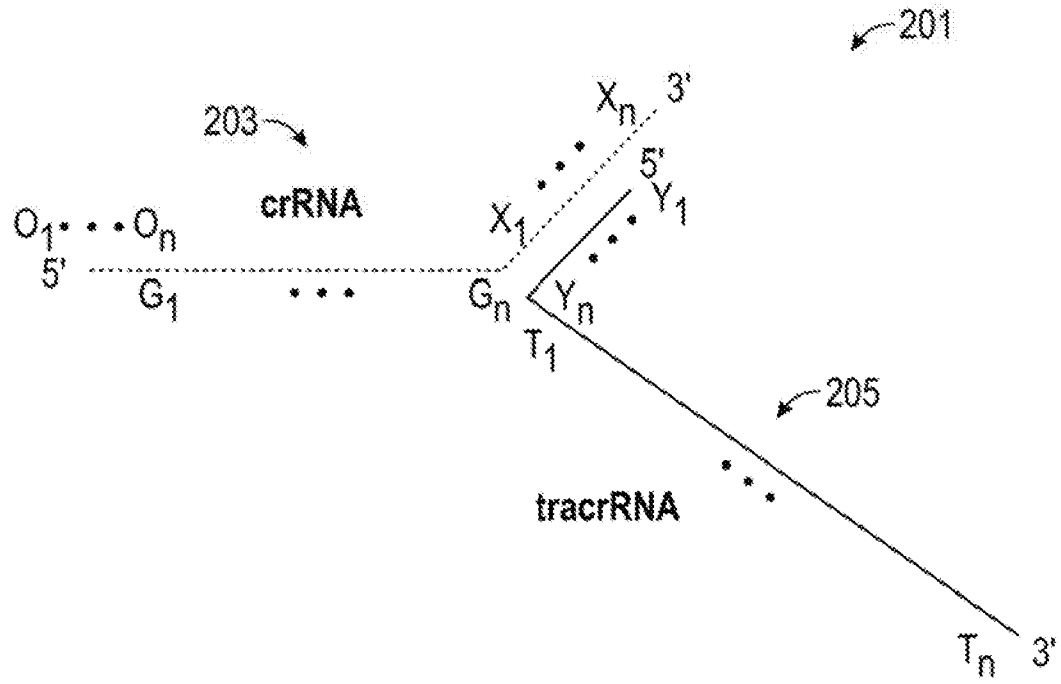

201
203
crRNA
$O_1 \cdots O_n$
5'
G_1
G_n
X_n 3'
5'
X_1
Y_1
Y_n
T_1
205
tracrRNA
T_n 3'

FIG. 2A

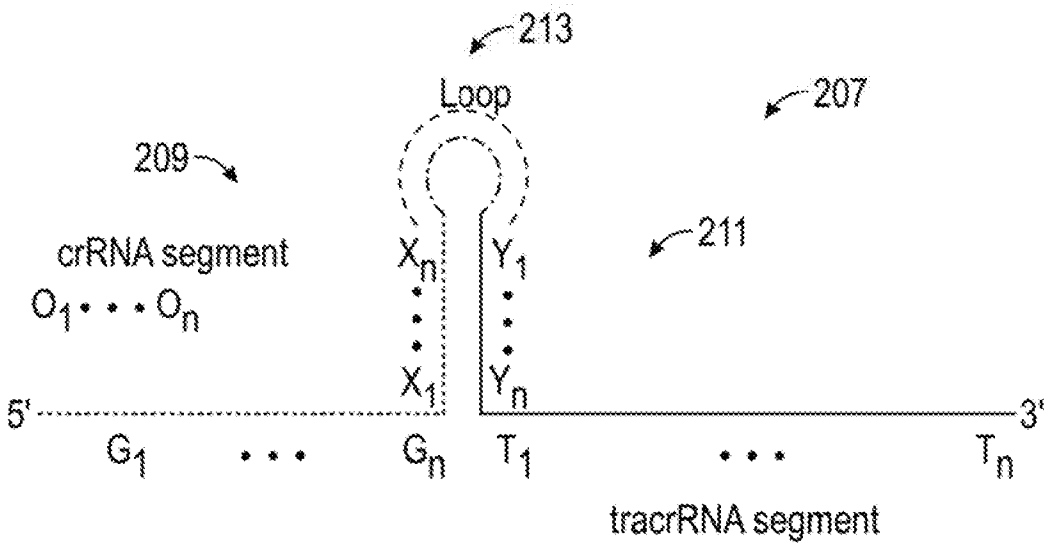

213
Loop
207
209
211
crRNA segment
$O_1 \cdots O_n$
X_n
X_1
Y_1
Y_n
5'
3'
G_1
G_n
T_1
T_n
tracrRNA segment

FIG. 2B

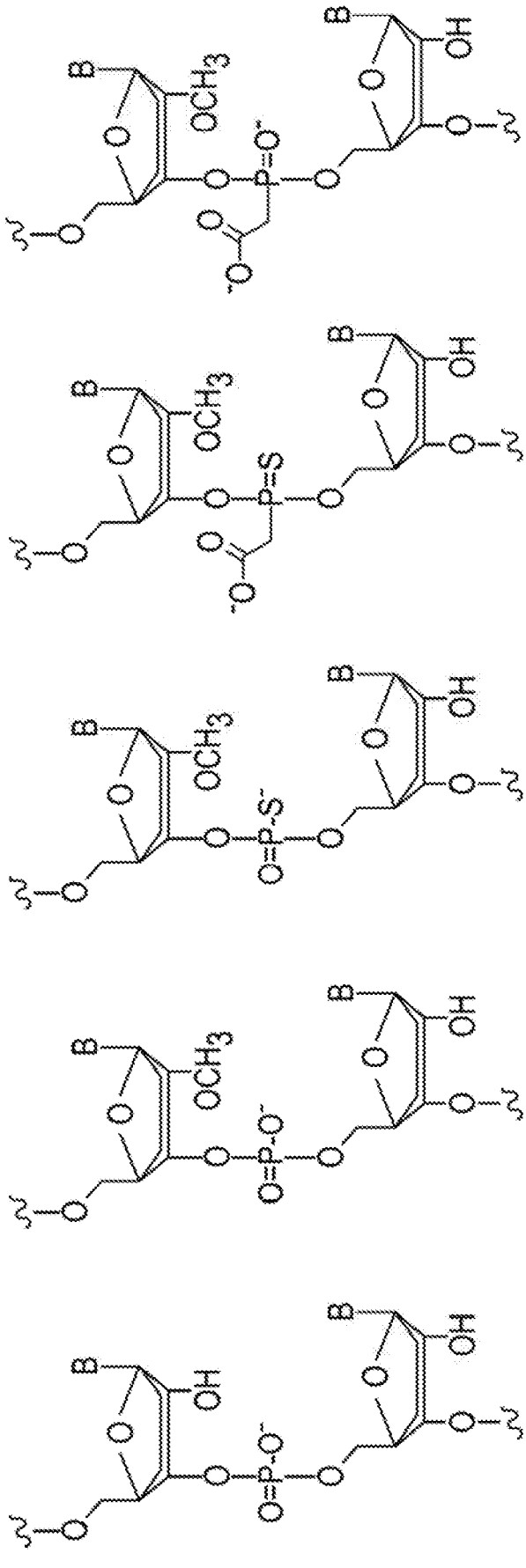


FIG. 3

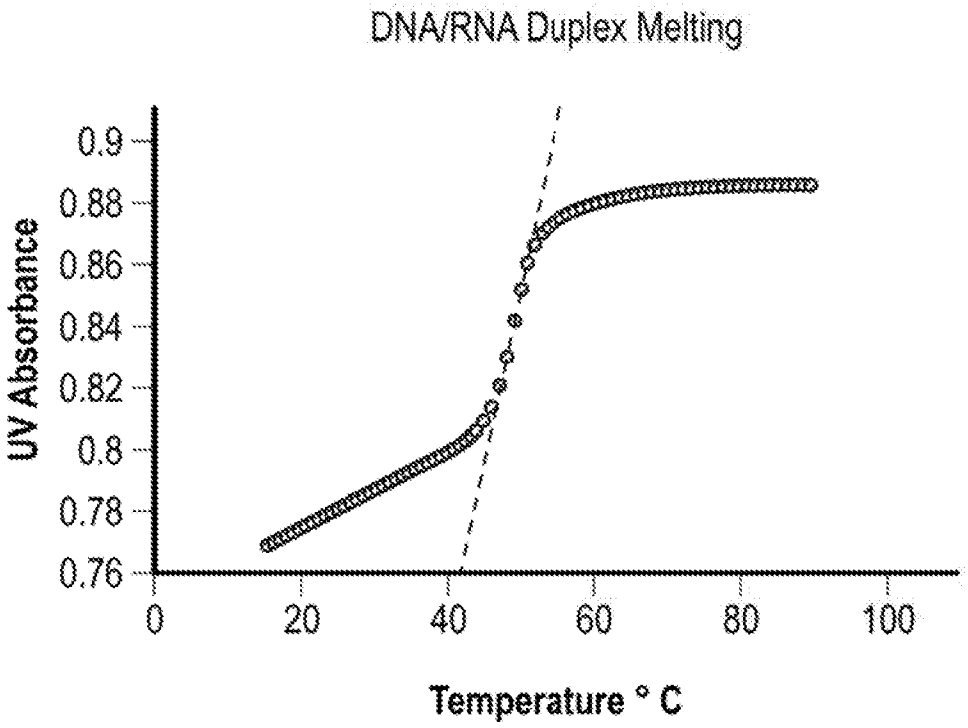

DNA/RNA Duplex Melting
UV Absorbance
0.9
0.88
0.86
0.84
0.82
0.8
0.78
0.76
0
20
40
60
80
100
Temperature ° C

FIG. 4

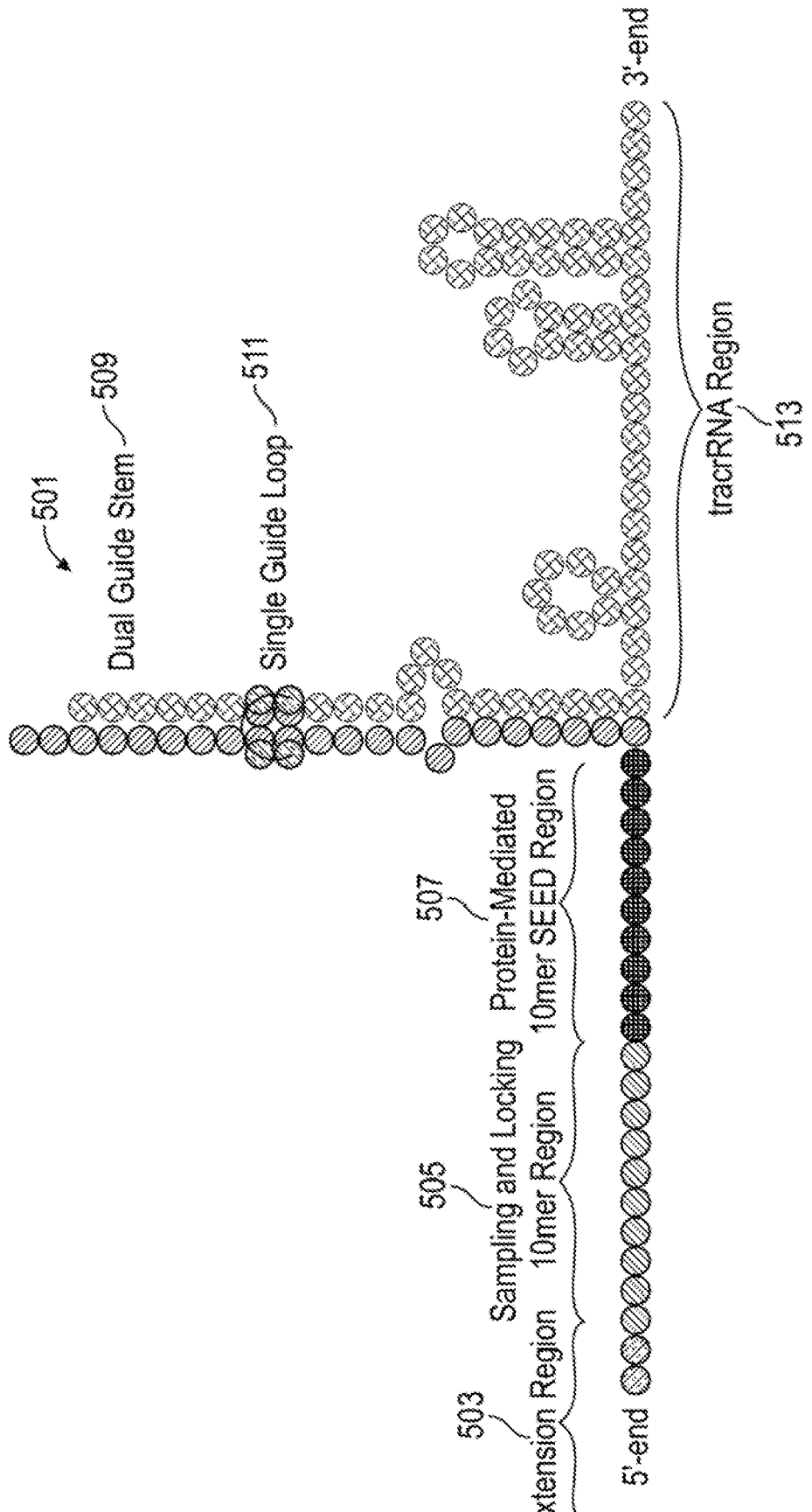

501
Dual Guide Stem
509
Single Guide Loop
511
3'-end
tracrRNA Region
513
507
Protein-Mediated
10mer SEED Region
505
Sampling and Locking
10mer Region
503
Extension Region
5'-end

FIG. 5A

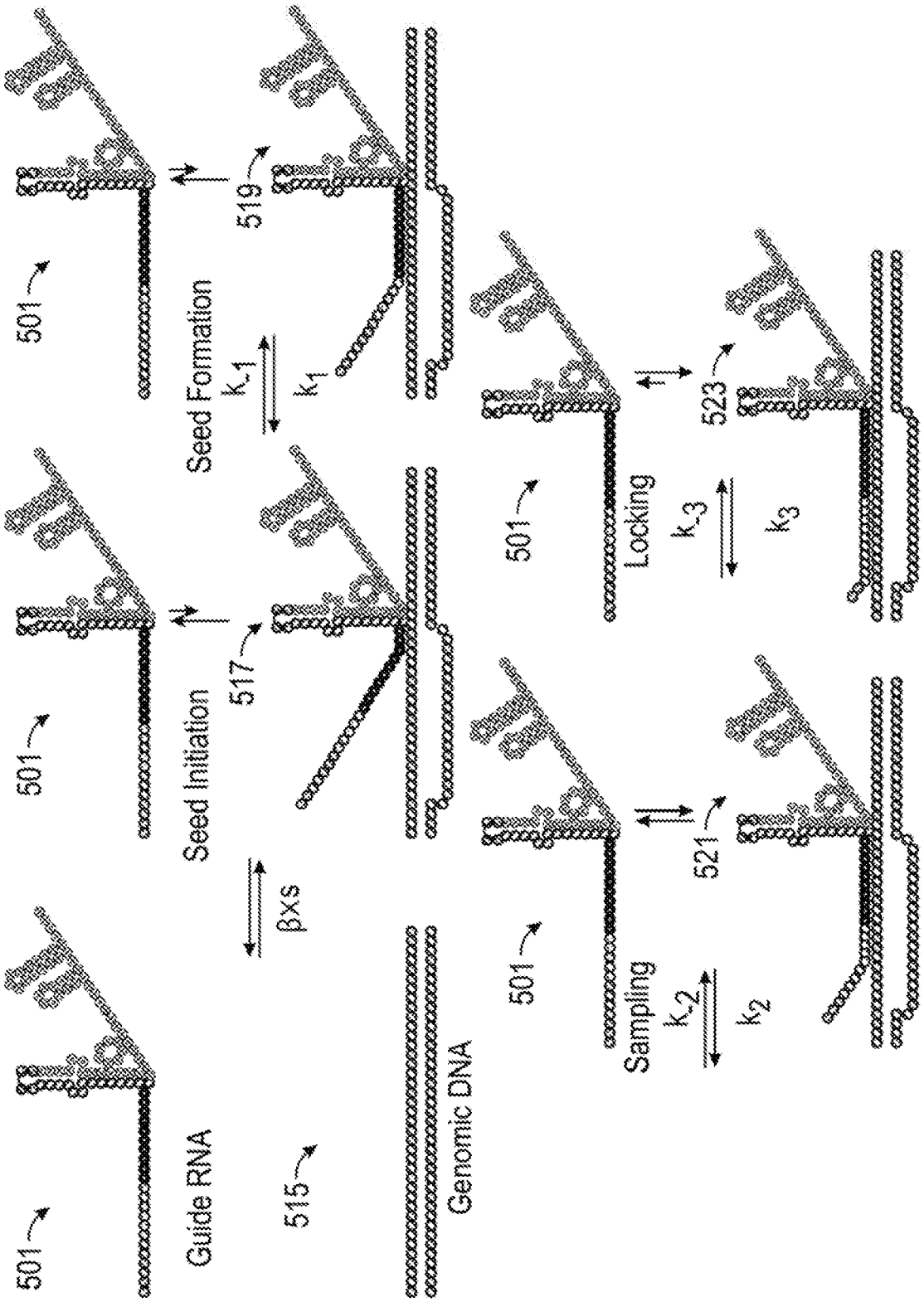

501
Guide RNA
515
Genomic DNA
Seed Initiation
βxs
517
Seed Formation
k-1
k1
519
Sampling
k-2
k2
521
Locking
k-3
k3
523

FIG. 5B

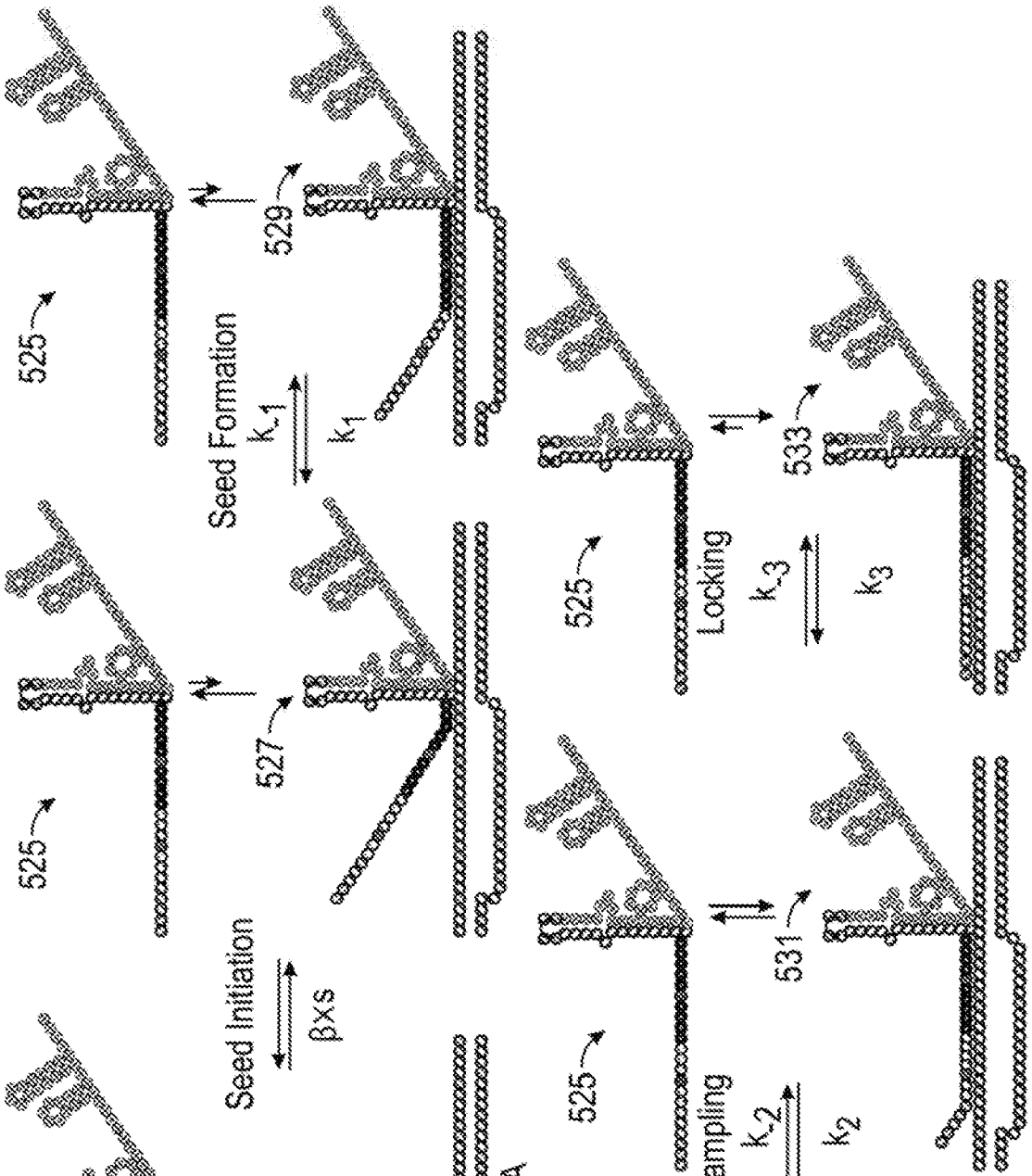

525
Chemical Modification
Guide RNA
515
Genomic DNA
Seed Initiation
βxs
527
Seed Formation
k_1
k_1
529
Locking
k_3
k_3
533
Sampling
k_2
k_2
531

FIG. 5C

WO 2017/214460

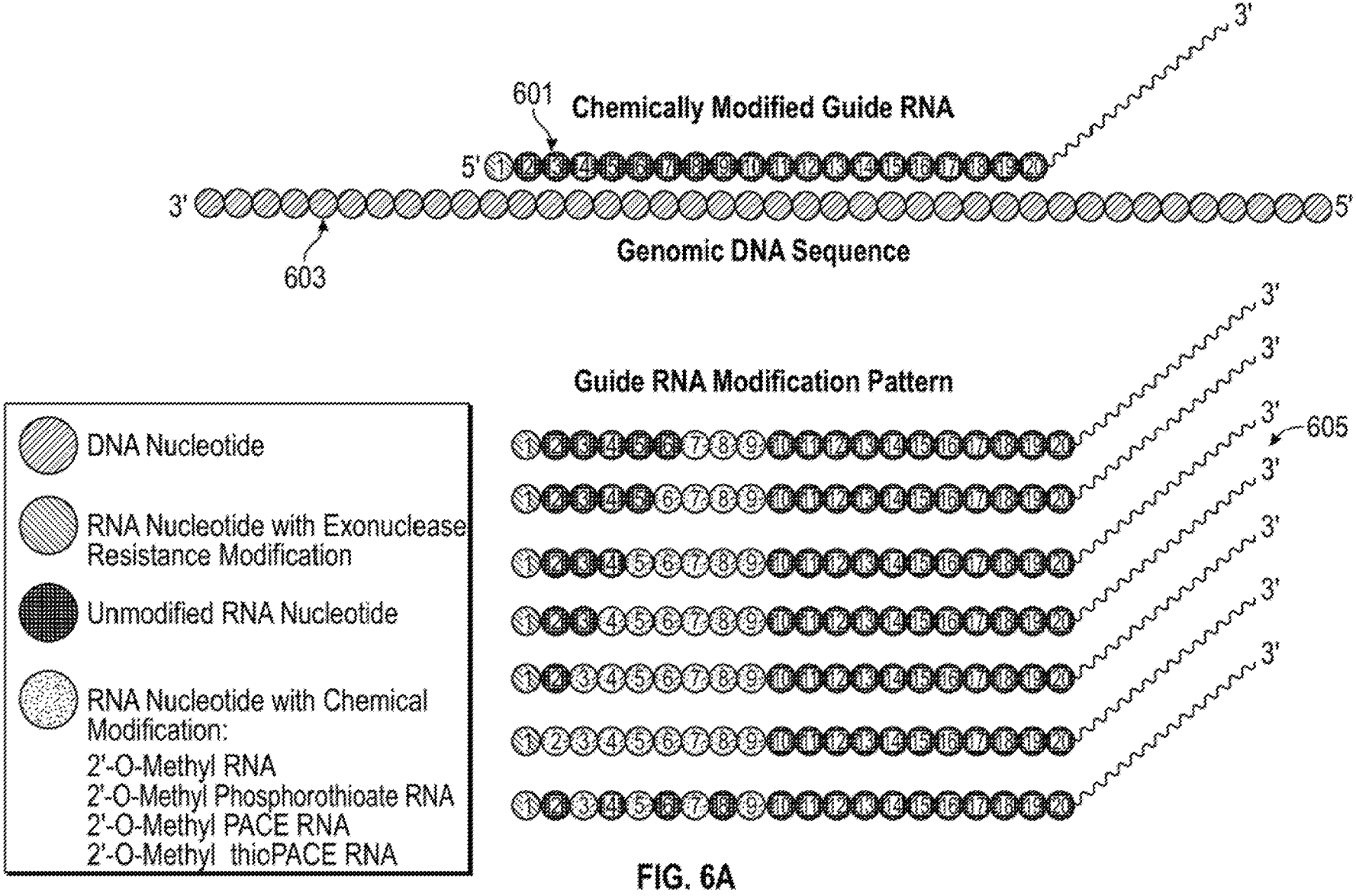


FIG. 6A

PCT/US2017/036648

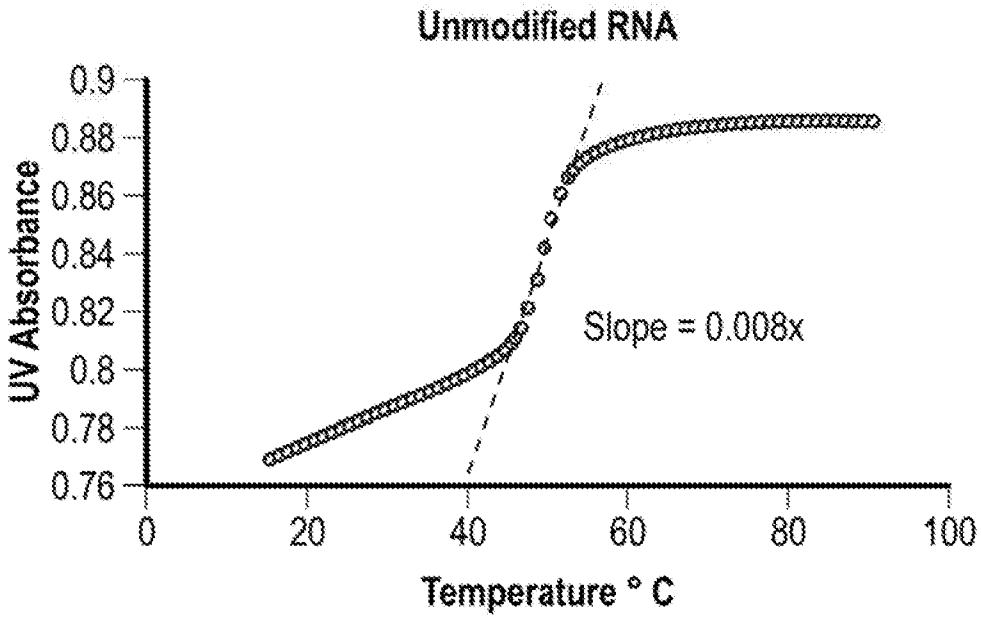

Unmodified RNA
UV Absorbance
0.9
0.88
0.86
0.84
0.82
0.8
0.78
0.76
Slope = 0.008x
0
20
40
60
80
100
Temperature ° C

FIG. 6B

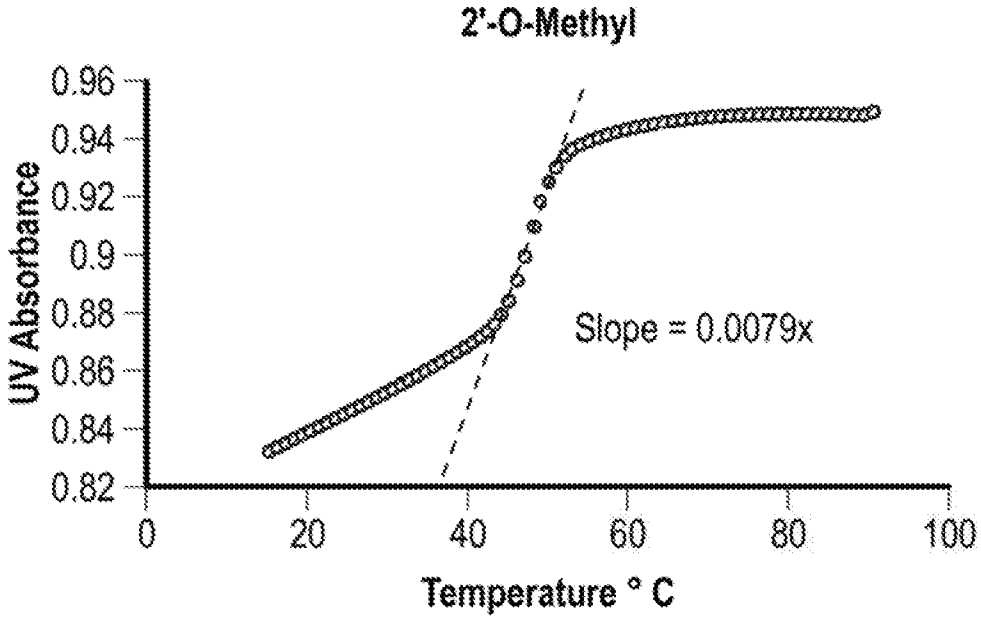

2'-O-Methyl
UV Absorbance
0.96
0.94
0.92
0.9
0.88
0.86
0.84
0.82
Slope = 0.0079x
0
20
40
60
80
100
Temperature ° C

FIG. 6C

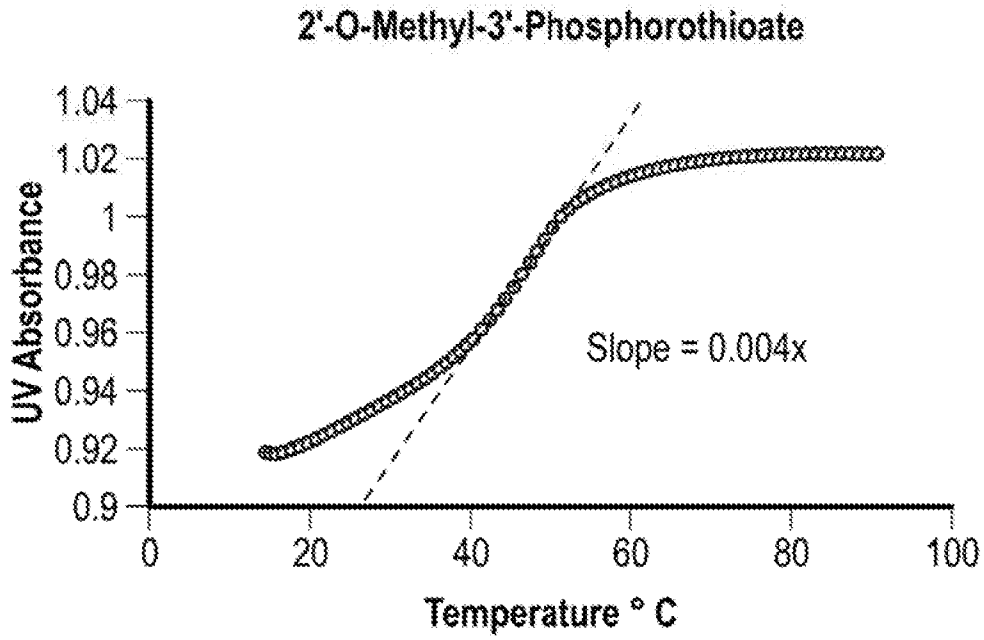

2'-O-Methyl-3'-Phosphorothioate
UV Absorbance
1.04
1.02
1
0.98
0.96
0.94
0.92
0.9
Slope = 0.004x
0
20
40
60
80
100
Temperature ° C

FIG. 6D

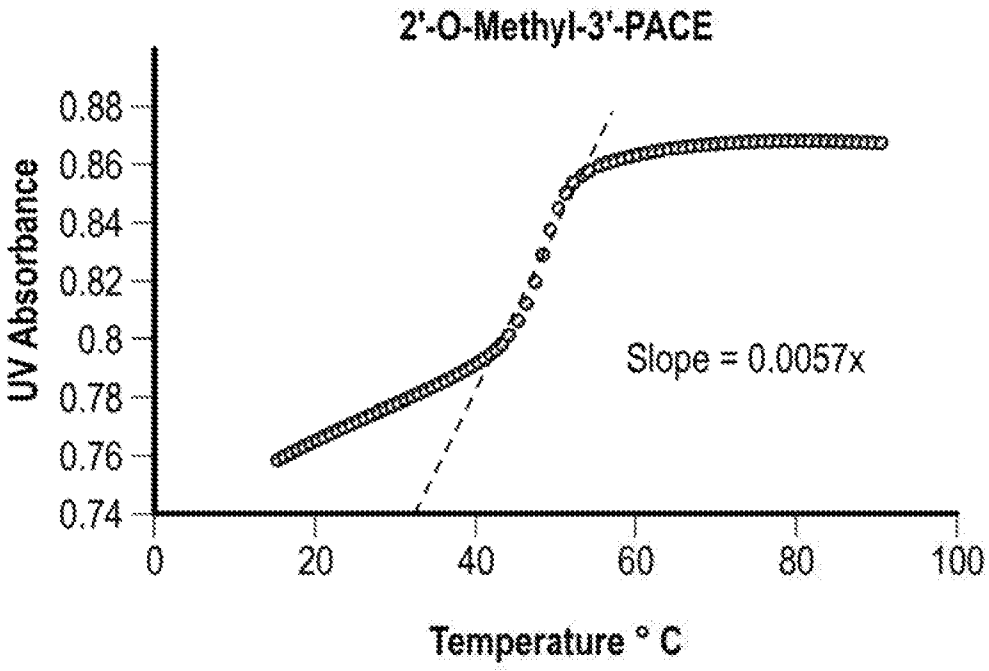

2'-O-Methyl-3'-PACE
UV Absorbance
0.88
0.86
0.84
0.82
0.8
0.78
0.76
0.74
Slope = 0.0057x
0
20
40
60
80
100
Temperature ° C

FIG. 6E

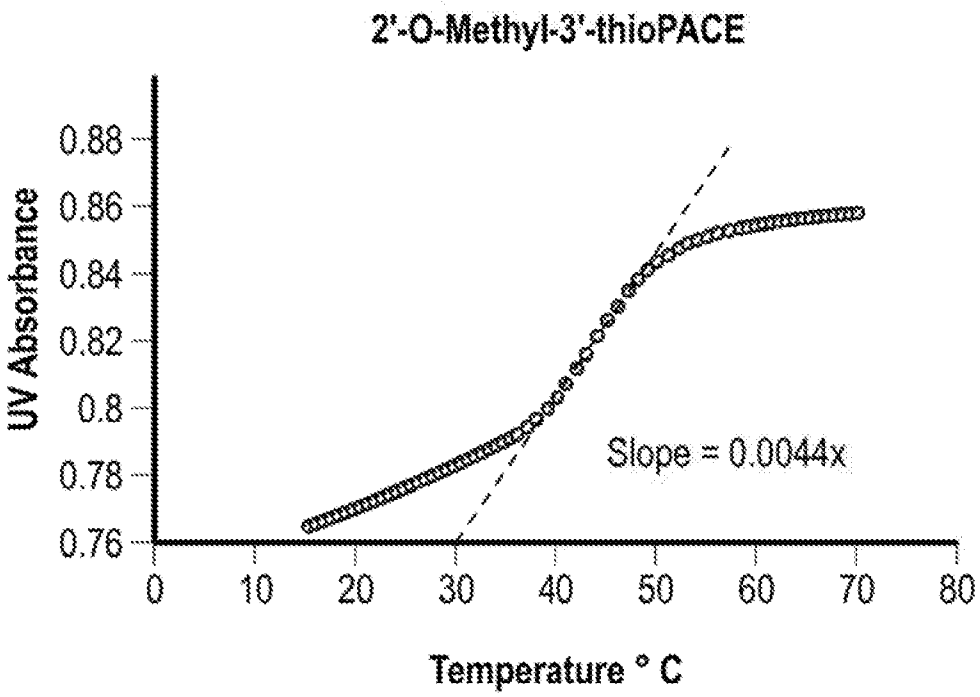

2'-O-Methyl-3'-thioPACE
UV Absorbance
0.88
0.86
0.84
0.82
0.8
0.78
0.76
Slope = 0.0044x
0
10
20
30
40
50
60
70
80
Temperature ° C

FIG. 6F

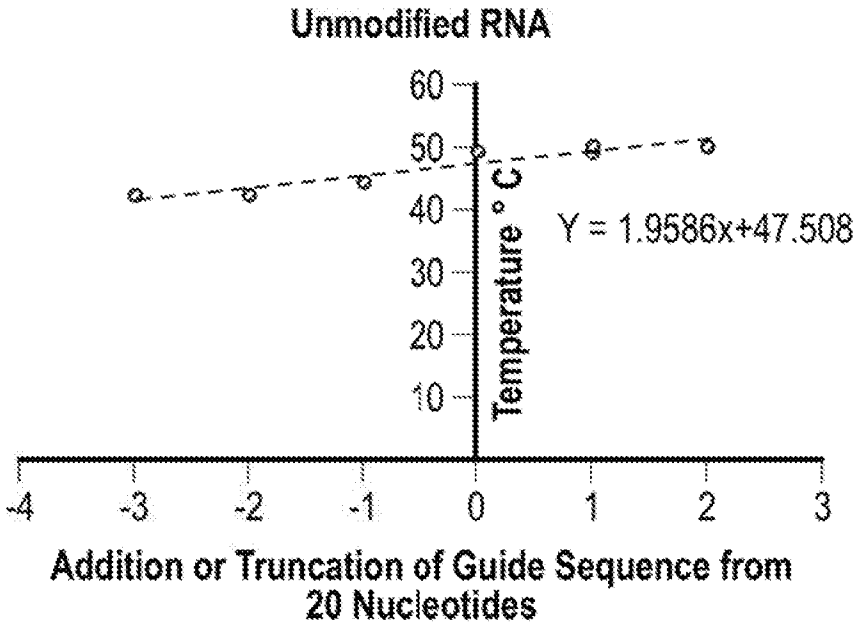

Unmodified RNA
60
50
40
30
20
10
Temperature ° C
Y = 1.9586x+47.508
-4
-3
-2
-1
0
1
2
3
Addition or Truncation of Guide Sequence from 20 Nucleotides

FIG. 7

Entry	Guide Sequence for CLTA1 target	% Target Cleaved		
		ON	OFF1	OFF3
1	AGUCCUCAUCUCCCUCAAGC	87%	82%	92%
2	A*sG*sU*CCUCAUCUCCCUCAAGC	84%	85%	91%
3	A*sG*sU*sCCUCAUCUCCCUCAAGC	84%	77%	83%
4	A*sGUCCUCAUCUCCCUCAAGC	92%	88%	94%
5	UCCUCAUCUCCCUCAAGC	78%	65%	81%
6	CCUCAUCUCCCUCAAGC	85%	32%	84%
7	G*AGUCCUCAUCUCCCUCAAGC	91%	88%	93%
8	G*G*AGUCCUCAUCUCCCUCAAGC	87%	89%	88%
9	A*GUCCUCAUCUCCCUCAAGC	82%	77%	83%
10	A*G*UCCUCAUCUCCCUCAAGC	91%	91%	92%
11	A*G*U*CCUCAUCUCCCUCAAGC	85%	77%	81%
12	A*G*U*C*CUCAUCUCCCUCAAGC	70%	20%	34%
13	A*G*U*C*C*UCAUCUCCCUCAAGC	40%	7%	16%
14	AGUCCUCAUCUCCCUCAAGC	84%	84%	92%
15	AGUCCUCAUCUCCCUCAAGC	86%	82%	90%
16	C*A*G*UCCUCAUCUCCCUCAAGC	82%	83%	83%
17	G*A*G*UCCUCAUCUCCCUCAAGC	71%	68%	70%
18	U*C*A*G*UCCUCAUCUCCCUCAAGC	56%	35%	51%
19	A*G*A*G*UCCUCAUCUCCCUCAAGC	58%	46%	54%
20	C*U*C*A*G*U*CCUCAUCUCCCUCAAGC	38%	8%	9%
21	G*A*G*A*G*U*CCUCAUCUCCCUCAAGC	36%	10%	10%
22	AGUCCUCAUCUCCCUCAAGC*	84%	80%	85%
23	AGUCCUCAUCUCCCUCAAG*C	83%	80%	84%
24	AGUCCUCAUCUCCCUCA*GC	85%	79%	83%
25	AGUCCUCAUCUCCCUC*AGC	89%	88%	93%
26	AGUCCUCAUCUCCCUC*A*GC	84%	91%	92%
27	AGUCCUCAUCUCCCUCAA*GC	86%	85%	88%
28	AGUCCUCAUCUCCCUCA*GC	83%	83%	85%
29	AGUCCUCAUCUCCCUC*AGC	86%	83%	88%
30	AGUCCUCAUCUCCCUC*AA*GC	84%	83%	87%
31	AGUCCUCAUCUCCCUCAAGC	22%	12%	5%
32	AGUCCUCAUCUCCCUCAAGC	89%	84%	93%

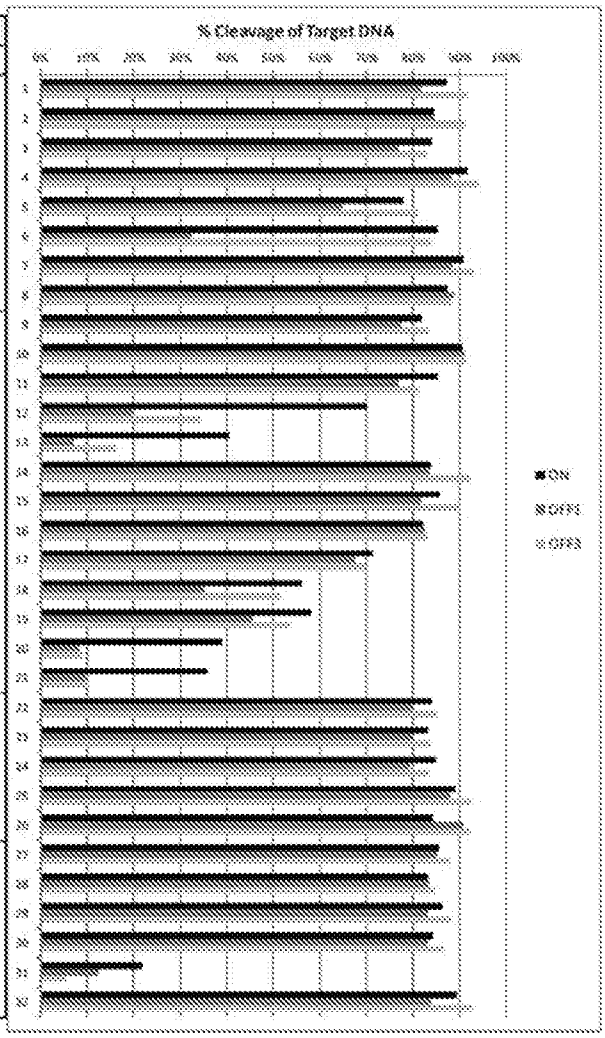


FIG. 8A

On- vs. Off-target Cleavage Ratios & Specificity Scores for in vitro cleavage of CLTA1					
Entry	Guide Sequence for CLTA1 target	ON : OFF1 Ratio	Specificity Score	ON : OFF3 Ratio	Specificity Score
1	AGUCCUCAUCUCCCUCAAGC-	1.07	0.93	0.95	0.83
2	AsGsUsCCUCAUCUCCCUCAAGC-	1.00	0.84	0.93	0.78
3	A*sG*sU*sCCUCAUCUCCCUCAAGC-	1.09	0.92	1.01	0.85
4	A*sGUCCUCAUCUCCCUCAAGC-	1.04	0.95	0.97	0.89
5	UCCUCAUCUCCCUCAAGC-	1.20	0.94	0.96	0.75
6	CCUCAUCUCCCUCAAGC-	2.63	2.24	1.02	0.87
7	G$_o$AGUCCUCAUCUCCCUCAAGC-	1.03	0.93	0.98	0.89
8	G$_o$G$_o$AGUCCUCAUCUCCCUCAAGC-	0.98	0.86	0.99	0.87
9	A*GUCCUCAUCUCCCUCAAGC-	1.06	0.87	0.98	0.81
10	A*G*UCCUCAUCUCCCUCAAGC-	0.99	0.90	0.99	0.90
11	A*G*U*CCUCAUCUCCCUCAAGC-	1.11	0.95	1.05	0.90
12	A*G*U*C*CUCAUCUCCCUCAAGC-	3.56	2.50	2.04	1.43
13	A*G*U*C*C*UCAUCUCCCUCAAGC-	5.75	2.32	2.49	1.01
14	AGUCCUCAUCUCCCUCAAGC-	1.00	0.84	0.91	0.76
15	AGUCCUCAUCUCCCUCAAGC-	1.05	0.90	0.95	0.81
16	C$_o$*A*G*UCCUCAUCUCCCUCAAGC-	0.99	0.82	0.99	0.81
17	G$_o$*A*G*UCCUCAUCUCCCUCAAGC-	1.06	0.75	1.02	0.73
18	U$_o$*C$_o$*A*G*U*CCUCAUCUCCCUCAAGC-	1.59	0.89	1.09	0.61
19	A$_o$*G$_o$*A*G*U*CCUCAUCUCCCUCAAGC-	1.27	0.74	1.08	0.63
20	C$_o$*U$_o$*C$_o$*A*G*U*C*CUCAUCUCCCUCAAGC-	4.70	1.84	4.32	1.69
21	G$_o$*A$_o$*G$_o$*A*G*U*C*CUCAUCUCCCUCAAGC-	3.69	1.33	3.50	1.26
22	AGUCCUCAUCUCCCUCAAGC*-	1.05	0.88	0.99	0.83
23	AGUCCUCAUCUCCCUCAAG*C-	1.04	0.87	0.99	0.83
24	AGUCCUCAUCUCCCUCAA*GC-	1.07	0.91	1.02	0.86
25	AGUCCUCAUCUCCCUCA*AGC-	1.01	0.90	0.96	0.86
26	AGUCCUCAUCUCCCUCA*A*GC-	0.93	0.78	0.91	0.77
27	AGUCCUCAUCUCCCUCAAGC-	1.01	0.86	0.97	0.83
28	AGUCCUCAUCUCCCUCAAGC-	1.00	0.83	0.98	0.82
29	AGUCCUCAUCUCCCUCAAGC-	1.04	0.90	0.98	0.84
30	AGUCCUCAUCUCCCUCAAGC-	1.01	0.85	0.97	0.82
31	AGUCCUCAUCUCCCUCAAGC-	1.76	0.39	4.11	0.90
32	AGUCCUCAUCUCCCUCAAGC-	1.06	0.95	0.96	0.86

FIG. 8B

Entry	Internal MP position(s)	Guide Sequence for CLTA4 target	% Target Cleaved			
			ON	OFF1	OFF2	OFF3
1	4	G*CAG*AUGUAGUGUUUCCACA-	87%	67%	45%	3%
2	5	G*CAG*UGUAGUGUUUCCACA-	90%	86%	43%	0%
3	6	G*CAGAU*GUAGUGUUUCCACA-	89%	93%	100%	85%
4	7	G*CAGAUG*UAGUGUUUCCACA-	73%	63%	65%	7%
5	8	G*CAGAUGU*AGUGUUUCCACA-	82%	85%	91%	73%
6	9	G*CAGAUGUA*GUGUUUCCACA-	85%	84%	97%	50%
7	10	G*CAGAUGUAG*UGUUUCCACA-	81%	79%	69%	83%
8	11	G*CAGAUGUAGU*GUUUCCACA-	71%	58%	65%	42%
9	12	G*CAGAUGUAGUG*UUUCCACA-	92%	93%	82%	44%
10	13	G*CAGAUGUAGUGU*UUCCACA-	93%	94%	100%	87%
11	14	G*CAGAUGUAGUGUU*UCCACA-	96%	97%	72%	33%
12	15	G*CAGAUGUAGUGUUU*CCACA-	17%	16%	5%	0%
13	16	G*CAGAUGUAGUGUUUC*CACA-	92%	93%	92%	47%
14	17	G*CAGAUGUAGUGUUUCC*ACA-	92%	97%	100%	90%
15	18	G*CAGAUGUAGUGUUUCCA*CA-	90%	91%	97%	77%
16	19	G*CAGAUGUAGUGUUUCCAC*A-	89%	89%	95%	79%
17	20	G*CAGAUGUAGUGUUUCCACA*-	90%	90%	100%	87%
18	control	GCAGAUGUAGUGUUUCCACA-	88%	86%	93%	64%
19	5-7	G*CAG*U*G*UAGUGUUUCCACA-	12%	0%	0%	0%
20	6-8	G*CAGU*G*U*AGUGUUUCCACA-	40%	28%	6%	0%
21	7-9	G*CAGUG*U*A*GUGUUUCCACA-	2%	1%	0%	0%
22	8-10	G*CAGUGU*A*G*UGUUUCCACA-	27%	12%	0%	2%
23	9-11	G*CAGUGUA*G*U*GUUUCCACA-	26%	13%	0%	0%
24	10-12	G*CAGUGUAG*U*G*UUUCCACA-	23%	19%	0%	0%
25	11-13	G*CAGUGUAGU*G*U*UUCCACA-	20%	3%	0%	2%
26	12-14	G*CAGUGUAGUG*U*U*UCCACA-	28%	1%	0%	0%
27	13-15	G*CAGUGUAGUGU*U*U*CCACA-	2%	0%	0%	0%
28	14-16	G*CAGUGUAGUGUU*U*C*CACA-	3%	0%	2%	0%

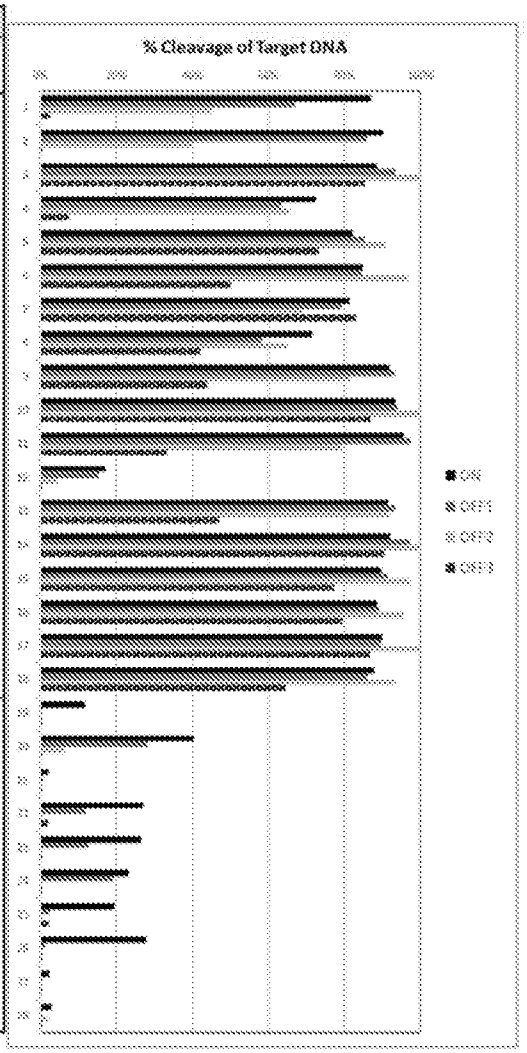


FIG. 9A

On- vs. Off-target Cleavage Ratios & Specificity Scores for in vitro cleavage of CLTA4								
Entry	Internal MP position(s)	Guide Sequence for CLTA4 target	ON : OFF1 Ratio	Specificity Score	ON : OFF2 Ratio	Specificity Score	ON : OFF3 Ratio	Specificity Score
1	4	G*CAG*AUGUAGUGUUUCCACA-	1.3	1.1	1.9	1.7	34.5	30.0
2	5	G*CAGA*UGUAGUGUUUCCACA-	1.1	0.9	2.3	2.0	large	165.9
3	6	G*CAGAU*GUAGUGUUUCCACA-	1.0	0.8	0.9	0.8	1.0	0.9
4	7	G*CAGAUG*UAGUGUUUCCACA-	1.1	0.8	1.1	0.8	9.7	7.1
5	8	G*CAGAUGU*AGUGUUUCCACA-	1.0	0.8	0.9	0.7	1.1	0.9
6	9	G*CAGAUGUA*GUGUUUCCACA-	1.0	0.9	0.9	0.7	1.7	1.4
7	10	G*CAGAUGUAG*UGUUUCCACA-	1.0	0.8	1.2	1.0	1.0	0.8
8	11	G*CAGAUGUAGU*GUUUCCACA-	1.2	0.9	1.1	0.8	1.7	1.2
9	12	G*CAGAUGUAGUG*UUUCCACA-	1.0	0.9	1.1	1.0	2.1	1.9
10	13	G*CAGAUGUAGUGU*UUCCACA-	1.0	0.9	0.9	0.9	1.1	1.0
11	14	G*CAGAUGUAGUGUU*UCCACA-	1.0	0.9	1.2	1.2	2.9	2.8
12	15	G*CAGAUGUAGUGUUU*CCACA-	1.1	0.2	3.7	0.6	large	6.0
13	16	G*CAGAUGUAGUGUUUC*CACA-	1.0	0.9	1.0	0.9	2.0	1.8
14	17	G*CAGAUGUAGUGUUUCC*ACA-	0.9	0.9	0.9	0.8	1.0	0.9
15	18	G*CAGAUGUAGUGUUUCCA*CA-	1.0	0.9	0.9	0.8	1.2	1.0
16	19	G*CAGAUGUAGUGUUUCCAC*A-	1.0	0.9	0.9	0.8	1.1	1.0
17	20	G*CAGAUGUAGUGUUUCCACA*-	1.0	0.9	0.9	0.8	1.0	0.9
18	control	GCAGAUGUAGUGUUUCCACA-	1.0	0.9	0.9	0.8	1.4	1.2
19	5–7	G*CAG*U*G*UAGUGUUUCCACA-	large	2.8	large	2.8	large	2.8
20	6–8	G*CAGU*G*U*AGUGUUUCCACA-	1.4	0.6	6.4	2.6	large	33.5
21	7–9	G*CAGAUG*U*A*GUGUUUCCACA-	2.5	0.1	large	0.1	large	0.1
22	8–10	G*CAGAUGU*A*G*UGUUUCCACA-	2.3	0.6	large	14.9	12.4	3.3
23	9–11	G*CAGAUGUA*G*U*GUUUCCACA-	2.1	0.6	large	14.3	large	14.3
24	10–12	G*CAGAUGUAG*U*G*UUUCCACA-	1.2	0.3	large	11.1	large	11.1
25	11–13	G*CAGAUGUAGU*G*U*UUCCACA-	7.6	1.5	large	7.8	8.3	1.6
26	12–14	G*CAGAUGUAGUG*U*U*UCCACA-	20.8	5.8	large	15.6	large	15.6
27	13–15	G*CAGAUGUAGUGU*U*U*CCACA-	large	0.1	large	0.1	large	0.1
28	14–16	G*CAGAUGUAGUGUU*U*C*CACA-	large	0.2	1.4	0.0	large	0.2

FIG. 9B

On- vs. Off-target Cleavage Ratios & Specificity Scores for in vitro cleavage of IL2RG						
Entry	Internal MP position(s)	Guide Sequence for IL2RG target	% Target Cleaved		Ratio	Specificity Score
			ON	OFF3	ON : OFF3	
1	2	U*G*GUAAUGAUGGCUUCAACA-	80%	75%	1.1	0.9
2	3	U*GG*UAAUGAUGGCUUCAACA-	85%	62%	1.4	1.2
3	4	U*GGU*AAUGAUGGCUUCAACA-	63%	8%	8.4	5.2
4	5	U*GGUA*AUGAUGGCUUCAACA-	76%	31%	2.5	1.9
5	6	U*GGUAA*UGAUGGCUUCAACA-	75%	69%	1.1	0.8
6	7	U*GGUAAU*GAUGGCUUCAACA-	65%	3%	18.8	12.3
7	8	U*GGUAAUG*AUGGCUUCAACA-	73%	53%	1.4	1.0
8	9	U*GGUAAUGA*UGGCUUCAACA-	73%	32%	2.3	1.6
9	10	U*GGUAAUGAU*GGCUUCAACA-	64%	48%	1.3	0.9
10	11	U*GGUAAUGAUG*GCUUCAACA-	65%	18%	3.7	2.4
11	12	U*GGUAAUGAUGG*CUUCAACA-	84%	10%	8.3	7.0
12	13	U*GGUAAUGAUGGC*UUCAACA-	95%	21%	4.5	4.2
13	14	U*GGUAAUGAUGGCU*UCAACA-	96%	10%	9.7	9.3
14	15	U*GGUAAUGAUGGCUU*CAACA-	29%	8%	3.8	1.1
15	16	U*GGUAAUGAUGGCUUC*AACA-	96%	9%	10.7	10.2
16	17	U*GGUAAUGAUGGCUUCA*ACA-	95%	12%	7.7	7.3
17	18	U*GGUAAUGAUGGCUUCAA*CA-	92%	17%	5.4	4.9
18	19	U*GGUAAUGAUGGCUUCAAC*A-	92%	25%	3.6	3.3
19	20	U*GGUAAUGAUGGCUUCAACA*-	87%	61%	1.4	1.2
20	control	U*GGUAAUGAUGGCUUCAACA-	84%	68%	1.2	1.0
21	4–6	U*GGU*A*A*UGAUGGCUUCAACA-	19%			
22	5–7	U*GGUA*A*U*GAUGGCUUCAACA-	11%			
23	6–8	U*GGUAA*U*G*AUGGCUUCAACA-	4%			
24	7–9	U*GGUAAU*G*A*UGGCUUCAACA-	2%	3%	0.5	0.0
25	8–10	U*GGUAAUG*A*U*GGCUUCAACA-	23%	4%	5.7	1.3
26	9–11	U*GGUAAUGA*U*G*GCUUCAACA-	50%	4%	12.1	6.0
27	10–12	U*GGUAAUGAU*G*G*CUUCAACA-	39%	6%	6.6	2.6
28	11–13	U*GGUAAUGAUG*G*C*UUCAACA-	35%	3%	10.7	3.7
29	12–14	U*GGUAAUGAUGG*C*U*UCAACA-	18%	3%	5.6	1.1
30	13–15	U*GGUAAUGAUGGC*U*U*CAACA-	4%	4%	1.1	0.0

FIG. 10

On- vs. Off-target Cleavage Ratios & Specificity Scores for in vitro cleavage of HBB						
Entry	Internal MP position(s)	Guide Sequence for HBB target	% Target Cleaved		Ratio	Specificity Score
			ON	OFF1	ON : OFF1	
1	4	C*UUG*CCCCACAGGGCAGUAA-	30%	3%	10.1	3.0
2	5	C*UUGC*CCCACAGGGCAGUAA-	53%	3%	16.7	8.9
3	6	C*UUGCC*CCACAGGGCAGUAA-	73%	37%	2.0	1.4
4	7	C*UUGCCC*CACAGGGCAGUAA-	39%	3%	14.1	5.5
5	8	C*UUGCCCC*ACAGGGCAGUAA-	40%	7%	6.1	2.4
6	9	C*UUGCCCCA*CAGGGCAGUAA-	59%	5%	11.9	7.0
7	10	C*UUGCCCCAC*AGGGCAGUAA-	58%	6%	10.1	5.9
8	11	C*UUGCCCCACA*GGGCAGUAA-	53%	3%	20.3	10.9
9	12	C*UUGCCCCACAG*GGCAGUAA-	75%	21%	3.6	2.7
10	13	C*UUGCCCCACAGG*GCAGUAA-	66%	20%	3.3	2.2
11	14	C*UUGCCCCACAGGG*CAGUAA-	76%	19%	3.9	3.0
12	15	C*UUGCCCCACAGGGC*AGUAA-	0%	2%	0.0	0.0
13	16	C*UUGCCCCACAGGGCA*GUAA-	65%	27%	2.4	1.6
14	17	C*UUGCCCCACAGGGCAG*UAA-	81%	47%	1.7	1.4
15	18	C*UUGCCCCACAGGGCAGU*AA-	76%	34%	2.2	1.7
16	19	C*UUGCCCCACAGGGCAGUA*A-	62%	23%	2.7	1.7
17	control	C*UUGCCCCACAGGGCAGUAA-	75%	38%	2.0	1.5

FIG. 11A

Entry	Guide Sequence for *HBB* target in vivo	% Target Cleaved				ON : OFF1 Ratio	Specificity Score
		ON	OFF1	OFF2	OFF3		
1	CUUGCCCCACAGGGCAGUAA-	19%	9%	0%	0%	2.1	0.4
2	CUUGCCCCACAGGGCAGUAA-	41%	34%	0%	0%	1.2	0.5
3	CsUsUsGCCCCACAGGGCAGUAA-	66%	55%	0%	0%	1.2	0.8
4	C*sU*sU*sGCCCCACAGGGCAGUAA-	61%	19%	0%	0%	3.2	1.9

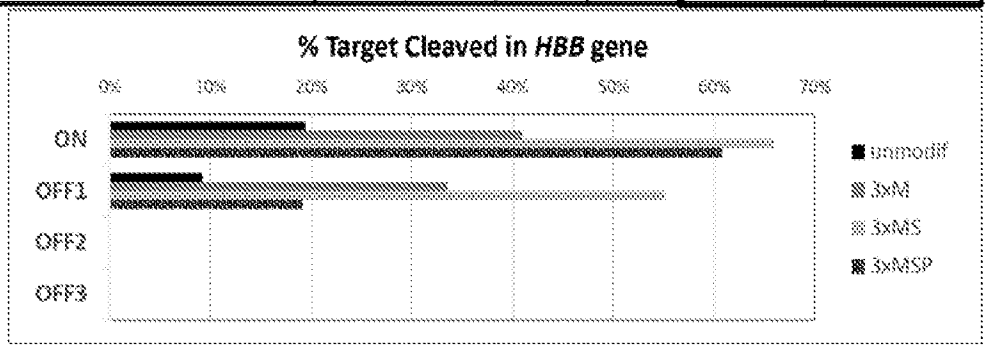


FIG. 11B

Entry	sgRNA Name	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
1	HBB_11MP_1xMP	53%	3%	20.3	10.9
2	HBB_5MP_1xMP	53%	3%	16.7	8.9
3	HBB_9MP_1xMP	59%	5%	11.9	7.0
4	HBB_10MP_1xMP	58%	6%	10.1	5.9
5	HBB_7MP_1xMP	39%	3%	14.1	5.5
6	HBB_14MP_1xMP	76%	19%	3.9	3.0
7	HBB_4MP_1xMP	30%	3%	10.1	3.0
8	HBB_12MP_1xMP	75%	21%	3.6	2.7
9	HBB_8MP_1xMP	40%	7%	6.1	2.4
10	HBB_13MP_1xMP	66%	20%	3.3	2.2
11	HBB_18MP_1xMP	78%	34%	2.2	1.7
12	HBB_19MP_1xMP	62%	23%	2.7	1.7
13	HBB_16MP_1xMP	65%	27%	2.4	1.6
14	HBB_1xMP (control)	75%	38%	2.0	1.5
15	HBB_6MP_1xMP	73%	37%	2.0	1.4
16	HBB_17MP_1xMP	81%	47%	1.7	1.4
17	HBB_15MP_1xMP	0%	2%	0.0	0.0
18	HBB_6,7MP_1xMP	50%	1%	50.0	25.0
19	HBB_10,17MP_1xMP	50%	1%	49.5	24.5
20	HBB_5,17MP_1xMP	46%	1%	46.1	21.3
21	HBB_5,16MP_1xMP	45%	1%	45.1	20.4
22	HBB_6,10MP_1xMP	61%	2%	34.5	20.9
23	HBB_10,16MP_1xMP	40%	1%	39.6	15.7
24	HBB_5,9MP_1xMP	65%	3%	23.5	15.3
25	HBB_9,16MP_1xMP	38%	1%	38.1	14.6
26	HBB_9,17MP_1xMP	37%	1%	37.5	14.0
27	HBB_6,8MP_1xMP	45%	2%	26.7	12.0
28	HBB_9,10MP_1xMP	50%	2%	24.0	12.0
29	HBB_10,13MP_1xMP	51%	2%	22.2	11.4
30	HBB_9,14MP_1xMP	34%	1%	33.5	11.3
31	HBB_9,17MP_1xMP	54%	3%	20.4	11.1
32	HBB_13,17MP_1xMP	32%	1%	31.9	10.2

Entry	sgRNA Name	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
33	HBB_7,14MP_1xMP	48%	2%	20.7	9.8
34	HBB_5,14MP_1xMP	46%	2%	19.8	9.2
35	HBB_5,6MP_1xMP	58%	4%	13.5	7.9
36	HBB_9,13MP_1xMP	54%	4%	12.8	7.0
37	HBB_6,11MP_1xMP	43%	3%	16.7	7.2
38	HBB_13,14MP_1xMP	75%	8%	9.6	7.2
39	HBB_6,9MP_1xMP	61%	6%	10.2	6.2
40	HBB_14,17MP_1xMP	23%	1%	23.3	5.4
41	HBB_7,17MP_1xMP	53%	6%	9.3	4.9
42	HBB_16,17MP_1xMP	21%	1%	20.9	4.4
43	HBB_8,14MP_1xMP	36%	2%	16.0	5.7
44	HBB_8,9MP_1xMP	27%	2%	17.5	4.7
45	HBB_8,13MP_1xMP	42%	4%	9.4	3.9
46	HBB_7,13MP_1xMP	39%	4%	10.2	3.9
47	HBB_8,16MP_1xMP	32%	3%	11.8	3.8
48	HBB_10,14MP_1xMP	26%	2%	14.0	3.7
49	HBB_7,16MP_1xMP	37%	4%	9.0	3.3
50	HBB_7,9MP_1xMP	32%	3%	10.1	3.3
51	HBB_5,8MP_1xMP	17%	1%	16.9	2.9
52	HBB_6,14MP_1xMP	64%	14%	4.5	2.9
53	HBB_6,13MP_1xMP	69%	16%	4.2	2.9
54	HBB_5,13MP_1xMP	35%	5%	7.7	2.7
55	HBB_6,16MP_1xMP	62%	17%	3.6	2.2
56	HBB_7,10MP_1xMP	21%	2%	8.4	1.7
57	HBB_6,17MP_1xMP	68%	37%	1.8	1.3
58	HBB_8,10MP_1xMP	16%	2%	7.8	1.2
59	HBB_1xMP (control)	69%	44%	1.6	1.1
60	HBB_13,16MP_1xMP	10%	1%	9.9	1.0
61	HBB_14,16MP_1xMP	9%	1%	9.0	0.8
62	HBB_5,10MP_1xMP	16%	3%	5.1	0.8
63	HBB_5,7MP_1xMP	18%	6%	2.9	0.5
64	HBB_5,11MP_1xMP	4%	3%	1.5	0.1

FIG. 12A

In K562 cells:

Entry	sgRNA Name or Type of Control	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
1	HBB_11MP_1xMP	69.6%	0.9%	80.2	55.8
2	HBB_5MP_1xMP	81.2%	3.0%	26.9	21.8
3	HBB_7MP_1xMP	53.5%	3.1%	17.2	9.2
4	HBB_10MP_1xMP	66.7%	9.1%	7.3	4.9
5	HBB_9MP_1xMP	65.0%	9.0%	7.2	4.7
6	HBB_13MP_1xMP	61.5%	19.6%	3.1	1.9
7	HBB_3xMS (control)	92.3%	52.6%	1.8	1.6
8	HBB_unmodif (control)	84.3%	46.5%	1.8	1.5
9	HBB_1xMP (control)	78.5%	44.4%	1.8	1.4
10	HBB_14MP_1xMP	34.9%	12.3%	2.8	1.0
11	HBB_17MP_1xMP	34.4%	22.0%	1.6	0.5
12	HBB_16MP_1xMP	9.1%	5.5%	1.7	0.2
13	HBB_6,10MP_1xMP	64.4%	4.4%	14.6	9.4
14	HBB_5,17MP_1xMP	25.8%	1.5%	17.4	4.5
15	HBB_6,7MP_1xMP	29.5%	2.7%	10.8	3.2
16	HBB_10,17MP_1xMP	24.9%	3.0%	8.3	2.1
17	HBB_5,16MP_1xMP	6.5%	0.2%	27.6	1.8
18	HBB_5,9MP_1xMP	12.6%	2.0%	6.3	0.8
19	HBB_9,17MP_1xMP	6.5%	0.8%	7.8	0.5
20	GFP transfection control	0.1%	0.1%		
21	Mock transfection control	0.2%	0.0%		

In iPS cells:

Entry	sgRNA Name or Type of Control	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
22	HBB_11MP_1xMP	45.1%	0.5%	96.1	43.3
23	HBB_5MP_1xMP	51.8%	1.8%	29.3	15.2
24	HBB_7MP_1xMP	39.2%	2.0%	19.9	7.8
25	HBB_10MP_1xMP	37.9%	6.1%	6.3	2.4
26	HBB_9MP_1xMP	32.5%	4.9%	6.6	2.1
27	HBB_13MP_1xMP	37.1%	19.7%	1.9	0.7
28	HBB_14MP_1xMP	25.2%	14.3%	1.8	0.4
29	HBB_3xMS (control)	31.3%	22.6%	1.4	0.4
30	HBB_1xMP (control)	34.0%	31.8%	1.1	0.4
31	HBB_unmodif (control)	25.3%	19.6%	1.3	0.3
32	HBB_16MP_1xMP	7.8%	6.2%	1.3	0.1
33	HBB_17MP_1xMP	18.4%	17.3%	1.0	0.2
34	HBB_6,10MP_1xMP	35.7%	2.0%	17.4	6.2
35	HBB_5,17MP_1xMP	16.2%	0.6%	29.1	4.7
36	HBB_5,16MP_1xMP	6.3%	0.1%	59.1	3.7
37	HBB_6,7MP_1xMP	21.2%	2.1%	10.8	2.1
38	HBB_10,17MP_1xMP	11.4%	1.1%	10.1	1.1
39	HBB_5,9MP_1xMP	9.8%	1.4%	6.5	0.6
40	HBB_9,17MP_1xMP	3.3%	0.6%	5.2	0.2
41	GFP transfection control	0.0%	0.0%		
42	Mock transfection control	0.0%	0.0%		

FIG. 12B

In K562 cells:

Entry	sgRNA Name or Type of Control	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
1	HBB_11MP_1xMP	69.6%	0.9%	80.2	55.8
2	HBB_5MP_1xMP	81.2%	3.0%	26.9	21.8
3	HBB_7MP_1xMP	53.5%	3.1%	17.2	9.2
4	HBB_10MP_1xMP	66.7%	9.1%	7.3	4.9
5	HBB_9MP_1xMP	65.0%	9.0%	7.2	4.7
6	HBB_13MP_1xMP	61.5%	19.6%	3.1	1.9
7	HBB_14MP_1xMP	34.9%	12.3%	2.8	1.0
8	HBB_3xMS (control)	92.3%	52.6%	1.8	1.6
9	HBB_unmodif (control)	84.3%	46.5%	1.8	1.5
10	HBB_1xMP (control)	78.5%	44.4%	1.8	1.4
11	HBB_16MP_1xMP	9.1%	5.5%	1.7	0.2
12	HBB_17MP_1xMP	34.4%	22.0%	1.6	0.5
13	HBB_5,16MP_1xMP	6.5%	0.2%	27.6	1.8
14	HBB_5,17MP_1xMP	25.8%	1.5%	17.4	4.5
15	HBB_6,10MP_1xMP	64.4%	4.4%	14.6	9.4
16	HBB_6,7MP_1xMP	29.5%	2.7%	10.8	3.2
17	HBB_10,17MP_1xMP	24.9%	3.0%	8.3	2.1
18	HBB_9,17MP_1xMP	6.5%	0.8%	7.8	0.5
19	HBB_5,9MP_1xMP	12.6%	2.0%	6.3	0.8
20	GFP transfection control	0.1%	0.1%		
21	Mock transfection control	0.2%	0.0%		

In iPS cells:

Entry	sgRNA Name or Type of Control	ON target	OFF1 target	ON : OFF1 ratio	Specificity Score
22	HBB_11MP_1xMP	45.1%	0.5%	96.1	43.3
23	HBB_5MP_1xMP	51.8%	1.8%	29.3	15.2
24	HBB_7MP_1xMP	39.2%	2.0%	19.9	7.8
26	HBB_9MP_1xMP	32.5%	4.9%	6.6	2.1
25	HBB_10MP_1xMP	37.9%	6.1%	6.3	2.4
27	HBB_13MP_1xMP	37.1%	19.7%	1.9	0.7
28	HBB_14MP_1xMP	25.2%	14.3%	1.8	0.4
29	HBB_3xMS (control)	31.3%	22.6%	1.4	0.4
31	HBB_unmodif (control)	25.3%	19.6%	1.3	0.3
32	HBB_16MP_1xMP	7.8%	6.2%	1.3	0.1
30	HBB_1xMP (control)	34.8%	31.8%	1.1	0.4
33	HBB_17MP_1xMP	18.4%	17.3%	1.0	0.2
34	HBB_5,16MP_1xMP	6.3%	0.1%	59.1	3.7
35	HBB_5,17MP_1xMP	16.2%	0.6%	29.1	4.7
36	HBB_6,10MP_1xMP	35.7%	2.0%	17.4	6.2
37	HBB_10,17MP_1xMP	11.4%	1.1%	10.1	1.1
38	HBB_6,7MP_1xMP	21.2%	2.1%	10.0	2.1
39	HBB_5,9MP_1xMP	9.0%	1.4%	6.5	0.6
40	HBB_9,17MP_1xMP	3.3%	0.6%	5.2	0.2
41	GFP transfection control	0.0%	0.0%		
42	Mock transfection control	0.0%	0.0%		

FIG. 12C

Entry	Internal MP position(s)	Guide Sequence	% Cleaved CLTA4		Specificity Score	% Cleaved CLTA4		Specificity Score	% Cleaved CLTA4		Specificity Score	% Cleaved IL2RG		Specificity Score	% Cleaved HBB		Specificity Score
			ON	OFF1		ON	OFF2		ON	OFF3		ON	OFF3		ON	OFF1	
1	4	N*NNNNNNNNNNNNNNNNNNN	87%	67%	1.1	87%	45%	1.7	87%	3%	30.0	63%	8%	5.2	30%	3%	3.0
2	5	N*NNNNNNNNNNNNNNNNNNN	90%	86%	0.9	90%	40%	2.0	90%	0%	165.9	76%	31%	1.9	53%	3%	8.9
3	6	N*NNNNNNNNNNNNNNNNNNN	89%	93%	0.8	89%	100%	0.8	89%	85%	0.9	75%	69%	0.8	73%	37%	1.4
4	7	N*NNNNNNNNNNNNNNNNNNN	73%	63%	0.8	73%	65%	0.8	73%	7%	7.1	65%	3%	12.3	39%	3%	5.5
5	8	N*NNNNNNNNNNNNNNNNNNN	82%	85%	0.8	82%	91%	0.7	82%	73%	0.9	73%	53%	1.0	40%	7%	2.4
6	9	N*NNNNNNNNNNNNNNNNNNN	85%	84%	0.9	85%	97%	0.7	85%	50%	1.4	73%	32%	1.6	59%	5%	7.0
7	10	N*NNNNNNNNNNNNNNNNNNN	81%	79%	0.8	81%	69%	1.0	81%	83%	0.8	64%	48%	0.9	58%	6%	5.9
8	11	N*NNNNNNNNNNNNNNNNNNN	71%	58%	0.9	71%	65%	0.8	71%	42%	1.2	65%	18%	2.4	53%	3%	10.9
9	12	N*NNNNNNNNNNNNNNNNNNN	92%	93%	0.9	92%	82%	1.0	92%	44%	1.9	84%	19%	7.0	75%	21%	2.7
10	13	N*NNNNNNNNNNNNNNNNNNN	93%	94%	0.9	93%	100%	0.9	93%	87%	1.0	95%	23%	4.2	66%	28%	2.2
11	14	N*NNNNNNNNNNNNNNNNNNN	96%	97%	0.9	96%	79%	1.2	96%	33%	2.8	96%	10%	9.3	76%	19%	3.0
12	15	N*NNNNNNNNNNNNNNNNNNN	17%	16%	0.2	17%	5%	0.6	17%	0%	6.0	29%	8%	1.1	0%	2%	0.0
13	16	N*NNNNNNNNNNNNNNNNNNN	92%	93%	0.9	92%	92%	0.9	92%	47%	1.8	96%	9%	10.2	65%	27%	1.6
14	17	N*NNNNNNNNNNNNNNNNNNN	92%	97%	0.9	92%	100%	0.8	92%	90%	0.9	95%	12%	7.3	81%	47%	1.4
15	18	N*NNNNNNNNNNNNNNNNNNN	90%	91%	0.9	90%	97%	0.8	90%	77%	1.0	92%	17%	4.9	76%	34%	1.7
16	19	N*NNNNNNNNNNNNNNNNNNN	89%	89%	0.9	89%	95%	0.8	89%	79%	1.0	92%	25%	3.3	62%	23%	1.7
17	20	N*NNNNNNNNNNNNNNNNNNN	90%	90%	0.9	90%	100%	0.8	90%	87%	0.9	87%	61%	1.2			
18	control	N*NNNNNNNNNNNNNNNNNNN	88%	86%	0.9	88%	93%	0.8	88%	64%	1.2	84%	68%	1.0	75%	38%	1.5

FIG. 13

Entry	Target Gene	sgRNA Name or Type of Control	ON target	OFF2 target	ON : OFF2 ratio	Specificity Score
1	*IL2RG*	IL2RG_unmodif (control)	98.5%	5.228%	18.8	18.6
2	*IL2RG*	IL2RG_1xMP	97.8%	0.094%	1,040	1,020
3	*IL2RG*	IL2RG_5MP_1xMP	93.2%	0.005%	18,600	17,400
4	*IL2RG*	IL2RG_11MP_1xMP	87.1%	0.008%	10,900	9,500
5	*VEGFA*	VEGFA_1xMP	74.1%	10.82%	6.8	5.1
6	*VEGFA*	VEGFA_5MP_1xMP	75.7%	0.19%	398	302
7	*VEGFA*	VEGFA_7MP_1xMP	16.5%	0.02%	825	136
8	*VEGFA*	VEGFA_9MP_1xMP	42.0%	0.22%	191	80
9	*VEGFA*	VEGFA_10MP_1xMP	23.7%	0.08%	296	70
10	*VEGFA*	VEGFA_11MP_1xMP	23.9%	0.11%	217	52

FIG. 14

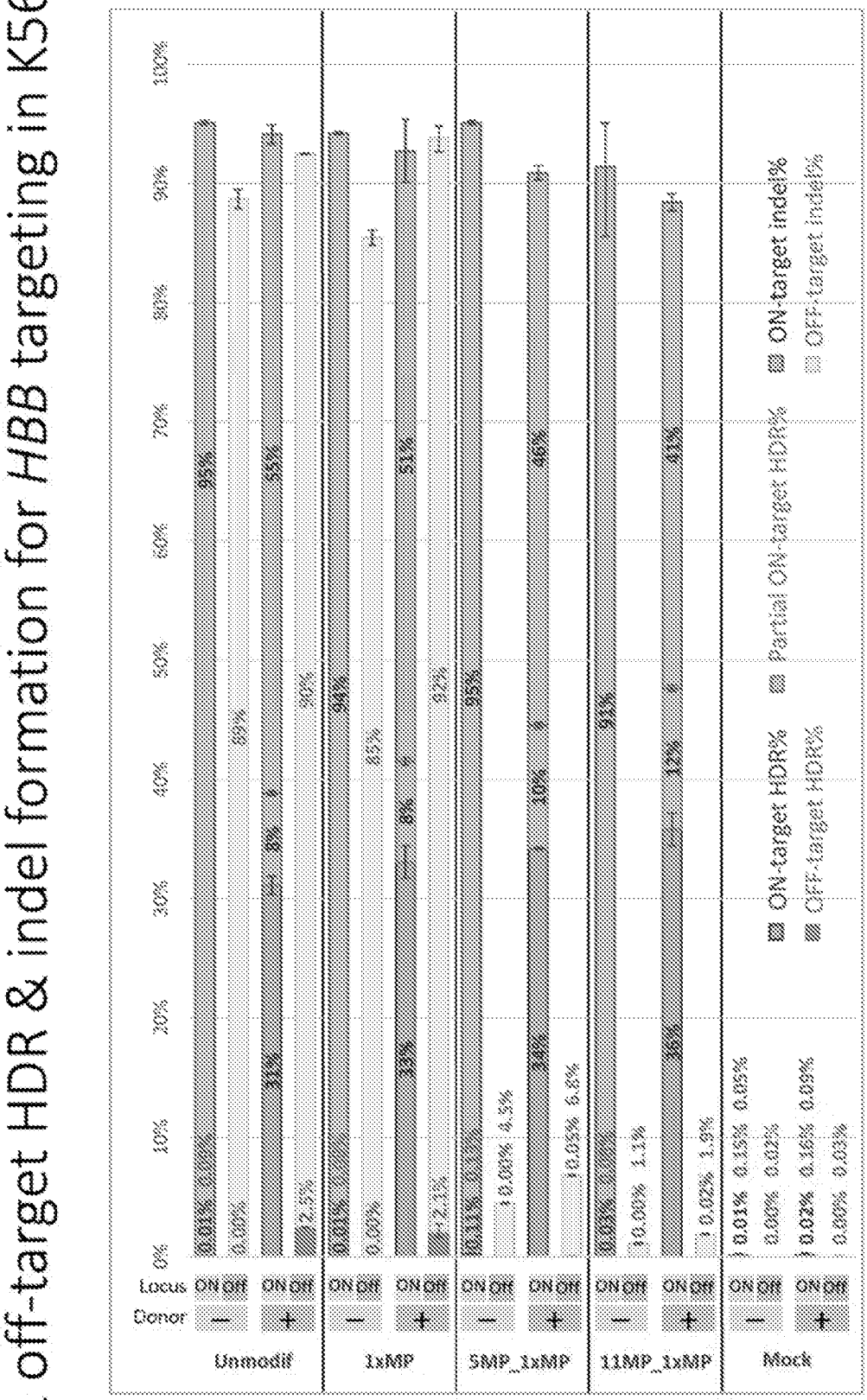

On- & off-target HDR & indel formation for HBB targeting in K562 cells
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Locus ON OFF ON OFF ON OFF ON OFF ON OFF
Donor – + – + – + – + –
Unmodif
1xMP
SMP_1xMP
11MP_1xMP
Mock
ON-target HDR%
Partial ON-target HDR%
ON-target indel%
OFF-target HDR%
OFF-target indel%

FIG. 15


On-target Editing of HBB and Off-target Editing of Similar Sequences in K562 Cells:
Deep Sequencing of PCR-amplified Loci from Treated Cells
% Indels Formed
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
HBB_unmodif
HBB_1xMP
HBB_5MP_1xMP
HBB_11MP_1xMP
Mock
HBB ON
OFF1
OFF2
OFF3
OFF4
OFF5
OFF6
OFF7
OFF8
OFF9
OFF10
OFF11
OFF12
OFF13
OFF14
OFF15
OFF16
On- or Off-target Sequence Name
89.1
89.4
87.2
84.3
76.0
82.6
0.03
1.50
0.84
0.03

FIG. 16

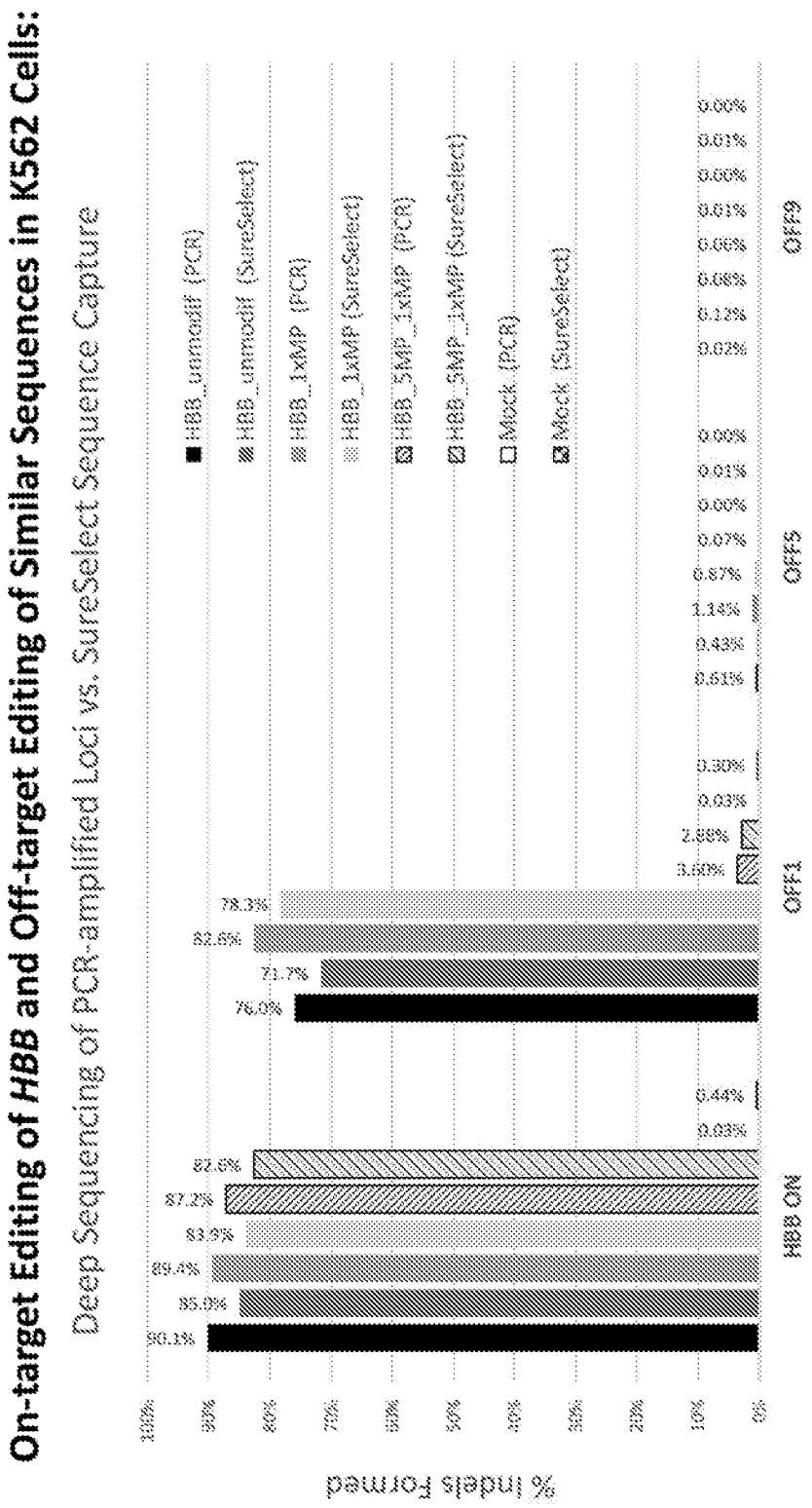

On-target Editing of HBB and Off-target Editing of Similar Sequences in K562 Cells:
Deep Sequencing of PCR-amplified Loci vs. SureSelect Sequence Capture
% Indels Formed
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
HBB_unmodif (PCR)
HBB_unmodif (SureSelect)
HBB_1xMP (PCR)
HBB_1xMP (SureSelect)
HBB_5MP_1xMP (PCR)
HBB_5MP_1xMP (SureSelect)
Mock (PCR)
Mock (SureSelect)
90.1%
85.0%
89.4%
83.9%
87.2%
82.6%
0.03%
0.44%
76.0%
71.7%
82.6%
78.3%
0.03%
0.30%
3.60%
2.88%
0.61%
0.43%
1.14%
0.67%
0.07%
0.00%
0.01%
0.00%
0.02%
0.12%
0.08%
0.06%
0.01%
0.00%
0.01%
0.00%
HBB ON
OFF1
OFF5
OFF9
On- or Off-target Sequence Name

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/036648

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/113(2010.01)i, C12N 15/10(2006.01)i, C12N 15/90(2006.01)i, C12N 9/22(2006.01)i, C12Q 1/44(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/113; C12N 15/90; A61P 25/28; C12N 9/22; A61K 47/48; C12N 15/85; C12N 15/10; C12Q 1/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: CRISPR RNA, guide RNA, CRISPR-Cas, modification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2016-0040189 A1 (AGILENT TECHNOLOGIES, INC.) 11 February 2016 See paragraphs [0029]-[0030], [0036], [0038], [0053], [0057]; and figures 3A-3D.	1-4
X	HENDEL et al., `Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells`, Nature Biotechnology, 29 June 2015(online), vol. 33, no. 9, pp. 985-989 See page 985; and figure 1.	1-4
A	FU et al., `Improving CRISPR-Cas nuclease specificity using truncated guide RNAs`, Nature Biotechnology, 26 January 2014(online), vol. 32, no. 3, pp. 279-284 See page 279.	1-4
A	WO 2015-139139 A1 (UNIVERSITE LAVAL) 24 September 2015 See abstract; and paragraph [0017].	1-4
A	US 2014-0273232 A1 (THE BROAD INSTITUTE, INC. et al.) 18 September 2014 See claims 1-9, 14-25.	1-4

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2017 (12.09.2017)	**12 September 2017 (12.09.2017)**

Name and mailing address of the ISA/KR
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Korean Intellectual Property Office
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/036648

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 10,13,15-16,18-19,24,33
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 10, 13, 15-16, 18-19, 24 and 33 are not clear because they refer to multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 5-9,11-12,14,17,20-23,25-32,34-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2017/036648

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016-0040189 A1	11/02/2016	WO 2016-022866 A1	11/02/2016
WO 2015-139139 A1	24/09/2015	AU 2015-234204 A1	06/10/2016
		CA 2942915 A1	24/09/2015
		EP 3126498 A1	08/02/2017
		JP 2017-514513 A	08/06/2017
US 2014-0273232 A1	18/09/2014	AU 2013-359123 A1	30/07/2015
		CA 2894701 A1	19/06/2014
		CN 105121648 A	02/12/2015
		EP 2771468 A1	03/09/2014
		EP 2771468 B1	11/02/2015
		HK 1202586 A1	02/10/2015
		IL 239344 A	30/07/2015
		PT 2771468 E	02/06/2015
		PT 2784162 E	27/08/2015
		US 2014-0242664 A1	28/08/2014
		US 2015-0232882 A1	20/08/2015
		US 2015-0247150 A1	03/09/2015
		US 8906616 B2	09/12/2014
		WO 2014-093712 A1	19/06/2014