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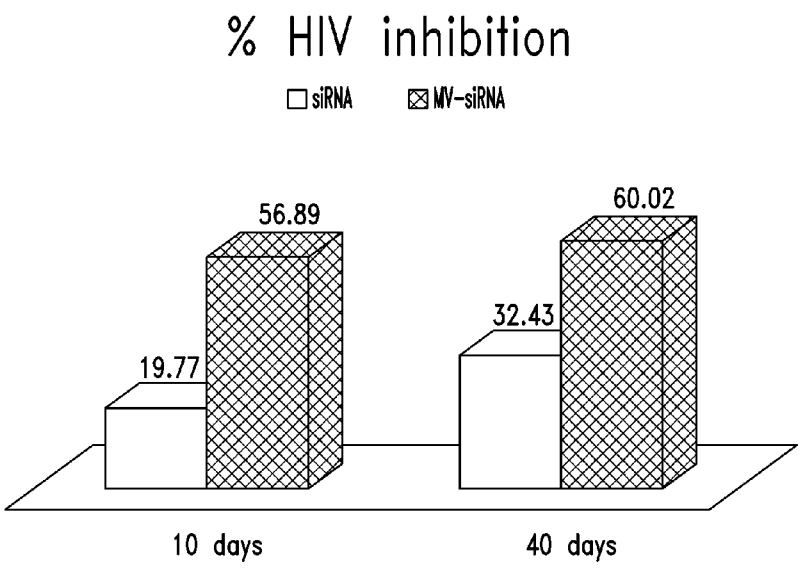

FIG. 9

(57) Abstract: The present invention includes bivalent or multivalent nucleic acid molecules or complexes of nucleic acid molecules having two or more target-specific regions, in which the target-specific regions are complementary to a single target gene at more than one distinct nucleotide site, and/or in which the target regions are complementary to more than one target gene or target sequence. Also included are compositions comprising such nucleic acid molecules and methods of using the same for multivalent RNA interference and the treatment of a variety of diseases and infections.

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POLYNUCLEOTIDES FOR MULTIVALENT RNA INTERFERENCE, COMPOSITIONS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application No. 61/183,011, filed June 1, 2009, which is incorporated by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 270038_405PC_SEQUENCE_LISTING.txt. The text file is 106 KB, was created on June 1, 2010, and is being submitted electronically via EFS-Web.

BACKGROUND

Technical Field

The present invention relates generally to precisely structured polynucleotide molecules, and methods of using the same for multivalent RNA interference and the treatment of disease.

Description of the Related Art

The phenomenon of gene silencing, or inhibiting the expression of a gene, holds significant promise for therapeutic and diagnostic purposes, as well as for the study of gene function itself. Examples of this phenomenon include antisense technology and dsRNA forms of posttranscriptional gene silencing (PTGS) which has become popular in the form of RNA interference (RNAi).

Antisense strategies for gene silencing have attracted much attention in recent years. The underlying concept is simple yet, in principle, effective: antisense nucleic acids (NA) base pair with a target RNA resulting in inactivation of the targeted RNA. Target RNA recognition by antisense RNA or DNA can be

considered a hybridization reaction. Since the target is bound through sequence complementarity, this implies that an appropriate choice of antisense NA should ensure high specificity. Inactivation of the targeted RNA can occur via different pathways, dependent on the nature of the antisense NA (either modified or unmodified DNA or RNA, or a hybrid thereof) and on the properties of the biological system in which inhibition is to occur.

RNAi based gene suppression is a widely accepted method in which a sense and an antisense RNA form double-stranded RNA (dsRNA), *e.g.*, as a long RNA duplex, a 19-24 nucleotide duplex, or as a short-hairpin dsRNA duplex (shRNA), which is involved in gene modulation by involving enzyme and/or protein complex machinery. The long RNA duplex and the shRNA duplex are pre-cursors that are processed into small interfering RNA (siRNA) by the endoribonuclease described as Dicer. The processed siRNA or directly introduced siRNA is believed to join the protein complex RISC for guidance to a complementary gene, which is cleaved by the RISC/siRNA complex.

However, many problems persist in the development of effective antisense and RNAi technologies. For example, DNA antisense oligonucleotides exhibit only short-term effectiveness and are usually toxic at the doses required; similarly, the use of antisense RNAs has also proved ineffective due to stability problems. Also, the siRNA used in RNAi has proven to result in significant off-target suppression due to either strand guiding cleaving complexes potential involvement in endogenous regulatory pathways. Various methods have been employed in attempts to improve antisense stability by reducing nuclease sensitivity and chemical modifications to siRNA have been utilized. These include modifying the normal phosphodiester backbone, *e.g.,* using phosphorothioates or methyl phosphonates, incorporating 2'-OMe-nucleotides, using peptide nucleic acids (PNAs) and using 3'-terminal caps, such as 3'-aminopropyl modifications or 3'-3' terminal linkages. However, these methods can be expensive and require additional steps. In addition, the use of non-naturally occurring nucleotides and modifications precludes the ability to express the antisense or siRNA sequences *in vivo*, thereby requiring them to be synthesized and administered afterwards.

Additionally, the siRNA duplex exhibits primary efficacy to a single gene and off-target to a secondary gene. This unintended effect is negative and is not a reliable RNAi multivalence.

Consequently, there remains a need for effective and sustained methods and compositions for the targeted, direct inhibition of gene function *in vitro* and *in vivo*, particularly in cells of higher vertebrates, including a single-molecule complex capable of multivalent gene inhibition.

BRIEF SUMMARY

The present invention provides novel compositions and methods, which include precisely structured oligonucleotides that are useful in specifically regulating gene expression of one or more genes simultaneously when the nucleotide target site sequence of each is not identical to the other.

In certain embodiments, the present invention includes an isolated precisely structured three-stranded polynucleotide complex comprising a region having a sequence complementary to a target gene or sequence at multiple sites or complementary to multiple genes at single sites.

In certain embodiments, the present invention includes an isolated precisely structured the polynucleotide comprising a region having a sequence complementary to a target gene or sequence at multiple sites or complementary to multiple genes at single sites; each having partially self-complementary regions. In particular embodiments, the oligonucleotide comprises two or more self-complementary regions. In certain embodiments, the polynucleotides of the present invention comprise RNA, DNA, or peptide nucleic acids.

Certain embodiments relate to polynucleotide complexes of at least three separate polynucleotides, comprising (a) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region; (b) a second polynucleotide comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (c) a third polynucleotide comprising a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region, wherein each

of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence, wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide, and wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

In certain embodiments, the first, second, and/or third polynucleotide comprises about 15-30 nucleotides. In certain embodiments, the first, second, and/or third polynucleotide comprises about 17-25 nucleotides. In certain embodiments, one or more of the self-complementary regions comprises about 5-10 nucleotide pairs. In certain embodiments, one or more of the self-complementary regions comprises about 7-8 nucleotide pairs.

In certain embodiments, each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA. In certain embodiments, at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

In certain embodiments, all or a portion of the 5' and/or 3' region of each polynucleotide is also complementary to the target sequence for that polynucleotide. In certain embodiments, one or more of the self-complementary regions comprises a 3' overhang.

Certain embodiments relate to self-hybridizing polynucleotide molecules, comprising (a) a first nucleotide sequence comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region, (b) a second nucleotide sequence comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (c) a third nucleotide sequence comprising a null region or a target-specific region that is complementary to a third target sequence, a 5' region, and a 3' region, wherein the target-specific regions of each of the first, second, and third nucleotide sequences

are complementary to a different target sequence, wherein the 5' region of the first nucleotide sequence is complementary to the 3' region of the third nucleotide sequence, wherein the 3' region of the first nucleotide sequence is complementary to the 5' region of the second nucleotide sequence, and wherein the 3' region of the second nucleotide sequence is complementary to the 5' region of the third nucleotide sequence, and wherein each of the 5' regions hybridizes to their complementary 3' regions to form a self-hybridizing polynucleotide molecule with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

In certain embodiments, the first, second, or third polynucleotide sequences comprise about 15-60 nucleotides. In certain embodiments, the target-specific region comprises about 15-30 nucleotides. In certain embodiments, one or more of the self-complementary regions comprises about 10-54 nucleotides. In certain embodiments, one or more of the self-complementary regions comprises a 3' overhang. In certain embodiments, one or more of the self-complementary regions forms a stem-loop structure. In certain embodiments, one or more of the self-complementary regions comprises a proximal box of dinucleotides AG/UU that is outside of the target specific region. In certain embodiments, one or more of the self-complementary regions comprises a distal box of 4 nucleotides that is outside of the target-specific region, wherein the third nucleotide of the distal box is not a G. Also included are vectors that encode a self-hybridizing polynucleotide molecule, as described herein.

In certain embodiments, each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA. In certain embodiments, at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

In certain embodiments, a self-complementary region comprises a stem-loop structure composed of a bi-loop, tetraloop or larger loop. In certain embodiments, the sequence complementary to a target gene sequence comprises at least 17 nucleotides, or 17 to 30 nucleotides, including all integers in between.

In certain embodiments, the self-complementary region (or double-stranded region) comprises at least 5 nucleotides, at least 6 nucleotides, at least 24 nucleotides, or 12 to 54 or 60 nucleotides, including all integers in between.

In certain embodiments, a loop region of a stem-loop structure comprises at least 1 nucleotide. In certain embodiments, the loop region comprises at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 nucleotides.

In further embodiments, a loop region of a stem-loop structure is comprised of a specific tetra-loop sequence NGNN or AAGU or UUUU or UUGA or GUUA, where these sequences are 5' to 3'.

In a further embodiment, the present invention includes an expression vector capable of expressing a polynucleotide of the present invention. In various embodiments, the expression vector is a constitutive or an inducible vector.

The present invention further includes a composition comprising a physiologically acceptable carrier and a polynucleotide of the present invention.

In other embodiments, the present invention provides a method for reducing the expression of a gene, comprising introducing a polynucleotide complex or molecule of the present invention into a cell. In various embodiments, the cell is plant, animal, protozoan, viral, bacterial, or fungal. In one embodiment, the cell is mammalian.

In some embodiments, the polynucleotide complex or molecule is introduced directly into the cell, while in other embodiments, the polynucleotide complex or molecule is introduced extracellularly by a means sufficient to deliver the isolated polynucleotide into the cell.

In another embodiment, the present invention includes a method for treating a disease, comprising introducing a polynucleotide complex or molecule of the present invention into a cell, wherein overexpression of the targeted gene is associated with the disease. In one embodiment, the disease is a cancer.

The present invention further provides a method of treating an infection in a patient, comprising introducing into the patient a polynucleotide complex or molecule of the present invention, wherein the isolated polynucleotide

mediates entry, replication, integration, transmission, or maintenance of an infective agent.

In yet another related embodiment, the present invention provides a method for identifying a function of a gene, comprising introducing into a cell a polynucleotide complex or molecule of the present invention, wherein the polynucleotide complex or molecule inhibits expression of the gene, and determining the effect of the introduction of the polynucleotide complex or molecule on a characteristic of the cell, thereby determining the function of the targeted gene. In one embodiment, the method is performed using high throughput screening.

In a further embodiment, the present invention provides a method of designing a polynucleotide sequence comprising two or more self-complementary regions for the regulation of expression of a target gene, comprising: (a) selecting the first three guide sequences 17 to 25 nucleotides in length and complementary to a target gene or multiple target genes; (b) selecting one or more additional sequences 4 to 54 nucleotides in length, which comprises self-complementary regions and which are not fully-complementary to the first sequence; and optionally (c) defining the sequence motif in (b) to be complementary, non-complementary, or replicate a gene sequence which are non-complementary to the sequence selected in step (a).

In another embodiment, the mutated gene is a gene expressed from a gene encoding a mutant p53 polypeptide. In another embodiment, the gene is viral, and may include one or more different viral genes. In specific embodiments, the gene is an HIV gene, such as gag, pol, env, or tat, among others described herein and known in the art. In other embodiments, the gene is ApoB.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figures 1 through 6 illustrate exemplary polynucleotide structures of the present invention.

Figure 1 shows a polynucleotide complex of three separate polynucleotide molecules. (A) indicates the region comprising sequence

complementary to a site on a target gene (hatched); (B) indicates the region comprising sequence complementary to a second site on the target gene or a site on a different gene (cross-hatched); (C) indicates the region comprising sequence complementary to a third site on the target gene or a site on a different gene (filled in black). The numbers 1, 2, and 3 indicate the 3' end of each oligonucleotide that guides gene silencing; (A) loads in the direction of 1, (B) in the direction 2, and (C) in the direction 3. The 3' and 5' regions of each molecule, which hybridize to each other to form their respective self-complementary or double-stranded regions, are indicated by connecting bars. Each polynucleotide comprises a two nucleotide 3' overhang.

Figure 2 shows a single, self-hybridizing polynucleotide of the invention, having three single-stranded regions and three self-complementary regions, which is a precursor for processing into a core molecule. The target specific regions are darkened. (D) indicates a self-complementary stem-loop region (filled in white) capped with a tetraloop of four nucleotides; (D) also indicates a stem-loop region having a 14/16 nucleotide cleavage site within the stem-loop structure; cleavage may occur by RNase III to remove the stem loop nucleotides shown in white); (E) indicates a distal box wherein the third nucleotide as determined 5' to 3' is not a G, since it is believed that the presence of a G would block RNase III cleavage required for removal of the stem-loop region; (F) indicates a proximal box of dinucleotides AG/UU, which is an in vivo determinante of RNase III recognition and binding of RNase III (Nichols 2000); (G) indicates a tetraloop. The polynucleotide molecule shown in Figure 2 is a longer transcript RNA that is 'pre-processed' in the cell by RNase III. The resulting RNA structure is identical to the structure depicted in Figure 1.

Figure 3 depicts a self-forming single-stranded oligonucleotide with tetraloop formats. (H) indicates a tetraloop; (I) indicates a tri-loop connecting two core strands when the leading strand incorporates a 2 nucleotide overhang. In this structure, tetraloops are used to mimic what would be a 3' hydroxyl/5' phosphate of the overhangs in the structure shown in Figure 1 and function more directly than those of the structure shown in Figure 2. As demonstrated in Example 2, this short

tetraloop format guides silencing directly without pre-processing. It is believed that the GUUA loop twists the nucleotides in the loop and expose the hydrogens (*see, e.g.*, Nucleic Acids Research, 2003, Vol. 31, No. 3, Fig. 6, page 1094). This structure is compatible with PAZ or RISC.

Figure 4 depicts a self-forming single stranded oligonucleotide for divalent use. (J) indicates a larger loop connecting two core strands; (K) indicates the key strand as completing the complex formation, but "null" to a target gene, *i.e.,* not-specific for a target gene. The two target specific regions are shaded. This structure is a composition for 'divalent' use when working with RNA transcripts. Since chemical modifications are not possible, the structure determines asymmetrically of loading and silencing activity. The first 19 nucleotides of the molecule is the PRIMARY strand, (K) indicates a KEY strand that is deactivated, and the SECONDARY strand is the last 21 nucleotides of the molecule. The first priority of loading into RISC and functioning is the SECONDARY strand by exposed 5'/3' ends. The next priority is the PRIMARY strand, which is exposed after RNase III pre-processing in the cell. The 3' end of the nullified KEY strand is not functional, since the large loop is not processed nor is compatible with loading into RISC itself.

Figure 5 depicts a polynucleotide complex of the present invention having modified RNA bases. (L), (M), and (N) illustrate regions (defined by hashed lines) in which the Tm can be incrementally increased by the use of modified RNA (*e.g.,* 2'-O-methyl RNA or 2'-fluoro RNA instead of 2'-OH RNA) to preference the annealing and/or the silencing order of ends 1, 2 or 3.

Figure 6 depicts two embodiments of oligonucleotide complexes of the present invention. (O) illustrates a blunt-ended DNA strand that deactivates the silencing function of this strand; and (P) illustrates an end that can be utilized for conjugation of a delivery chemistry, ligand, antibody, or other payload or targeting molecule.

Figure 7 shows the results of suppression of GFP expression by multivalent-siRNA molecules of the invention, as compared to standard shRNA molecules (*see* Example 1). Figure 7A shows increased suppression of GFP by

MV clone long I (108%) and MV clone long II (119%), relative to shRNA control (set at 100%). Figure 7B shows increased suppression of GFP expression by synthetic MV-siRNA GFP I (127%), relative to shRNA control (set at 100%), which is slightly reduced when one of the strands of the synthetic MV-siRNA complex is replaced by a DNA strand (MF-siRNA GFP I DNA (116%)).

Figure 8 shows exemplary targeting regions (underlined) for the GFP coding sequence (SEQ ID NO:8). Figure 8A shows the regions that were targeted by the MV-siRNA molecules of Tables 1 and 2 in Example 1. Figures 8B and 8C show additional exemplary targeting regions.

Figure 9 shows the inhibitory effects of MV-siRNA molecules on HIV replication, in which a di-valent MV-siRNA targeted to both gag and tat has a significantly greater inhibitory effect on HIV replication than an siRNA targeted to gag only. The di-valent MV-siRNA exhibited 56.89% inhibition at 10 days and 60.02% inhibition at 40 days, as compared to the siRNA targeted to gag alone, which exhibited 19.77% inhibition at 10 days and 32.43% inhibition at 40 days.

Figure 10 shows the nucleotide sequence of an exemplary HIV genome (SEQ ID NO:9), which can be targeted according to the MV-siRNA molecules of the present invention. This sequence extends from Figure 10A through Figure 10D.

Figure 11 shows the nucleotide sequence of the env gene (SEQ ID NO:4), derived from the HIV genomic sequence of Figure 10.

Figure 12 provides addition HIV sequences. Figure 12A shows the nucleotide sequence of the gag gene (SEQ ID NO:2), and Figure 12B shows the nucleotide sequence of the tat gene (SEQ ID NO:3), both of which are derived from the HIV genomic sequence of Figure 10.

Figure 13 shows the coding sequence of murine apolipoprotein B (ApoB) (SEQ ID NO:10), which can be targeted using certain MV-siRNAs provided herein. This sequence extends from Figure 13A through Figure 13E.

Figure 14 shows the mRNA sequence of human apolipoprotein B (apoB) (SEQ ID NO:1), which can be targeted using certain MV-siRNAs provided herein. This sequence extends from Figure 14A through Figure 14E.

DETAILED DESCRIPTION

The present invention provides novel compositions and methods for inhibiting the expression of a gene at multiple target sites, or for inhibiting the expression of multiple genes at one or more target sites, which sites are not of equivalent nucleotide sequences, in eukaryotes *in vivo* and *in vitro*. In particular, the present invention provides polynucleotide complexes and polynucleotide molecules comprising two, three, or more regions having sequences complementary to regions of one or more target genes, which are capable of targeting and reducing expression of the target genes. In various embodiments, the compositions and methods of the present invention may be used to inhibit the expression of a single target gene by targeting multiple sites within the target gene or its expressed RNA. Alternatively, they may be used to target two or more different genes by targeting sites within two or more different genes or their expressed RNAs.

The present invention offers significant advantages over traditional siRNA molecules. First, when polynucleotide complexes or molecules of the present invention target two or more regions within a single target gene, they are capable of achieving greater inhibition of gene expression from the target gene, as compared to an RNAi agent that targets only one region within a target gene. In addition, polynucleotide complexes or molecules of the present invention that target two or more different target genes may be used to inhibit the expression of multiple target genes associated with a disease or disorder using a single polynucleotide complex or molecule. Furthermore, polynucleotide complexes and molecules of the present invention do not require the additional non-targeting strand present in conventional double-stranded RNAi agents, so they do not have off-target effects caused by the non-targeting strand. Accordingly, the polynucleotide complexes and molecules of the present invention offer surprising advantages over polynucleotide inhibitors of the prior art, including antisense RNA and RNA interference molecules, including increased potency and increased effectiveness against one or more target genes.

The present invention is also based upon the recognition of the polynucleotide structure guiding a protein complex for cleavage using only one, two, or three of the guide strands, which are complementary to one, two, or three distinct nucleic sequences of the target genes. This multivalent function results in a markedly broader and potent inhibition of a target gene or group of target genes than that of dsRNA, while utilizing many of the same endogenous mechanisms.

Certain embodiments of the present invention are also based upon the recognition of the polynucleotide structure directionally by presentation of the 3' overhangs and 5' phosphate resulting in a sense strand free complex, which contributes to greater specificity than that of dsRNA-based siRNA.

Given their effectiveness, the compositions of the present invention may be delivered to a cell or subject with an accompanying guarantee of specificity predicted by the single guide strand complementary to the target gene or multiple target genes.

Multivalent siRNAs

The present invention includes polynucleotide complexes and molecules that comprise two or more targeting regions complementary to regions of one or more target genes. The polynucleotide complexes and molecules of the present invention may be referred to as multivalent siRNAs (mv-siRNAs), since they comprise at least two targeting regions complementary to regions of one or more target genes. Accordingly, the compositions and methods of the present invention may be used to inhibit or reduce expression of one or more target genes, either by targeting two or more regions within a single target gene, or by targeting one or more regions within two or more target genes.

In certain embodiments, polynucleotide complexes of the present invention comprise three or more separate oligonucleotides, each having a 5' and 3' end, with two or more of the oligonucleotides comprising a targeting region, which oligonucleotides hybridize to each other as described herein to form a complex. Each of the strands is referred to herein as a "guide strand." In other embodiments, polynucleotide molecules of the present invention are a single

polynucleotide that comprises three or more guide strands, with two or more of the guide strands comprising a targeting region, which polynucleotide hybridizes to itself through self-complementary regions to form a structure described herein. The resulting structure may then be processed, *e.g.*, intracellularly, to remove loop structures connecting the various guide strands. Each guide strand, which may be present in different oligonucleotides or within a single polynucleotide, comprises regions complementary to other guide strands.

In certain embodiments, the present invention provides polynucleotide complexes and molecules that comprise at least three guide strands, at least two of which comprise regions that are complementary to different sequences within one or more target genes. In various embodiments, the polynucleotide complexes of the present invention comprise two, three or more separate polynucleotides each comprising one or more guide strands, which can hybridize to each other to form a complex. In other embodiments, the polynucleotide molecules of the present invention comprise a single polynucleotide that comprises three or more guide strands within different regions of the single polynucleotide.

Certain embodiments of the present invention are directed to polynucleotide complexes or molecules having at least three guide strands, two or more of which are partially or fully complementary to one or more target genes; and each having about 4 to about 12, about 5 to about 10, or preferably about 7 to about 8, nucleotides on either end that are complementary to each other (*i.e.*, complementary to a region of another guide strand), allowing the formation of a polynucleotide complex (*see, e.g.,* Figure 1). For example, each end of a guide strand may comprise nucleotides that are complementary to nucleotides at one end of another of the guide strands of the polynucleotide complex or molecule. Certain embodiments may include polynucleotide complexes that comprise 4, 5, 6 or more individual polynucleotide molecules or guide strands.

In certain embodiments, a polynucleotide complex of the present invention comprises at least three separate polynucleotides, which include: (1) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region; (2) a second polynucleotide

comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (3) a third polynucleotide comprising either a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region, wherein each of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence, wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide, and wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

As described above, in particular embodiments, a polynucleotide complex of the present invention comprises at least three separate oligonucleotides, each having a 5' end and a 3' end. As depicted in Figure 1, a region at the 5' end of the first oligonucleotide anneals to a region at the 3' end of the third oligonucleotide; a region at the 5' end of the third oligonucleotide anneals to a region at the 3' end of the second oligonucleotide; and a region at the 5' end of the second oligonucleotide anneals to a region at the 3' end of the first oligonucleotide. If additional oligonucleotides are present in the complex, then they anneal to other oligonucleotides of the complex in a similar manner. The regions at the ends of the oligonucleotides that anneal to each other may include the ultimate nucleotides at either or both the 5' and/or 3' ends. Where the regions of both the hybridizing 3' and 5' ends include the ultimate nucleotides of the oligonucleotides, the resulting double-stranded region is blunt-ended. In particular embodiments, the region at the 3' end that anneals does not include the ultimate and/or penultimate nucleotides, resulting in a double-stranded region having a one or two nucleotide 3' overhang.

In certain embodiments, the guide strands are present in a single polynucleotide molecule, and hybridize to form a single, self-hybridizing

polynucleotide with three single-stranded regions and three self-complementary regions (or double-stranded regions), and at least two target-specific regions (*see, e.g.,* Figure 2). In related embodiments, a single molecule may comprise at least 3, at least 4, at least 5 or at least 6 guide strands, and forms a single, self-hybridizing polynucleotide with at least 3, at least 4, at least 5, or at least 6 self-complementary regions (or double-stranded regions), and at least 2, at least 3, at least 4, or at least 5 target-specific regions, respectively. In particular embodiments, this single, self-hybridizing polynucleotide is a precursor molecule that may be processed by the cell to remove the loop regions and, optionally, an amount of proximal double-stranded region, resulting in an active mv-siRNA molecule (*see, e.g.*, Figure 2).

Thus, in particular embodiments, the present invention includes a self-hybridizing polynucleotide molecule, comprising: (1) a first nucleotide sequence comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region, (2) a second nucleotide sequence comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and (3) a third nucleotide sequence comprising a null region or a target-specific region that is complementary to a third target sequence, a 5' region, and a 3' region, wherein the target-specific regions of each of the first, second, and third nucleotide sequences are complementary to a different target sequence, wherein the 5' region of the first nucleotide sequence is complementary to the 3' region of the third nucleotide sequence, wherein the 3' region of the first nucleotide sequence is complementary to the 5' region of the second nucleotide sequence, and wherein the 3' region of the second nucleotide sequence is complementary to the 5' region of the third nucleotide sequence, and wherein each of the 5' regions hybridizes to their complementary 3' regions to form a self-hybridizing polynucleotide molecule with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

In particular embodiments, a single, self-hybridizing polynucleotide of the present invention may comprise one or more cleavable nucleotides in the single-stranded loops that form when the polynucleotide is annealed to itself. Once the single, self-hybridizing polynucleotide is annealed to itself, the cleavable

nucleotides may be cleaved to result in a polynucleotide complex comprising three or more separate oligonucleotides. Examples of cleavable nucleotides that may be used according to the present invention include, but are not limited to, photocleavable nucleotides, such as pcSpacer (Glen Research Products, Sterling, VA, USA), or phosphoramadite nucleotides.

As used herein, polynucleotides complexes and molecules of the present invention include isolated polynucleotides comprising three single-stranded regions, at least two of which are complementary to two or more target sequences, each target sequence located within one or more target genes, and comprising at least two or three self-complementary regions interconnecting the 5' or 3' ends of the single-stranded regions, by forming a double-stranded region, such as a stem-loop structure. The polynucleotides may also be referred to herein as the oligonucleotides.

In certain embodiments, the polynucleotide complexes and molecules of the present invention comprise two or more regions of sequence complementary to a target gene. In particular embodiments, these regions are complementary to the same target genes or genes, while in other embodiments, they are complementary to two or more different target genes or genes.

Accordingly, the present invention includes one or more self-complementary polynucleotides that comprise a series of sequences complementary to one or more target genes or genes. In particular embodiments, these sequences are separated by regions of sequence that are non-complementary or semi-complementary to a target gene sequence and non-complementary to a self-complementary region. In other embodiments of the polynucleotide comprising multiple sequences that are complementary to target genes or genes, the polynucleotide comprises a self-complementary region at the 5' end, 3 end', or both ends of one or more regions of sequence complementary to a target gene. In a particular embodiment, a polynucleotide comprises two or more regions of sequence complementary to one or more target genes, with self-complementary regions located at the 5' and 3' end of each guide strand that is complementary to a target gene. In certain embodiments, all or a portion of these

3' and 5' regions may be complementary to the target sequence, in addition to being complementary to their corresponding 3' or 5' regions.

The term "complementary" refers to nucleotide sequences that are fully or partially complementary to each other, according to standard base pairing rules. The term "partially complementary" refers to sequences that have less than full complementarity, but still have a sufficient number of complementary nucleotide pairs to support binding or hybridization within the stretch of nucleotides under physiological conditions.

In particular embodiments, the region of a guide strand complementary to a target gene (*i.e.*, the targeting region) may comprise one or more nucleotide mismatches as compared to the target gene. Optionally, the mismatched nucleotide(s) in the guide strand may be substituted with an unlocked (UNA) nucleic acid or a phosphoramidite nucleic acid (*e.g.*, rSpacer, Glen Research , Sterling, VA, USA), to allow base-pairing, *e.g.*, Watson-Crick base pairing, of the mismatched nucleotide(s) to the target gene.

As used herein, the term "self-complementary" or "self-complementary region" may refer to a region of a polynucleotide molecule of the invention that binds or hybridizes to another region of the same molecule to form A-T(U) and G-C hybridization pairs, thereby forming a double stranded region; and/or it may refer to a region of a first nucleotide molecule that binds to a region of a second or third nucleotide molecule to form a polynucleotide complex of the invention (*i.e.,* an RNAi polynucleotide complex), wherein the complex is capable of RNAi interference activity against two or more target sites. The two regions that bind to each other to form the self-complementary region may be contiguous or may be separated by other nucleotides. Also, as in an RNAi polynucleotide complex, the two regions may be on separate nucleotide molecules.

In certain embodiments, a "self complementary region" comprises a "3' region" of a first defined nucleotide sequence that is bound or hybridized to a "5' region" of a second or third defined nucleotide sequence, wherein the second or third defined sequence is within the same molecule – to form a self-hybridizing polynucleotide molecule. In certain embodiments, a "self complementary region"

comprises a "3' region" of a first polynucleotide molecule that is bound or hybridized to a "5' region" of a separate polynucleotide molecule, to form a polynucleotide complex. These 3' and '5 regions are typically defined in relation to their respective target-specific region, in that the 5' regions are on the 5' end of the target-specific region and the 3' regions are on the 3' end of the target specific region. In certain embodiments, one or both of these 3' and 5' regions not only hybridize to their corresponding 3' or 5' regions to form a self-complementary region, but may be designed to also contain full or partial complementarity their respective target sequence, thereby forming part of the target-specific region. In these embodiments, the target-specific region contains both a single-stranded region and self-complementary (*i.e.,* double-stranded) region.

In certain embodiments, these "self-complementary regions" comprise about 5-12 nucleotide pairs, preferably 5-10 or 7-8 nucleotide pairs, including all integers in between. Likewise, in certain embodiments, each 3' region or 5' region comprises about 5-12 nucleotides, preferably 5-10 or 7-8 nucleotides, including all integers in between.

The term "non-complementary" indicates that in a particular stretch of nucleotides, there are no nucleotides within that align with a target to form A-T(U) or G-C hybridizations. The term "semi-complementary" indicates that in a stretch of nucleotides, there is at least one nucleotide pair that aligns with a target to form an A-T(U) or G-C hybridizations, but there are not a sufficient number of complementary nucleotide pairs to support binding within the stretch of nucleotides under physiological conditions.

The term "isolated" refers to a material that is at least partially free from components that normally accompany the material in the material's native state. Isolation connotes a degree of separation from an original source or surroundings. Isolated, as used herein, *e.g.,* related to DNA, refers to a polynucleotide that is substantially away from other coding or non-coding sequences, and that the DNA molecule can contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally

isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In various embodiments, a polynucleotide complex or molecule of the present invention comprises RNA, DNA, or peptide nucleic acids, or a combination of any or all of these types of molecules. In addition, a polynucleotide may comprise modified nucleic acids, or derivatives or analogs of nucleic acids. General examples of nucleic acid modifications include, but are not limited to, biotin labeling, fluorescent labeling, amino modifiers introducing a primary amine into the polynucleotide, phosphate groups, deoxyuridine, halogenated nucleosides, phosphorothioates, 2'-O-Methyl RNA analogs, chimeric RNA analogs, wobble groups, universal bases, and deoxyinosine.

A "subunit" of a polynucleotide or oligonucleotide refers to one nucleotide (or nucleotide analog) unit. The term may refer to the nucleotide unit with or without the attached intersubunit linkage, although, when referring to a "charged subunit", the charge typically resides within the intersubunit linkage (*e.g.*, a phosphate or phosphorothioate linkage or a cationic linkage). A given synthetic MV-siRNA may utilize one or more different types of subunits and/or intersubunit linkages, mainly to alter its stability, Tm, RNase sensitivity, or other characteristics, as desired. For instance, certain embodiments may employ RNA subunits with one or more 2'-O-methyl RNA subunits.

The cyclic subunits of a polynucleotide or an oligonucleotide may be based on ribose or another pentose sugar or, in certain embodiments, alternate or modified groups. Examples of modified oligonucleotide backbones include, without limitation, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, phosphorodiamidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-

3' or 2'-5' to 5'-2'. Also contemplated are peptide nucleic acids (PNAs), locked nucleic acids (LNAs), 2'-O-methyl oligonucleotides (2'-OMe), 2'-methoxyethoxy oligonucleotides (MOE), among other oligonucleotides known in the art.

The purine or pyrimidine base pairing moiety is typically adenine, cytosine, guanine, uracil, thymine or inosine. Also included are bases such as pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trime115thoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetyltidine, 5-(carboxyhydroxymethyl)uridine, 5´-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, β-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonyhnethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, β-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, Biochemistry, 35, 14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucleotide bases other than adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U), as illustrated above; such bases can be used at any position in the antisense molecule. Persons skilled in the art will appreciate that depending on the uses or chemistries of the oligomers, Ts and Us are interchangeable. For instance, with other antisense chemistries such as 2'-O-methyl antisense oligonucleotides that are more RNA-like, the T bases may be shown as U.

As noted above, certain polynucleotides or oligonucleotides provided herein include one or more peptide nucleic acid (PNAs) subunits. Peptide nucleic acids (PNAs) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone, consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing

natural pyrimidine and purine bases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition (Egholm, Buchardt *et al.* 1993). The backbone of PNAs is formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications (see structure below). A backbone made entirely of PNAs is uncharged, resulting in PNA/DNA or PNA/RNA duplexes that exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases.

PNAs may be produced synthetically using any technique known in the art. PNA is a DNA analog in which a polyamide backbone replaces the traditional phosphate ribose ring of DNA. Despite a radical structural change to the natural structure, PNA is capable of sequence-specific binding in a helix form to DNA or RNA. Characteristics of PNA include a high binding affinity to complementary DNA or RNA, a destabilizing effect caused by single-base mismatch, resistance to nucleases and proteases, hybridization with DNA or RNA independent of salt concentration and triplex formation with homopurine DNA. Panagene™ has developed its proprietary Bts PNA monomers (Bts; benzothiazole-2-sulfonyl group) and proprietary oligomerisation process. The PNA oligomerisation using Bts PNA monomers is composed of repetitive cycles of deprotection, coupling and capping. Panagene's patents to this technology include US 6969766, US 7211668, US 7022851, US 7125994, US 7145006 and US 7179896. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, Science, 1991, 254, 1497.

Also included are "locked nucleic acid" subunits (LNAs). The structures of LNAs are known in the art: for example, Wengel, *et al.*, Chemical Communications (1998) 455; Tetrahedron (1998) 54, 3607, and Accounts of Chem. Research (1999) 32, 301); Obika, *et al.*, Tetrahedron Letters (1997) 38, 8735; (1998) 39, 5401, and Bioorganic Medicinal Chemistry (2008)16, 9230.

Polynucleotides and oligonucleotides may incorporate one or more LNAs; in some cases, the compounds may be entirely composed of LNAs. Methods

for the synthesis of individual LNA nucleoside subunits and their incorporation into oligonucleotides are known in the art: U.S. Patents 7,572,582; 7,569,575; 7,084,125; 7,060,809; 7,053,207; 7,034,133; 6,794,499; and 6,670,461. Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed. One embodiment includes an LNA containing compound where each LNA subunit is separated by a RNA or a DNA subunit (*i.e.*, a deoxyribose nucleotide). Further exemplary compounds may be composed of alternating LNA and RNA or DNA subunits where the intersubunit linker is phosphorothioate.

Certain polynucleotides or oligonucleotides may comprise morpholino-based subunits bearing base-pairing moieties, joined by uncharged or substantially uncharged linkages. The terms "morpholino oligomer" or "PMO" (phosphoramidate- or phosphorodiamidate morpholino oligomer) refer to an oligonucleotide analog composed of morpholino subunit structures, where (i) the structures are linked together by phosphorus-containing linkages, one to three atoms long, preferably two atoms long, and preferably uncharged or cationic, joining the morpholino nitrogen of one subunit to a 5' exocyclic carbon of an adjacent subunit, and (ii) each morpholino ring bears a purine or pyrimidine or an equivalent base-pairing moiety effective to bind, by base specific hydrogen bonding, to a base in a polynucleotide.

Variations can be made to this linkage as long as they do not interfere with binding or activity. For example, the oxygen attached to phosphorus may be substituted with sulfur (thiophosphorodiamidate). The 5' oxygen may be substituted with amino or lower alkyl substituted amino. The pendant nitrogen attached to phosphorus may be unsubstituted, monosubstituted, or disubstituted with (optionally substituted) lower alkyl. The purine or pyrimidine base pairing moiety is typically adenine, cytosine, guanine, uracil, thymine or inosine. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in U.S. Patent Nos. 5,698,685, 5,217,866, 5,142,047, 5,034,506, 5,166,315, 5,521,063, and 5,506,337, and PCT Appn. Nos. PCT/US07/11435 (cationic linkages) and

US08/012804 (improved synthesis), all of which are incorporated herein by reference.

In one aspect of the invention, MV-siRNA comprise at least one ligand tethered to an altered or non-natural nucleobase. Included are payload molecules and targeting molecules. A large number of compounds can function as the altered base. The structure of the altered base is important to the extent that the altered base should not substantially prevent binding of the oligonucleotide to its target, *e.g.*, mRNA. In certain embodiments, the altered base is difluorotolyl, nitropyrrolyl, nitroimidazolyl, nitroindolyl, napthalenyl, anthrancenyl, pyridinyl, quinolinyl, pyrenyl, or the divalent radical of any one of the non-natural nucleobases described herein. In certain embodiments, the non-natural nucleobase is difluorotolyl, nitropyrrolyl, or nitroimidazolyl. In certain embodiments, the non-natural nucleobase is difluorotolyl.

A wide variety of ligands are known in the art and are amenable to the present invention. For example, the ligand can be a steroid, bile acid, lipid, folic acid, pyridoxal, B12, riboflavin, biotin, aromatic compound, polycyclic compound, crown ether, intercalator, cleaver molecule, protein-binding agent, or carbohydrate. In certain embodiments, the ligand is a steroid or aromatic compound. In certain instances, the ligand is cholesteryl.

In other embodiments, the polynucleotide or oligonucleotide is tethered to a ligand for the purposes of improving cellular targeting and uptake. For example, an MV-siRNA agent may be tethered to an antibody, or antigen binding fragment thereof. As an additional example, an MV-siRNA agent may be tethered to a specific ligand binding molecule, such as a polypeptide or polypeptide fragment that specifically binds a particular cell-surface receptor, or that more generally enhances cellular uptake, such as an arginine-rich peptide.

The term “analog” as used herein refers to a molecule, compound, or composition that retains the same structure and/or function *(e.g.,* binding to a target) as a polynucleotide herein. Examples of analogs include peptidomimetic and small and large organic or inorganic compounds.

The term “derivative” or “variant” as used herein refers to a polynucleotide that differs from a naturally occurring polynucleotide *(e.g.,* target

gene sequence) by one or more nucleic acid deletions, additions, substitutions or side-chain modifications. In certain embodiments, variants have at least 70%, at least 80% at least 90%, at least 95%, or at least 99% sequence identity to a region of a target gene sequence. Thus, for example, in certain embodiments, an oligonucleotide of the present invention comprises a region that is complementary to a variant of a target gene sequence.

Polynucleotide complexes and molecules of the present invention comprise a sequence region, or two or more sequence regions, each of which is complementary, and in particular embodiments completely complementary, to a region of a target gene or polynucleotide sequences (or a variant thereof). In particular embodiments, a target gene is a mammalian gene, *e.g.*, a human gene, or a gene of a microorganism infecting a mammal, such as a virus. In certain embodiments, a target gene is a therapeutic target. For example, a target gene may be a gene whose expression or overexpression is associated with a human disease or disorder. This may be a mutant gene or a wild type or normal gene. A variety of therapeutic target genes have been identified, and any of these may be targeted by polynucleotide complexes and molecules of the present invention. Therapeutic target genes include, but are not limited to, oncogenes, growth factor genes, translocations associated with disease such as leukemias, inflammatory protein genes, transcription factor genes, growth factor receptor genes, anti-apoptotic genes, interleukins, sodium channel genes, potassium channel genes, such as, but not limited to the following genes or genes encoding the following proteins: apolipoprotein B (ApoB), apolipoprotein B-100 (ApoB-100), bcl family members, including bcl-2 and bcl-x, MLL-AF4, Huntington gene, AML-MT68 fusion gene, IKK-B, Aha1, PCSK9, Eg5, transforming growth factor beta (TGFbeta), Nav1.8, RhoA, HIF-1alpha, Nogo-L, Nogo-R, toll-like receptor 9 (TLR9), vascular endothelial growth factor (VEGF), SNCA, beta-catenin, CCR5, c-myc, p53, interleukin-1, interleukin 2, interleukin-12, interleukin-6, interleukin-17a (IL-17a), interleukin-17f (IL-17f), Osteopontin (OPN) gene, psoriasis gene, and tumor necrosis factor gene.

In particular embodiments, polynucleotide complexes or molecules of the present invention comprise guide strands or target-specific regions targeting two or more genes, *e.g.*, two or more genes associated with a particular disease or disorder. For example, they may include guide strands complementary to interleukin-1 gene or mRNA and tumor necrosis factor gene or mRNA; complementary to interleukin-1 gene or mRNA and interleukin-12 gene or mRNA; or complementary to interleukin-1 gene or mRNA, interleukin-12 gene or mRNA and tumor necrosis factor gene or mRNA, for treatment of rheumatoid arthritis. In one embodiment, they include guide strands complementary to osteopontin gene or mRNA and TNF gene or mRNA.

Other examples of therapeutic target genes include genes and mRNAs encoding viral proteins, such as human immunodeficiency virus (HIV) proteins, HTLV virus proteins, hepatitis C virus (HCV) proteins, Ebola virus proteins, JC virus proteins, herpes virus proteins, human polyoma virus proteins, influenza virus proteins, and Rous sarcoma virus proteins. In particular embodiments, polynucleotide complexes or molecules of the present invention include guide strands complementary to two or more genes or mRNAs expressed by a particular virus, *e.g.*, two or more HIV protein genes or two or more herpes virus protein genes. In other embodiments, they include guide strands having complementary to two or more herpes simplex virus genes or mRNAs, *e.g.*, the UL29 gene or mRNA and the Nectin-1 gene or mRNA of HSV-2, to reduce HSV-2 expression, replication or activity. In one embodiment, the polynucleotide complexes or molecules having regions targeting two or more HSV-2 genes or mRNAs are present in a formulation for topical delivery.

In particular embodiments, polynucleotide complexes and molecules of the present invention comprise one, two, three or more guide strands or target-specific regions that target an apolipoprotein B (ApoB) gene or mRNA, *e.g.*, the human ApoB gene or mRNA or the mouse ApoB gene or mRNA. Accordingly, in particular embodiments, they comprise one, two, three or more regions comprising a region complementary to a region of the human ApoB sequence set forth in SEQ ID NO:1. In other embodiments, they comprise one, two, three or more regions

comprising a region complementary to a region of the mouse ApoB sequence set forth in SEQ ID NO:10. In particular embodiments, they comprise two or more guide sequences having the specific sequences set forth in the accompanying Examples.

In certain embodiments, polynucleotide complexes and molecules of the present invention comprise one, two, three or more guide strands or regions that target HIV genes. In particular embodiments, they target one, two, three or more HIV genes or mRNAs encoding one or more proteins selected from HIV gag, HIV tat, HIV env, HIV gag-pol, HIV vif, and HIV nef proteins. Accordingly, in particular embodiments, they comprise one, two, three or more regions complementary to a region of the HIV gag sequence set forth in SEQ ID NO:2; one, two, three or more regions complementary to a region of the HIV tat sequence set forth in SEQ ID NO:3, one, two, three or more regions complementary to a region of the HIV env sequence set forth in SEQ ID NO:4, one, two, three or more regions complementary to a region of the HIV gag-pol sequence set forth in SEQ ID NO:5, one, two, three or more regions comprising a region complementary to a region of the HIV vif sequence set forth in SEQ ID NO:6, one, two, three or more regions comprising a region complementary to a region of the HIV nef sequence set forth in SEQ ID NO:7. In particular embodiments, they comprise two or more guide sequences having the specific HIV sequences set forth in the accompanying Examples.

In certain embodiments, selection of a sequence region complementary to a target gene (or gene) is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability and cell specificity. Such sequences may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce structural integrity of the polynucleotide or prohibit specific binding to the target gene in a host cell.

Preferred target regions of the target gene or mRNA may include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the gene or mRNA. These

secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.,* Nucleic Acids Res. 1997, 25(17):3389-402) or Oligoengine Workstation 2.0.

In one embodiment, target sites are preferentially not located within the 5' and 3' untranslated regions (UTRs) or regions near the start codon (within approximately 75 bases), since proteins that bind regulatory regions may interfere with the binding of the polynucleotide. In addition, potential target sites may be compared to an appropriate genome database, such as BLASTN 2.0.5, available on the NCBI server at www.ncbi.nlm, and potential target sequences with significant homology to other coding sequences eliminated.

In another embodiment, the target sites are located within the 5' or 3' untranslated region (UTRs). In addition, the self-complementary region of the polynucleotide may be composed of a particular sequence found in the gene of the target.

The target gene may be of any species, including, for example, plant, animal (*e.g.* mammalian), protozoan, viral (*e.g.,* HIV), bacterial or fungal. In certain embodiments, the polynucleotides of the present invention may comprise or be complementary to the GFP sequences in Example 1, the HIV sequences in Example 2, or the ApoB sequences in Example 3.

As noted above, the target gene sequence and the complementary region of the polynucleotide may be complete complements of each other, or they may be less than completely complementary, as long as the strands hybridize to each other under physiological conditions.

The polynucleotide complexes and molecules of the present invention comprise at least one, two, or three regions complementary to one or more target genes, as well as one or more self-complementary regions and/or interconnecting loops. Typically, the region complementary to a target gene is 15 to 17 to 24 nucleotides in length, including integer values within these ranges. This region may be at least 16 nucleotides in length, at least 17 nucleotides in length, at least 20 nucleotides in length, at least 24 nucleotides in length, between 15 and 24

nucleotides in length, between 16 and 24 nucleotides in length, or between 17 and 24 nucleotides in length, inclusive of the end values, including any integer value within these ranges.

The self-complementary region is typically between 2 and 54 nucleotides in length, at least 2 nucleotides in length, at least 16 nucleotides in length, or at least 20 nucleotides in length, including any integer value within any of these ranges. Hence, in one embodiment, a self-complementary region may comprise about 1-26 nucleotide pairs. A single-stranded region can be about 3-15 nucleotides, including all integers in between. A null region refers to a region that is not-specific for any target gene, at least by design. A null region or strand may be used in place of a target-specific region, such as in the design of a bi-valent polynucleotide complex or molecule of the invention (*see, e.g.,* Figure IV(K)).

In certain embodiments, a self-complementary region is long enough to form a double-stranded structure. In certain embodiments, a 3' region and a 5' region may hybridize to for a self-complementary region (*i.e.,* a double-stranded region) comprising a stem-loop structure. Accordingly, in one embodiment, the primary sequence of a self-complementary region comprises two stretches of sequence complementary to each other separated by additional sequence that is not complementary or is semi-complementary. While less optimal, the additional sequence can be complementary in certain embodiments. The additional sequence forms the loop of the stem-loop structure and, therefore, must be long enough to facilitate the folding necessary to allow the two complementary stretches to bind each other. In particular embodiments, the loop sequence comprises at least 3, at least 4, at least 5 or at least 6 bases. In one embodiment, the loop sequence comprises 4 bases. The two stretches of sequence complementary to each other (within the self-complementary region; *i.e.,* the stem regions) are of sufficient length to specifically hybridize to each other under physiological conditions. In certain embodiments, each stretch comprises 4 to 12 nucleotides; in other embodiments, each stretch comprises at least 4, at least 5, at least 6, at least 8, or at least 10 nucleotides, or any integer value within these ranges. In a particular embodiment, a self-complementary region comprises two stretches of at

least 4 complementary nucleotides separated by a loop sequence of at least 4 nucleotides. In certain embodiments, all or a portion of a self-complementary region may or may not be complementary to the region of the polynucleotide that is complementary to the target gene or gene.

In particular embodiments, self-complementary regions possess thermodynamic parameters appropriate for binding of self-complementary regions, *e.g.,* to form a stem-loop structure.

In one embodiment, self-complementary regions are dynamically calculated by use of RNA via free-energy analysis and then compared to the energy contained within the remaining "non self-complementary region" or loop region to ensure that the energy composition is adequate to form a desired structure, *e.g.,* a stem-loop structure. In general, different nucleotide sequences of the gene targeting region are considered in determining the compositions of the stem-loops structures to ensure the formation of such. The free-energy analysis formula may again be altered to account for the type of nucleotide or pH of the environment in which it is used. Many different secondary structure prediction programs are available in the art, and each may be used according to the invention. Thermodynamic parameters for RNA and DNA bases are also publicly available in combination with target sequence selection algorithms, of which several are available in the art.

In one embodiment, the polynucleotide complex or molecule comprises or consists of (a) three oligonucleotides comprising 17 to 24 nucleotides in length (including any integer value in-between), which is complementary to and capable of hybridizing under physiological conditions to at least a portion of an gene molecule, flanked optionally by (b) self-complementary sequences comprising 16 to 54 nucleotides in length (including any integer value in-between) or (c) 2 to 12 nucleotides capable of forming a loop. In one embodiment, each self-complementary sequence is capable of forming a stem-loop structure, one of which is located at the 5' end and one of which is located at the 3' end of the secondary guide strands.

In certain embodiments, the self-complementary region functions as a structure to recruit enzymatic cleavage of itself and/or bind to particular regions of proteins involved in the catalytic process of gene modulation. In addition, the loop may be of a certain 4-nucleotide (e.g., tetraloop NGNN, AAGU, UUGA, or GUUA) structure to promote the cleavage of the self-complementary region by an RNase such as RNase III. In addition, the self-complementary region can be cleaved by RNase III 11/13 or 14/16 nucleotides into the duplex region leaving a 2 nucleotide 3' end. In certain embodiments, the tetraloop has the sequence GNRA or GNYA, where N indicates any nucleotide or nucleoside, R indicates a purine nucleotide or nucleoside; and Y indicates a pyrimidine nucleotide or nucleoside.

In certain embodiments, the self-complementary polynucleotide that has been enzymatically cleaved as described above will load onto the protein region of RISC complexes. In certain embodiments, the self-complementary region containing a loop greater than 4 nucleotides can prevent the cleavage of the self-complementary region by RNase such as RNase III. In preferred embodiments, the polynucleotide of the present invention binds to and reduces expression of a target gene. A target gene may be a known gene target, or, alternatively, a target gene may be not known, *i.e.,* a random sequence may be used. In certain embodiments, target gene levels are reduced at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, or at least 95%.

In one embodiment of the invention, the level of inhibition of target gene expression (*i.e.,* gene expression) is at least 90%, at least 95%, at least 98%, and at least 99% or is almost 100%, and hence the cell or organism will in effect have the phenotype equivalent to a so-called "knock out" of a gene. However, in some embodiments, it may be preferred to achieve only partial inhibition so that the phenotype is equivalent to a so-called "knockdown" of the gene. This method of knocking down gene expression can be used therapeutically or for research (*e.g.*, to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery).

The polynucleotide complexes and molecules of the invention can be used to target and reduce or inhibit expression of genes (inclusive of coding and non-coding sequences), cDNAs, mRNAs, or microRNAs. In particular embodiments, their guide strands or targeting regions bind to mRNAs or microRNAs.

The invention further provides arrays of the polynucleotide of the invention, including microarrays. Microarrays are miniaturized devices typically with dimensions in the micrometer to millimeter range for performing chemical and biochemical reactions and are particularly suited for embodiments of the invention. Arrays may be constructed via microelectronic and/or microfabrication using essentially any and all techniques known and available in the semiconductor industry and/or in the biochemistry industry, provided that such techniques are amenable to and compatible with the deposition and/or screening of polynucleotide sequences.

Microarrays of the invention are particularly desirable for high throughput analysis of multiple polynucleotides. A microarray typically is constructed with discrete region or spots that comprise the polynucleotide of the present invention, each spot comprising one or more the polynucleotide, preferably at positionally addressable locations on the array surface. Arrays of the invention may be prepared by any method available in the art. For example, the light-directed chemical synthesis process developed by Affymetrix (see, U.S. Pat. Nos. 5,445,934 and 5,856,174) may be used to synthesize biomolecules on chip surfaces by combining solid-phase photochemical synthesis with photolithographic fabrication techniques. The chemical deposition approach developed by Incyte Pharmaceutical uses pre-synthesized cDNA probes for directed deposition onto chip surfaces (see, *e.g.,* U.S. Pat. No. 5,874,554).

In certain embodiments, a polynucleotide molecule of the present invention is chemically synthesized using techniques widely available in the art, and annealed as a three stranded complex. In a related embodiment, the three or more guide strands of a polynucleotide complex of the present invention may be

individually chemically synthesized and annealed to produce the polynucleotide complex.

In other embodiments, it is expressed *in vitro* or *in vivo* using appropriate and widely known techniques, such as vectors or plasmid constructs. Accordingly, in certain embodiments, the present invention includes *in vitro* and *in vivo* expression vectors comprising the sequence of a polynucleotide of the present invention interconnected by either stem-loop or loop forming nucleotide sequences. Methods well known to those skilled in the art may be used to construct expression vectors containing sequences encoding a polynucleotide, as well as appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y.

A vector or nucleic acid construct system can comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. In the present case, the vector or nucleic acid construct is preferably one which is operably functional in a mammalian cell. The vector can also include a selection marker such as an antibiotic or drug resistance gene, or a reporter gene (*i.e.,* green fluorescent protein, luciferase), that can be used for selection or identification of suitable transformants or transfectants. Exemplary delivery systems may include viral vector systems (*i.e.,* viral-mediated transduction) including, but not limited to, retroviral (*e.g.*, lentiviral) vectors, adenoviral vectors, adeno-associated viral vectors, and herpes viral vectors, among others known in the art.

As noted above, certain embodiments employ retroviral vectors such as lentiviral vectors. The term “lentivirus” refers to a genus of complex retroviruses that are capable of infecting both dividing and non-dividing cells. Examples of

lentiviruses include HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2), visna-maedi, the caprine arthritis-encephalitis virus, equine infectious anemia virus, feline immunodeficiency virus (FIV), bovine immune deficiency virus (BIV), and simian immunodeficiency virus (SIV). Lentiviral vectors can be derived from any one or more of these lentiviruses (*see, e.g.,* Evans et al., *Hum Gene* Ther.10:1479-1489, 1999; Case et al., *PNAS USA* 96:2988-2993, 1999; Uchida et al., *PNAS USA* 95:11939-11944, 1998; Miyoshi et al., *Science* 283:682-686, 1999; Sutton et al., *J Virol* 72:5781-5788, 1998; and Frecha et al., *Blood*. 112:4843-52, 2008, each of which is incorporated by reference in its entirety).

In certain embodiments the retroviral vector comprises certain minimal sequences from a lentivirus genome, such as the HIV genome or the SIV genome. The genome of a lentivirus is typically organized into a 5' long terminal repeat (LTR) region, the gag gene, the pol gene, the env gene, the accessory genes (*e.g.,* nef, vif, vpr, vpu, tat, rev) and a 3' LTR region. The viral LTR is divided into three regions referred to as U3, R (repeat) and U5. The U3 region contains the enhancer and promoter elements, the U5 region contains the polyadenylation signals, and the R region separates the U3 and U5 regions. The transcribed sequences of the R region appear at both the 5' and 3' ends of the viral RNA (*see, e.g.,* "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, 2000); O Narayan , *J. Gen. Virology*. 70:1617-1639, 1989; Fields et al., Fundamental Virology Raven Press., 1990; Miyoshi et al., *J Virol*. 72:8150-7,1998; and U.S. Pat. No. 6,013,516, each of which is incorporated by reference in its entirety). Lentiviral vectors may comprise any one or more of these elements of the lentiviral genome, to regulate the activity of the vector as desired, or, they may contain deletions, insertions, substitutions, or mutations in one or more of these elements, such as to reduce the pathological effects of lentiviral replication, or to limit the lentiviral vector to a single round of infection.

Typically, a minimal retroviral vector comprises certain 5'LTR and 3'LTR sequences, one or more genes of interest (to be expressed in the target cell), one or more promoters, and a cis-acting sequence for packaging of the RNA. Other regulatory sequences can be included, as described herein and known in the

art. The viral vector is typically cloned into a plasmid that may be transfected into a packaging cell line, such as a eukaryotic cell (*e.g.,* 293-HEK), and also typically comprises sequences useful for replication of the plasmid in bacteria.

In certain embodiments, the viral vector comprises sequences from the 5' and/or the 3' LTRs of a retrovirus such as a lentivirus. The LTR sequences may be LTR sequences from any lentivirus from any species. For example, they may be LTR sequences from HIV, SIV, FIV or BIV. Preferably the LTR sequences are HIV LTR sequences.

In certain embodiments, the viral vector comprises the R and U5 sequences from the 5' LTR of a lentivirus and an inactivated or "self-inactivating" 3' LTR from a lentivirus. A "self-inactivating 3' LTR" is a 3' long terminal repeat (LTR) that contains a mutation, substitution or deletion that prevents the LTR sequences from driving expression of a downstream gene. A copy of the U3 region from the 3' LTR acts as a template for the generation of both LTR's in the integrated provirus. Thus, when the 3' LTR with an inactivating deletion or mutation integrates as the 5' LTR of the provirus, no transcription from the 5' LTR is possible. This eliminates competition between the viral enhancer/promoter and any internal enhancer/promoter. Self-inactivating 3' LTRs are described, for example, in Zufferey et al., *J Virol.* 72:9873-9880, 1998; Miyoshi et al., *J Virol.* 72:8150-8157, 1998; and Iwakuma et al., *Virology* 261:120-132, 1999, each of which is incorporated by reference in its entirety. Self-inactivating 3' LTRs may be generated by any method known in the art. In certain embodiments, the U3 element of the 3' LTR contains a deletion of its enhancer sequence, preferably the TATA box, Spl and/or NF-kappa B sites. As a result of the self-inactivating 3' LTR, the provirus that is integrated into the host cell genome will comprise an inactivated 5' LTR.

Expression vectors typically include regulatory sequences, which regulate expression of the polynucleotide. Regulatory sequences present in an expression vector include those non-translated regions of the vector, *e.g.,* enhancers, promoters, 5' and 3' untranslated regions, which interact with host cellular proteins to carry out transcription and translation. Such elements may vary

in their strength and specificity. Depending on the vector system and cell utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. In addition, tissue- or-cell specific promoters may also be used.

For expression in mammalian cells, promoters from mammalian genes or from mammalian viruses are generally preferred. In addition, a number of viral-based expression systems are generally available. For example, in cases where an adenovirus is used as an expression vector, sequences encoding a polypeptide of interest may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptide in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Certain embodiments may employ the one or more of the RNA polymerase II and III promoters. A suitable selection of RNA polymerase III promoters can be found, for example, in Paule and White. *Nucleic Acids Research.*, Vol 28, pp 1283-1298, 2000, which is incorporated by reference in its entirety. RNA polymerase II and III promoters also include any synthetic or engineered DNA fragments that can direct RNA polymerase II or III, respectively, to transcribe its downstream RNA coding sequences. Further, the RNA polymerase II or III (Pol II or III) promoter or promoters used as part of the viral vector can be inducible. Any suitable inducible Pol II or III promoter can be used with the methods of the invention. Exemplary Pol II or III promoters include the tetracycline responsive promoters provided in Ohkawa and Taira, *Human Gene Therapy*, Vol. 11, pp 577-585, 2000; and Meissner et al., *Nucleic Acids Research*, Vol. 29, pp 1672-1682, 2001, each of which is incorporated by reference in its entirety.

Non-limiting examples of constitutive promoters that may be used include the promoter for ubiquitin, the CMV promoter (*see, e.g.,* Karasuyama et al., *J. Exp. Med*. 169:13, 1989), the β-actin (*see, e.g.,* Gunning et al., *PNAS USA*

84:4831-4835, 1987), and the pgk promoter (*see, e.g.,*, Adra et al., *Gene* 60:65-74, 1987); Singer-Sam *et al*., *Gene* 32:409-417, 1984; and Dobson et al., *Nucleic Acids Res*. 10:2635-2637, 1982, each of which is incorporated by reference). Non-limiting examples of tissue specific promoters include the lck promoter (*see, e.g.*, Garvin et al., *Mol. Cell Biol*. 8:3058-3064, 1988; and Takadera et al., *Mol. Cell Biol*. 9:2173-2180, 1989), the myogenin promoter (Yee et al., *Genes and Development* 7:1277-1289. 1993), and the thy1 (*see, e.g.,* Gundersen et al., *Gene* 113:207-214, 1992).

Additional examples of promoters include the ubiquitin-C promoter, the human μ heavy chain promoter or the Ig heavy chain promoter (*e.g.,* MH-b12), and the human κ light chain promoter or the Ig light chain promoter (*e.g.,* EEK-b12), which are functional in B-lymphocytes. The MH-b12 promoter contains the human μ heavy chain promoter preceded by the iEμ enhancer flanked by matrix association regions, and the EEK-b12 promoter contains the κ light chain promoter preceded an intronic enhancer (iEκ), a matrix associated region, and a 3' enhancer (3'Eκ) (*see, e.g.,* Luo et al., *Blood*. 113:1422-1431, 2009, herein incorporated by reference). Accordingly, certain embodiments may employ one or more of these promoter or enhancer elements.

In certain embodiments, the invention provides for the conditional expression of a polynucleotide. A variety of conditional expression systems are known and available in the art for use in both cells and animals, and the invention contemplates the use of any such conditional expression system to regulate the expression or activity of a polynucleotide. In one embodiment of the invention, for example, inducible expression is achieved using the REV-TET system. Components of this system and methods of using the system to control the expression of a gene are well documented in the literature, and vectors expressing the tetracycline-controlled transactivator (tTA) or the reverse tTA (rtTA) are commercially available (*e.g.,* pTet-Off, pTet-On and ptTA-2/3/4 vectors, Clontech, Palo Alto, CA). Such systems are described, for example, in U.S. Patents No. 5650298, No. 6271348, No. 5922927, and related patents, which are incorporated by reference in their entirety.

In certain embodiments, the viral vectors (*e.g.,* retroviral, lentiviral) provided herein are "pseudo-typed" with one or more selected viral glycoproteins or envelope proteins, mainly to target selected cell types. Pseudo-typing refers to generally to the incorporation of one or more heterologous viral glycoproteins onto the cell-surface virus particle, often allowing the virus particle to infect a selected cell that differs from its normal target cells. A "heterologous" element is derived from a virus other than the virus from which the RNA genome of the viral vector is derived. Typically, the glycoprotein-coding regions of the viral vector have been genetically altered such as by deletion to prevent expression of its own glycoprotein. Merely by way of illustration, the envelope glycoproteins gp41 and/or gp120 from an HIV-derived lentiviral vector are typically deleted prior to pseudo-typing with a heterologous viral glycoprotein.

Generation of viral vectors can be accomplished using any suitable genetic engineering techniques known in the art, including, without limitation, the standard techniques of restriction endonuclease digestion, ligation, transformation, plasmid purification, PCR amplification, and DNA sequencing, for example as described in Sambrook et al. (Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, N.Y. (1989)), Coffin et al. (Retroviruses. Cold Spring Harbor Laboratory Press, N.Y. (1997)) and "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)).

Any variety of methods known in the art may be used to produce suitable retroviral particles whose genome comprises an RNA copy of the viral vector. As one method, the viral vector may be introduced into a packaging cell line that packages the viral genomic RNA based on the viral vector into viral particles with a desired target cell specificity. The packaging cell line typically provides in trans the viral proteins that are required for packaging the viral genomic RNA into viral particles and infecting the target cell, including the structural gag proteins, the enzymatic pol proteins, and the envelope glycoproteins.

In certain embodiments, the packaging cell line may stably express certain of the necessary or desired viral proteins (*e.g.,* gag, pol) (*see, e.g.,* U.S. Pat. No. 6,218,181, herein incorporated by reference). In certain embodiments, the

packaging cell line may be transiently transfected with plasmids that encode certain of the necessary or desired viral proteins (*e.g.,* gag, pol, glycoprotein), including the measles virus glycoprotein sequences described herein. In one exemplary embodiment, the packaging cell line stably expresses the gag and pol sequences, and the cell line is then transfected with a plasmid encoding the viral vector and a plasmid encoding the glycoprotein. Following introduction of the desired plasmids, viral particles are collected and processed accordingly, such as by ultracentrifugation to achieve a concentrated stock of viral particles. Exemplary packaging cell lines include 293 (ATCC CCL X), HeLa (ATCC CCL 2), D17 (ATCC CCL 183), MDCK (ATCC CCL 34), BHK (ATCC CCL-10) and Cf2Th (ATCC CRL 1430) cell lines.

In one particular embodiment, the polynucleotides are expressed using a vector system comprising a pSUPER vector backbone and additional sequences corresponding to the polynucleotide to be expressed. The pSUPER vectors system has been shown useful in expressing shRNA reagents and downregulating gene expression (Brummelkamp, T.T. *et al.,* Science 296:550 (2002) and Brummelkamp, T.R. *et al.,* Cancer Cell, published online August 22, 2002). PSUPER vectors are commercially available from OligoEngine, Seattle, WA.

Methods of Regulating Gene Expression

The polynucleotides of the invention may be used for a variety of purposes, all generally related to their ability to inhibit or reduce expression of one or more target genes. Accordingly, the invention provides methods of reducing expression of one or more target genes comprising introducing a polynucleotide complex or molecule of the present invention into a cell comprising said one or more target genes. In particular embodiments, the polynucleotide complex or molecule comprises one or more guide strands that collectively target the one or more target genes. In one embodiment, a polynucleotide of the invention is introduced into a cell that contains a target gene or a homolog, variant or ortholog

thereof, targeted by either one, two, or three of the guide strands or targeting regions.

In addition, the polynucleotides of the present invention may be used to reduce expression indirectly. For example, a polynucleotide complex or molecule of the present invention may be used to reduce expression of a transactivator that drives expression of a second gene (*i.e.*, the target gene), thereby reducing expression of the second gene. Similarly, a polynucleotide may be used to increase expression indirectly. For example, a polynucleotide complex or molecule of the present invention may be used to reduce expression of a transcriptional repressor that inhibits expression of a second gene, thereby increasing expression of the second gene.

In various embodiments, a target gene is a gene derived from the cell into which a polynucleotide is to be introduced, an endogenous gene, an exogenous gene, a transgene, or a gene of a pathogen that is present in the cell after transfection thereof. Depending on the particular target gene and the amount of the polynucleotide delivered into the cell, the method of this invention may cause partial or complete inhibition of the expression of the target gene. The cell containing the target gene may be derived from or contained in any organism (*e.g.*, plant, animal, protozoan, virus, bacterium, or fungus). As used herein, "target genes" include genes, mRNAs, and microRNAs.

Inhibition of the expression of the target gene can be verified by means including, but not limited to, observing or detecting an absence or observable decrease in the level of protein encoded by a target gene, an absence or observable decrease in the level of a gene product expressed from a target gene (*e.g.*, mRNA0, and/or a phenotype associated with expression of the gene, using techniques known to a person skilled in the field of the present invention.

Examples of cell characteristics that may be examined to determine the effect caused by introduction of a polynucleotide complex or molecule of the present invention include, cell growth, apoptosis, cell cycle characteristics, cellular differentiation, and morphology.

A polynucleotide complex or molecule of the present invention may be directly introduced to the cell (*i.e.*, intracellularly), or introduced extracellularly into a cavity or interstitial space of an organism, *e.g.*, a mammal, into the circulation of an organism, introduced orally, introduced by bathing an organism in a solution containing the polynucleotide, or by some other means sufficient to deliver the polynucleotide into the cell.

In addition, a vector engineered to express a polynucleotide may be introduced into a cell, wherein the vector expresses the polynucleotide, thereby introducing it into the cell. Methods of transferring an expression vector into a cell are widely known and available in the art, including, *e.g.,* transfection, lipofection, scrape-loading, electroporation, microinjection, infection, gene gun, and retrotransposition. Generally, a suitable method of introducing a vector into a cell is readily determined by one of skill in the art based upon the type of vector and the type of cell, and teachings widely available in the art. Infective agents may be introduced by a variety of means readily available in the art, including, *e.g.,* nasal inhalation.

Methods of inhibiting gene expression using the oligonucleotides of the invention may be combined with other knockdown and knockout methods, *e.g.,* gene targeting, antisense RNA, ribozymes, double-stranded RNA (*e.g.*, shRNA and siRNA) to further reduce expression of a target gene.

In different embodiments, target cells of the invention are primary cells, cell lines, immortalized cells, or transformed cells. A target cell may be a somatic cell or a germ cell. The target cell may be a non-dividing cell, such as a neuron, or it may be capable of proliferating *in vitro* in suitable cell culture conditions. Target cells may be normal cells, or they may be diseased cells, including those containing a known genetic mutation. Eukaryotic target cells of the invention include mammalian cells, such as, for example, a human cell, a murine cell, a rodent cell, and a primate cell. In one embodiment, a target cell of the invention is a stem cell, which includes, for example, an embryonic stem cell, such as a murine embryonic stem cell.

The polynucleotide complexes, molecules, and methods of the present invention may be used to treat any of a wide variety of diseases or disorders, including, but not limited to, inflammatory diseases, cardiovascular diseases, nervous system diseases, tumors, demyelinating diseases, digestive system diseases, endocrine system diseases, reproductive system diseases, hemic and lymphatic diseases, immunological diseases, mental disorders, muscoloskeletal diseases, neurological diseases, neuromuscular diseases, metabolic diseases, sexually transmitted diseases, skin and connective tissue diseases, urological diseases, and infections.

In certain embodiments, the methods are practiced on an animal, in particular embodiments, a mammal, and in certain embodiments, a human.

Accordingly, in one embodiment, the present invention includes methods of using a polynucleotide complex or molecule of the present invention for the treatment or prevention of a disease associated with gene deregulation, overexpression, or mutation. For example, a polynucleotide complex or molecule of the present invention may be introduced into a cancerous cell or tumor and thereby inhibit expression of a gene required for or associated with maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected that is, *e.g.,* required for initiation or maintenance of a disease/pathology. Treatment may include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

In addition, the polynucleotides of the present invention are used to treat diseases or disorders associated with gene mutation. In one embodiment, a polynucleotide is used to modulate expression of a mutated gene or allele. In such embodiments, the mutated gene is a target of the polynucleotide complex or molecule, which will comprise a region complementary to a region of the mutated gene. This region may include the mutation, but it is not required, as another region of the gene may also be targeted, resulting in decreased expression of the mutant gene or gene. In certain embodiments, this region comprises the mutation, and, in related embodiments, the polynucleotide complex or molecule specifically inhibits expression of the mutant gene or gene but not the wild type gene or gene.

Such a polynucleotide is particularly useful in situations, *e.g.,* where one allele is mutated but another is not. However, in other embodiments, this sequence would not necessarily comprise the mutation and may, therefore, comprise only wild-type sequence. Such a polynucleotide is particularly useful in situations, *e.g.,* where all alleles are mutated. A variety of diseases and disorders are known in the art to be associated with or caused by gene mutation, and the invention encompasses the treatment of any such disease or disorder with a the polynucleotide.

In certain embodiments, a gene of a pathogen is targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. In addition, the target gene may be a pathogen gene or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (*i.e.*, prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, are included in the present invention. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of a the polynucleotide according to the invention (*see* Examples 1 and 2 for targeting sequences). Thus, in one embodiment, polynucleotide complexes or molecules of the present invention that target one or more HIV proteins are used to treat or inhibit HIV infection or acquired immune deficiency syndrome (AIDS).

In other specific embodiments, the present invention is used for the treatment or development of treatments for cancers of any type. Examples of tumors that can be treated using the methods described herein include, but are not limited to, neuroblastomas, myelomas, prostate cancers, small cell lung cancer, colon cancer, ovarian cancer, non-small cell lung cancer, brain tumors, breast cancer, leukemias, lymphomas, and others.

In one embodiment, polynucleotide complexes or molecules of the present invention that target apolipoprotein B (apoB) are used to treat, reduce, or

inhibit atherosclerosis or heart disease. ApoB is the primary apolipoprotein of low-density lipoproteins (LDLs), which is responsible for carrying cholesterol to tissues. ApoB on the LDL particle acts as a ligand for LDL receptors, and high levels of ApoB can lead to plaques that cause vascular disease (atherosclerosis), leading to heart disease.

The polynucleotide complexes, molecules and expression vectors (including viral vectors and viruses) may be introduced into cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or they may be directly introduced to a patient by *in vivo* administration. Thus, the invention provides methods of gene therapy, in certain embodiments. Compositions of the invention may be administered to a patient in any of a number of ways, including parenteral, intravenous, systemic, local, topical, oral, intratumoral, intramuscular, subcutaneous, intraperitoneal, inhalation, or any such method of delivery. In one embodiment, the compositions are administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In a specific embodiment, the liposomal compositions are administered by intravenous infusion or intraperitoneally by a bolus injection.

Compositions of the invention may be formulated as pharmaceutical compositions suitable for delivery to a subject. The pharmaceutical compositions of the invention will often further comprise one or more buffers (*e.g.,* neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.,* glucose, mannose, sucrose, dextrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The amount of the oligonucleotides administered to a patient can be readily determined by a physician based upon a variety of factors, including, *e.g.,* the disease and the level of the oligonucleotides expressed from the vector being used (in cases where a vector is administered). The amount administered per dose

is typically selected to be above the minimal therapeutic dose but below a toxic dose. The choice of amount per dose will depend on a number of factors, such as the medical history of the patient, the use of other therapies, and the nature of the disease. In addition, the amount administered may be adjusted throughout treatment, depending on the patient's response to treatment and the presence or severity of any treatment-associated side effects.

Methods of Determining Gene Function

The invention further includes a method of identifying gene function in an organism comprising the use of a polynucleotide complex or molecule of the present invention to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics envisions determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention may be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (*e.g.*, nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

In one embodiment, a polynucleotide of the present invention is used to inhibit gene expression based upon a partial sequence available from an expressed sequence tag (EST), *e.g.,* in order to determine the gene's function or biological activity. Functional alterations in growth, development, metabolism,

disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which a polynucleotide can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, solutions containing the polynucleotide that are capable of inhibiting different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. In one embodiment, the polynucleotides of the invention are used for chemocogenomic screening, *i.e.,* testing compounds for their ability to reverse a disease modeled by the reduction of gene expression using a polynucleotide of the invention.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, a polynucleotide can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined.

The present invention is also useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of a the polynucleotide at specific times of development and locations in the organism without introducing permanent mutations into the target genome. Similarly, the invention contemplates the use of inducible or conditional vectors that express a the polynucleotide only when desired.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. A the polynucleotide that targets the gene that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the gene occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined. If decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

Methods of Designing and Producing Polynucleotide Complexes and Molecules

The polynucleotide complexes and molecules of the present invention comprise a novel and unique set of functional sequences, arranged in a manner so as to adopt a secondary structure containing one or more double-stranded regions (sometimes adjoined by stem-loop or loop structures), which imparts the advantages of the polynucleotide. Accordingly, in certain embodiments, the present invention includes methods of designing the polynucleotide complexes and molecules of the present invention. Such methods typically involve appropriate selection of the various sequence components of the polynucleotide complexes and molecules. The terms “primary strand”, “secondary strand”, and “key strand” refer to the various guide strands present within a polynucleotide complex or molecule of the present invention.

In one embodiment, the basic design of the polynucleotide complex is as follows:

DESIGN MOTIFS:

(primary strand)(UU)(secondary strand)(UU)(key strand)(UU)

Accordingly, in a related embodiment, a the polynucleotide is designed as follows:

II. (secondary strand)(UU)(UU)(key strand)(UU)(primary strand)

III. (secondary strand)(UU)(loop or stem-loop)(key strand)(UU)(loop or stem-loop)(primary strand)(UU)

SET PARAMETERS

Set seed size for self complementarity at approx 38-43%. For a 19 nucleotide targets, a range or 7 or 8 nucleotides is preferred as SEED_SIZE.

For each gene, define a PRIMARY and SECONDARY target gene.

DEFINE PRIMARY STRANDS

Start with one or more target gene sequences. For each gene, build a list of PRIMARY target sequences 17-24 nucleotide motifs that meet criteria of G/C content, specificity, and poly-A or poly-G free. For each, find also a SECONDARY and KEY strand.

FIND SECONDARY AND KEY STRANDS

d. For each target sequence on each gene, clustal align base 1 through SEED_SIZE the reverse of each sequence to the SECONDARY gene

Record sequence with a perfect alignment. The target sequence on the SECONDARY gene is the alignment start, minus the length of the motif, plus SEED_SIZE to alignment start, plus SEED_SIZE. The SECONDARY strand is the reverse compliment.

To find each KEY strand, define SEED_A as base 1 through SEED_SIZE of the PRIMARY strand, define SEED_B as bases at motif length minus SEED_SIZE to motif length of the SECONDARY strand. Set a MID_SECTION as characters "|" repeated of length motif sequence length minus SEED_A length plus SEED_B length. Set key alignment sequence as SEED_A, MID_SECTION, SEED_B. Clustal align to the target gene for the key segment. Record KEY target sequence as bases at alignment hit on key target gene to bases alignment hit plus motif length. The KEY strand is the reverse compliment.

CONSTRUCT OPTIONAL POLYNUCLEOTIDE

g. Build candidate Stem A & B with (4-24) nucleotides that have melting temperature dominant to equal length region of target. Stem strands have

A-T, G-C complementarity to each other. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure.

h. Build candidate Stem C & D with (4-24) nucleotides that have melting temperature dominant to equal length region of target. Stem strands have A-T, G-C complementarity to each other, but no complementarity to Stem A & B. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure.

i. Build loop candidates with (4-12) A-T rich nucleotides into loop A & B. Length and composition depend upon which endoribonuclease is chosen for pre-processing of the stem-loop structure. Tetraloops as described are suggested for longer stems processed by RNase III or Pac1 RNase III endoribonucleases as drawn in (Fig. A.). Larger loops are suggested for preventing RNase III or Pac1 processing and placed onto shorter stems as drawn in (Fig. C, Fig. D.).

j. Form a contiguous sequence for each motif candidate.

k. Fold candidate sequence using software with desired parameters.

l. From output, locate structures with single stranded target regions which are flanked at either one or both ends with a desired stem/loop structure.

In one embodiment, a method of designing a polynucleotide sequence comprising one or more self-complementary regions for the regulation of expression of a target gene (*i.e.,* a the polynucleotide), includes: (a) selecting a first sequence 17 to 30 nucleotides in length and complementary to a target gene; and (b) selecting one or more additional sequences 12 to 54 nucleotides in length, which comprises self-complementary regions and which are non-complementary to the first sequence.

These methods, in certain embodiments, include determining or predicting the secondary structure adopted by the sequences selected in step (b), *e.g.,* in order to determine that they are capable of adopting a stem-loop structure.

Similarly, these methods can include a verification step, which comprises testing the designed polynucleotide sequence for its ability to inhibit expression of a target gene, *e.g.,* in an *in vivo* or *in vitro* test system.

The invention further contemplates the use of a computer program to select sequences of a polynucleotide, based upon the complementarity characteristics described herein. The invention, thus, provides computer software programs, and computer readable media comprising said software programs, to be used to select the polynucleotide sequences, as well as computers containing one of the programs of the present invention.

In certain embodiments, a user provides a computer with information regarding the sequence, location or name of a target gene. The computer uses this input in a program of the present invention to identify one or more appropriate regions of the target gene to target, and outputs or provides complementary sequences to use in the polynucleotide of the invention. The computer program then uses this sequence information to select sequences of the one or more self-complementary regions of the polynucleotide. Typically, the program will select a sequence that is not complementary to a genomic sequence, including the target gene, or the region of the polynucleotide that is complementary to the target gene. Furthermore, the program will select sequences of self-complementary regions that are not complementary to each other. When desired, the program also provides sequences of gap regions. Upon selection of appropriate sequences, the computer program outputs or provides this information to the user.

The programs of the present invention may further use input regarding the genomic sequence of the organism containing the target gene, *e.g.,* public or private databases, as well as additional programs that predict secondary structure and/or hybridization characteristics of particular sequences, in order to ensure that the polynucleotide adopts the correct secondary structure and does not hybridize to non-target genes.

The present invention is based, in part, upon the surprising discovery that the polynucleotide, as described herein, is extremely effective in reducing target gene expression of one or more genes. The polynucleotide offer significant

advantages over previously described antisense RNAs, including increased potency, and increased effectiveness to multiple target genes. Furthermore, the polynucleotide of the invention offer additional advantages over traditional dsRNA molecules used for siRNA, since the use of the polynucleotide substantially eliminates the off-target suppression associated with dsRNA molecules and offers multivalent RNAi.

It is understood that the compositions and methods of the present invention may be used to target a variety of different target genes. The term "target gene" may refer to a gene, an mRNA, or a microRNA. Accordingly, target sequences provided herein may be depicted as either DNA sequences or RNA sequences. One of skill the art will appreciate that the compositions of the present invention may include regions complementary to either the DNA or RNA sequences provided herein. Thus, where either a DNA or RNA target sequence is provided, it is understood that the corresponding RNA or DNA target sequence, respectively, may also be targeted.

The practice of the present invention will employ a variety of conventional techniques of cell biology, molecular biology, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are fully described in the literature. See, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press, 1989); and DNA Cloning, Volumes I and II (D. N. Glover ed. 1985).

All of the patents, patent applications, and non-patent references referred to herein are incorporated by reference in their entirety, as if each one was individually incorporated by reference.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of

the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

EXAMPLES

EXAMPLE 1

TRIVOID ANTI-GFP

Multivalent siRNA were designed against a single gene, the green fluorescent protein (GFP). A multivalent synthetic RNA MV-siRNA complex directed against GFP was tested to compare suppression activity in relation to that of a single shRNA clone. Also, to test the effect of deactivating one of the strands of the synthetic MV-siRNA complex, one strand was replaced with DNA (T1-19_C_dna); as shown below. This replacement resulted in a relative drop in suppression by ~30%. Additionally, 'short' and 'long' forms of the MV-siRNA self-complementary clones described herein were tested and compared to the suppression of GFP expression in relation to that of a published shRNA clone.

Oligomer sequences for the synthetic MV-siRNA, and the DNA replacement strand, are shown below in Table 1. The targeted regions of the GFP coding sequence are illustrated in Figure 8A.

Table 1: Oligos for Synthetic MV-siRNA:

Name	Sequence	SEQ ID NO:
TI-19/7_A	GGGCAGCUUGCCGGUGGUGUU	11
TI-19/7_B	CACCACCCCGGUGAACAGCUU	12
TI-19/7_C	GCUGUUCACGUCGCUGCCCUU	13
TI-19/7_C_dna	GCTGTTCACGTCGCTGCCC	14

To prepare the synthetic multivalent-siRNAs (MV-siRNAs), each tube of the individual oligos above was resuspended in RNase-free water to obtain a final concentration of 50 µM (50pmoles/µL). The individual oligos were then combined as (a) TI-19/7_A, TI-19/7_B, and TI-19/7_C (MV-siRNA GFP I), or as (b) TI-19/7_A, TI-19/7_B, and TI-19/7_C_dna (MV-siRNA GFP I DNA), and annealed as follows. 30 µL of each one of the resupended oligos were combined with 10 µL of 10x annealing buffer (100mM Tris-HCl pH7.5, 1M NaCl, 10mM EDTA), vortexed, heated for 5 minutes at 94°C, and step cooled to 70°C over 30 minutes. The final concentration of the annealed MV-siRNA was about 15 µM.

To prepare the multivalent-siRNA clones and shRNA control, the sequences in Table 2 below were cloned into the pSUPER vector, according to the pSUPER manual. The first sequence for each named clone (*e.g.,* TI, T1_long, TII) represents the sequence of the self-complementary multivalent siRNA that was expressed in the cell as an RNA transcript (comparable to the sequence of the synthetic MV-siRNAs in Table 1), and the sequence referred to as "_as" is part of the coding sequence for that molecule.

Table 2: Oligos for MV-siRNA expressing clones:

Name	Sequence	SEQ ID NO:
TI	GATCCCCCACCACCCCGGTGAACAGCgttaGCTGTTCACGTCGCT GCCCgttaGGGCAGCTTGCCGGTGGTGttTTTTTA	15
TI_as	AGCTTAACACCACCGGCAAGCTGCCCTAACGGGCAGCGACGTG AACAGCTAACGCTGTTCACCGGGGTGGTGGGG	16
T1_long	GATCCCCCACCACCCCGGTGAACAGCTTGTAGGTGGCATCGCA GAAGCGATGCCACCTACAAGCTGTTCACGTCGCTGCCCTTGTAG GTGGCATCGCAGAAGCGATGCCACCTACAAGGGCAGCTTGCCG GTGGTGttTTTTTA	17
T1_long_as	AGCTTAACACCACCGGCAAGCTGCCCTTGTAGGTGGCATCGCTT CTGCGATGCCACCTACAAGGGCAGCGACGTGAACAGCTTGTAG GTGGCATCGCTTCTGCGATGCCACCTACAAGCTGTTCACCGGG GTGGTGGGG	18
TII	GATCCCCCGTGCTGCTTCATGTGGTCGTTgttaCGACCACAATGG CGACAACCTTgttaGGTTGTCGGGCAGCAGCACGTTttTTTTTA	19
TII_as	AGCTTAAAACGTGCTGCTGCCCGACAACCTAACAAGGTTGTCGC CATTGTGGTCGTAACAACGACCACATGAAGCAGCACGGGG	20
TII_long	GATCCCCCGTGCTGCTTCATGTGGTCGTTGTAGGTGGCATCGCA GAAGCGATGCCACCTACAACGACCACAATGGCGACAACCTTGTA GGTGGCATCGCAGAAGCGATGCCACCTACAAGGTTGTCGGGCA GCAGCACGttTTTTTA	21
TII_long_as	AGCTTAACGTGCTGCTGCCCGACAACCTTGTAGGTGGCATCGCT TCTGCGATGCCACCTACAAGGTTGTCGCCATTGTGGTCGTTGTA GGTGGCATCGCTTCTGCGATGCCACCTACAACGACCACATGAA	22

	GCAGCACGGGG	
shRNA	GATCCCCGCAAGCTGACCCTGAAGTTCTTCAAGAGAGAACTTCA GGGTCAGCTTGCTTTTTA	23
shRNA_as	AGCTTAAAAAGCAAGCTGACCCTGAAGTTCTCTCTTGAAGAACTT CAGGGTCAGCTTGCGGG	24

To test the effects on GFP-expression, the annealed MV-siRNA molecules (at a final concentration of 7.5 nM per well) and pSUPER vectors containing the MV-siRNA clones or shRNA control were transfected with Lipofectamine 2000 into 293 cells that constitutively express GFP. GFP fluorescence was measure by flow cytometry 24 hour after transfection.

The results for one experiment are shown in Table 3 below, and summarized in Figure 7A. In Figure 7A, the MV-siRNA long I and long II clones demonstrate significantly increased suppression of GFP activity compared to the shRNA control (referred to in that Figure as “siRNA”).

Table 3:

Well	Transfected:	Mean Fluorescence	% GFP
Positive shRNA	shRNA	330	66%
	shRNA	302	60%
Synthetic:	MV-siRNA	305	61%
Clone:	MV-siRNA short TI	360	72%
	MV-siRNA long TI	218	43%
	MV-siRNA long TII	245	49%
Negative	Blank	502	100%
	non-GFP 293 cells	0.5	0%

Figure 7B shows the results of an experiment in which the synthetic MV-siRNA GFP I complex demonstrated increased suppression of GFP activity compared to the shRNA clone (referred to in that Figure as “siRNA”). However, the suppression activity for the MV-siRNA GFP I complex was slightly reduced when one strand was replaced with DNA, as shown for the synthetic MV-siRNA GFP I DNA complex.

Exemplary synthetic MV-siRNAs directed to GFP can also be designed as in Table 4 below, in which the 3 oligos of T1.A-C can be annealed as described above. Similarly, the 3 oligos of T2.A-C can be annealed as described above.

Table 4: Exemplary synthetic siRNA sets T1 and T2.

Name	Sequence	SEQ ID NO:
T1.A	CUGCUGGUAGUGGUCGGCGUU	25
T1.B	CGCCGACUUCGUGACGUGCUU	26
T1.C	GCACGUCGCCGUCCAGCAGUU	27
T2.A	GUUGCCGUCGUCCUUGAAGUU	28
T2.B	CUUCAAGUGGAACUACGGCUU	29
T2.C	GCCGUAGGUAGGCGGCAACUU	30

MV-siRNA clones directed to GFP can also be designed as in Table 5 below. As illustrated above, these sequences can be cloned into the pSuper vector, or any other vector system.

Table 5: Exemplary MV-siRNA clones

Name	Sequence	SEQ ID NO:
T1_transcript	CGCCGACUUCGUGACGUGCUUGUGCACGUCGCCGUCCAGC AGUUGUCUGCUGGUAGUGGUCGGCGUU	31
T1	GATCCCCCGCCGACTTCGTGACGTGCTTGTGCACGTCGCCGT CCAGCAGTTGTCTGCTGGTAGTGGTCGGCGTTTTTTTA	32
T1_as	AGCTTAAAAAAACGCCGACCACTACCAGCAGACAACTGCTGG ACGGCGACGTGCACAAGCACGTCACGAAGTCGGCGGGG	33
T1_long transcript	CGCCGACUUCGUGACGUGCUUGUAGGUGGCAUCGCAGAAG CGAUGCCACCUACAAGCACGUCGCCGUCCAGCAGUUGUAGG UGGCAUCGCAGAAGCGAUGCCACCUACAACUGCUGGUAGUG GUCGGCGUU	34
T1_long	GATCCCCCGCCGACTTCGTGACGTGCTTGTAGGTGGCATCGC AGAAGCGATGCCACCTACAAGCACGTCGCCGTCCAGCAGTTG TAGGTGGCATCGCAGAAGCGATGCCACCTACAACTGCTGGTA GTGGTCGGCGTTTTTA	35
T1_long_as	AGCTTAAAAACGCCGACCACTACCAGCAGTTGTAGGTGGCAT CGCTTCTGCGATGCCACCTACAACTGCTGGACGGCGACGTGC TTGTAGGTGGCATCGCTTCTGCGATGCCACCTACAAGCACGT CACGAAGTCGGCGGGG	36
T2_transcript	CUUCAAGUGGAACUACGGCUUGUGCCGUAGGUAGGCGGCAA CUUGUGUUGCCGUCGUCCUUGAAGUU	37
T2	GATCCCCGGATCCGACATCCACGTTCTTCAAGAGAGAACGTG GATGTCGGATCCTTTTTA	38
T2_as	AGCTTAAAAAGGATCCGACATCCACGTTCTCTCTTGAAGAACG TGGATGTCGGATCCGGG	39
T2_long transcript	CUUCAAGUGGAACUACGGCUUGUAGGUGGCAUCGCAGAAGC GAUGCCACCUACAAGCCGUAGGUAGGCGGCAACUUGUAGGU GGCAUCGCAGAAGCGAUGCCACCUACAAGUUGCCGUCGUCC UUGAAGUU	40
T2_long	GATCCCCCTTCAAGTGGAACTACGGCTTGTAGGTGGCATCGC AGAAGCGATGCCACCTACAAGCCGTAGGTAGGCGGCAACTTG	41

	TAGGTGGCATCGCAGAAGCGATGCCACCTACAAGTTGCCGTC GTCCTTGAAGTTTTTA	
T2_long_as	AGCTTAAAAACTTCAAGGACGACGGCAACTTGTAGGTGGCATC GCTTCTGCGATGCCACCTACAAGTTGCCGCCTACCTACGGCTT GTAGGTGGCATCGCTTCTGCGATGCCACCTACAAGCCGTAGT TCCACTTGAAGGGG	42

EXAMPLE 2

TRIVOID ANTI-HIV

Multivalent-siRNA can be designed against multiple genes at unrelated sites. In this example, a cloned MV-siRNA was tested against HIV. These results show that a di-valent MV-siRNA molecule against HIV's Gag and Tat (hv_sB) genes was significantly more efficient in inhibiting HIV replication than an siRNA directed against Gag alone (hv_s).

The oligos shown in Table 6 were cloned into pSUPER.neo+gfp vector according to manufacturers guidelines. The hv_s is targeted to Gag only, and the hv_sB is targeted to both Gag and Tat.

Table 6: Anti-HIV MV-siRNA clones

Name	Sequence	SEQ ID NO:
hv_s	GATCCCCGTGAAGGGGAACCAAGAGATTgaTCTCTTGTTAATATCAG CTTgaGCTGATATTTCTCCTTCACTTTTTA	43
hv_s_as	AGCTTAAAAAGTGAAGGAGAAATATCAGCTCAAGCTGATATTAACAA GAGATCAATCTCTTGGTTCCCCTTCACGGG	44
hv_sB	GATCCCCCAAGCAGTTTTAGGCTGACgTTaGTCAGCCTCATTGACAC AGgTTaCTGTGTCAGCTGCTGCTTGTTTTTTTA	45
hv_sB_As	AGCTTAAAAAAACAAGCAGCAGCTGACACAGTAACCTGTGTCAATGA GGCTGACTAACGTCAGCCTAAAACTGCTTGGGG	46

The vector constructs encoding the MV-siRNA clones were transfected into cells, and the analyses were carried out on days 10 and 40 post infection with HIV-1 (pNL4.3 strain) with an MOI of 1.0. Figure 9 shows that at 10 days post transfection, inhibition of HIV replication by the MV-siRNA targeted to both Gag and Tat was about 3 times greater than inhibition by the siRNA molecule targeted only to Gag.

Multivalent-siRNA can be designed to target 1, 2, or 3 different genes of HIV. The sequence of an exemplary HIV genome is provided in Figure 10. A sequence of an env gene is provided in Figure 11, a gag gene in Figure 12A, and a

tat gene in Figure 12B. The various genes or regions of HIV can be generally defined and targeted by their range of nucleotide sequence as follows: 5' LTR: 1-181; GAG: 336-1838; POL: 1631-4642; VIF: 4587/4662-5165; VPR: 5105-5395 (including mutations at 5157, 5266, and 5297); TAT: 5376-7966; REV: 5515-8195; VPU: 5607-5852; ENV: 5767-8337; NEF: 8339-8959; and 3' LTR: 8628-9263. Based on these target genes, exemplary MV-RNA oligo sequences for HIV are provided in Table 7 below.

Table 7: Exemplary Trivalent MV-siRNA Sequences

No.	Sequence	Target Gene	SEQ ID NO:
1	GCCUUCCCUUGUGGGAAGGUU	1649	47
2	CCUUCCCUUGUGGGAAGGCUU	1648	48
3	GCCUUCCUUGUGGGAAGGCUU	1648	49
4	UUCUGCACCUUACCUCUUAUU	6259	50
5	UAAGAGGAAGUAUGCUGUUUU	4062	51
6	AACAGCAGUUGUUGCAGAAUU	5291	52
7	CCAGACAAUAAUUGUCUGGUU	7387	53
8	CCAGACAAUAAUUGUCUGGUU	7387	53
9	CCAGACAAUAAUUGUCUGGUU	7387	53
10	CUCCCAGGCUCAGAUCUGGUU	16	54
11	CCAGAUCUUCCCUAAAAAAUU	1630	55
12	UUUUUUAUCUGCCUGGGAGUU	7011	56
13	UGGGUUCCCUAGUUAGCCAUU	40	57
14	UGGCUAAGAUCUACAGCUGUU	8585	58
15	CAGCUGUCCCAAGAACCCAUU	7325	59
16	AUCCUUUGAUGCACACAAUUU	591	60

No	Sequence	Target Gene	SEQ ID NO:
17	AUUGUGUCACUUCCUUCAGUU	6988	61
18	CUGAAGGAAGCUAAAGGAUUU	1785	62
19	UCCUGUGUCAGCUGCUGCUUU	685	63
20	AGCAGCAUUGUUAGCUGCUUU	8481	64
21	AGCAGCUUUAUACACAGGAUU	9046	65
22	ACCAACAAGGUUUCUGUCAUU	1284	66
23	UGACAGAUCUAAUUACUACUU	6573	67
24	GUAGUAAUUAUCUGUUGGUUU	6311	68
25	CUGAGGGAAGCUAAAGGAUUU	1785	69
26	AUCCUUUGAUGCACACAAUUU	591	60
27	AUUGUGUCACUUCCCUCAGUU	6988	61
28	CAAAGCUAGAUGAAUUGCUUU	3534	70
29	AGCAAUUGGUACAAGCAGUUU	5432	71
30	ACUGCUUGUUAGAGCUUUGUU	2952	72
31	AGGUCAGGGUCUACUUGUGUU	4872	73
32	CACAAGUGCUGAUAUUUCUUU	5779	74
33	AGAAAUAAUUGUCUGACCUUU	7384	75
34	CUAAGUUAUGGAGCCAUAUUU	5212	76
35	AUAUGGCCUGAUGUACCAUUU	758	77
36	AUGGUACUUCUGAACUUAGUU	4736	78
37	UGGCUCCAUUUCUUGCUCUUU	5365	79
38	AGAGCAACCCCAAAUCCCCUU	7544	80

No	Sequence	Target Gene	SEQ ID NO:
39	GGGGAUUUAGGGGGAGCCAUU	4191	81
40	AUCUCCACAAGUGCUGAUAUU	5784	82
41	UAUCAGCAGUUCUUGAAGUUU	8942	83
42	ACUUCAAAUUGUUGGAGAUUU	8158	84
43	AGACUGUGACCCACAAUUUUU	5862	85
44	AAAUUGUGGAUGAAUACUGUU	4310	86
45	CAGUAUUUGUCUACAGUCUUU	499	87
46	ACAGGCCUGUGUAAUGACUUU	6362	88
47	AGUCAUUGGUCUUAAAGGUUU	8559	89
48	ACCUUUAGGACAGGCCUGUUU	6371	90
49	UCAGUGUUAUUUGACCCUUUU	6973	91
50	AAGGGUCUGAGGGAUCUCUUU	135	92
51	AGAGAUCUUUCCACACUGAUU	158	93
52	CAUAGUGCUUCCUGCUGCUUU	7337	94
53	AGCAGCAUUGUUAGCUGCUUU	8481	95
54	AGCAGCUAACAGCACUAUGUU	8190	96
55	GCUGCUUAUAUGCAGGAUCUU	9044	97
56	GAUCCUGUCUGAAGGGAUGUU	531	98
57	CAUCCCUGUUAAAAGCAGCUU	7118	99
58	UGGUCUAACCAGAGAGACCUU	9081	100
59	GGUCUCUUUUAACAUUUGCUU	928	101
60	GCAAAUGUUUUCUAGACCAUU	7557	102

No	Sequence	Target Gene	SEQ ID NO:
61	CUCCCAGGCUCAGAUCUGGUU	9097	103
62	CCAGAUCUUCCCUAAAAAAUU	1630	55
63	UUUUUUAUCUGCCUGGGAGUU	7011	56
64	UGGGUUCCCUAGUUAGCCAUU	9121	104
65	UGGCUAAGAUCUACAGCUGUU	8585	58
66	CAGCUGUCCCAAGAACCCAUU	7325	59

To Make MV-siRNA complexes targeted to HIV from the sequences in Table 7 above, the individual oligos can be combined and annealed as follows. 1) MV-siRNA_1649/1648/1648; Anneal sequences 1 & 2, and 3. 2) MV-siRNA_6259/4062/5291; Anneal sequences 4 & 5, and 6. 3) MV-siRNA_7387/7387/7387; Anneal sequences 7 & 8, and 9. 4) MV-siRNA_16/1630/7011; Anneal sequences 10 & 11, and 12. 5) MV-siRNA_40/8585/7325; Anneal sequences 13 & 14, and 15. 6) MV-siRNA_591/6988/1785; Anneal sequences 16 & 17, and 18. 7) MV-siRNA_685/8481/9046; Anneal sequences 19 & 20, and 21. 8) MV-siRNA_1284/6573/6311; Anneal sequences 21 & 22, and 23. 9) MV-siRNA_1785/591/6988; Anneal sequences 24 & 25, and 26. 10) MV-siRNA_3534/5432/2952; Anneal sequences 27 & 28, and 29. 11) MV-siRNA_4872/5779/7384; Anneal sequences 30 & 31, and 32. 12) MV-siRNA_5212/758/4736; Anneal sequences 33 & 34, and 35. 13) MV-siRNA_5365/7544/4191; Anneal sequences 36 & 37, and 38. 14) MV-siRNA_5784/8942/8158; Anneal sequences 39 & 40, and 41. 15) MV-siRNA_5862/4310/499; Anneal sequences 42 & 43, and 44. 16) MV-siRNA_6362/8559/6371; Anneal sequences 45 & 46, and 47. 17) MV-siRNA_6973/135/158; Anneal sequences 48 & 49, and 50. 18) MV-siRNA_7337/8481/8190; Anneal sequences 51 & 52, and 53. 19) MV-

siRNA_9044/531/7118; Anneal sequences 54 & 55, and 56. 20) MV-siRNA_9081/928/7557; Anneal sequences 57 & 58, and 59. 21) MV-siRNA_9097/1630/7011; Anneal sequences 60 & 61, and 62. 22) MV-siRNA_9121/8585/7325; Anneal sequences 63 & 64, and 65.

EXAMPLE 3

TRIVOID ANTI-APOB

Multivalent siRNA can be designed to suppress large genes by targeting in 2-3 locations on a single gene. The MV-siRNA can also employ alternative RNA chemistries to enhance the Tm during annealing. In this example, as shown in Table 8 below, a series of MV-siRNA are designed to target the apolipoprotein B (ApoB) gene, and the presence of optional 2'-O methyl RNA subunits is indicated within parenthesis.

Table 8: Trivalent MV-siRNA to ApoB

No	Sequence	Target Gene	SEQ ID NO
1	(UGGAACU)UUCAGCUUCAUAUU	ApoB @ 268	105
2	(UAUGAAG)GCACCAUGAUGUUU	ApoB @ 9905	106
3	(ACAUCAU)CUUCC(AGUUCCA)UU	ApoB @ 1703	107
4	(ACUCUUC)AGAGUUCUUGGUUU	ApoB @ 448	108
5	(ACCAAGA)CCUUGGAGACACUU	ApoB @ 2288	109
6	(GUGUCUC)AGUUG(GAAGAGU)UU	ApoB @ 6609	110
7	(ACCUGGA)CAUGGCAGCUGCUU	ApoB @ 469	111
8	(GCAGCUG)CAAACUCUUCAGUU	ApoB @ 458	112
9	(CUGAAGA)CGUAU(UCCAGGU)UU	ApoB @ 12263	113
10	(CAGGGUA)AAGAACAAUUUGUU	ApoB @ 520	114
11	(CAAAUUG)CUGUAGACAUUUUU	ApoB @ 4182	115
12	(AAAUGUC)CAGCG(UACCCUG)UU	ApoB @ 12548	116
13	(CCCUGGA)CACCGCUGGAACUUUU	ApoB @ 279	117
14	(AAGUUCC)AAUAACUUUUCCAUUU	ApoB @ 9161	118

No.	Sequence	Target Gene	SEQ ID NO:
15	(AUGGAAA)AGGCAAG(UCCAGGG)UU	ApoB @ 9968	119
16	(CCCUGGA)CACCGCUGGAACUUUUU	ApoB @ 278	120
17	(AAAGUUC)CAAUAACUUUUCCAUUU	ApoB @ 9161	121
18	(AUGGAAA)AUGGCAAG(UCCAGGG)UU	ApoB @ 9968	122

To make synthetic MV-siRNA trivalent complexes from the sequences in Table 8 above, the individual oligos can be combined and annealed as follows. 1) MV-siRNA_268/9950/1703; Anneal sequences 1 & 2, and then 3. 2) MV-siRNA_448/2288/6609; Anneal sequences 4 & 5, and then 6. 3) MV-siRNA_469/458/12263; Anneal sequences 7 & 8, and then 9. 4) MV-siRNA_520/4182/12548; Anneal sequences 10 & 11, and then 12. 5) MV-siRNA_279/9161/9986; Anneal sequences 13 & 14, and then 15. 6) MV-siRNA_278/9161/9986; Anneal sequences 16 & 17, and then 18.

Multivalent siRNA that are designed with potent primary and secondary strands can also employ wobble or universal bases to complete target complimentarity, or blunt ended DNA to deactivate the strand from silencing any target. Exemplary oligos directed to ApoB are shown in Table 9 below, in which (*) indicates an optional wobble or universal base.

Table 9: Exemplary Bivalent MV-siRNA to ApoB

No.	Sequence	Target Gene	SEQ ID NO:
19	UGAAUCGAGUUGCAUCUUUUU	ApoB @ 223	123
20	AAAGAUGCUGCUCAUCACAUU	ApoB @ 883	124
21	UGUGAUGACACUCGAUUCAUU	ApoB @ 10116 (G/A pairs)	125
22	U*UGAU*ACACUCGAUUCAUU	ApoB @ 10116 (univ. base)	126
23	TGTGATGACACTCGATTCA	null @ 10116	127
24	CAGCUUGAGUUCGUACCUGUU	ApoB @ 483	128
25	CAGGUACAGAGAACUCCAAUU	ApoB @ 11596	129
26	UUGGAGUCUGACCAAGCUGUU	ApoB @ 2454	130
27	UUGGAGUCUGAC*AAGCU*UU	ApoB @ 2454	131

No.	Sequence	Target Gene	SEQ ID NO:
28	TTGGAGTCTGACCAAGCTG	null @ 2454	132

To make synthetic MV-siRNA bivalent complexes from the sequences in Table 9 above, the individual oligos can be combined and annealed as follows. 7a) MV-siRNA_223/883/10116); Anneal sequences 19, 20, and 21. 7b) MV-siRNA_223/883/10116*); Anneal sequences 19, 20, and 22. 7c) MV-siRNA_223/883/null); Anneal sequences 19, 20, and 23. 8a) MV-siRNA_483/11596/2454); Anneal sequences 24, 25, and 26. 8b) MV-siRNA_483/11596/2454*); Anneal sequences 24, 25, and 26. 8c) MV-siRNA_483/11596/null); Anneal sequences 24, 25, and 26.

Multivalent-siRNAs can also be designed to suppress large genes by targeting 2-3 locations on a single gene. As noted, above, certain embodiments of the instant MV-siRNAs can also employ alternative RNA chemistries to enhance the Tm during annealing. In Table 10 below, optional 2'-O methyl RNA 2'-fluoro bases are indicated within parenthesis. Among other examples of alternate bases, 5-methyl can also increase Tm of MV-siRNA structure, if desired.

Table 10: Exemplary Trivalent MV-siRNA to ApoB

No.	Sequence	Target Gene	SEQ ID NO:
1	UGG(AA)CUUUCAGCUUCAUAUU	ApoB @ 268	105
2	U(AU)GAAGGCACCAUGAUGUUU	ApoB @ 9905	106
3	(ACAUCAU)CUUCCAGUUCCAUU	ApoB @ 1703	107
4	AC(U)CUUCAGAGUUCUUGGUUU	ApoB @ 448	108
5	(ACCAAGA)CCUUGGAGACACUU	ApoB @ 2288	109
6	G(U)GUCUCAGUUGGAAGAGUUU	ApoB @ 6609	110
7	(ACCUGGA)CAUGGCAGCUGCUU	ApoB @ 469	111
8	GC(A)GCUGCAAACUCUUCAGUU	ApoB @ 458	112
9	(CUGAAGA)CGUAU(UCCAGGU)UU	ApoB @ 12263	113
10	(CAGGGUA)AAGAACAAUUUGUU	ApoB @ 520	114

No	Sequence	Target Gene	SEQ ID NO:
11	(CAAAUU)GCUGUAGACA(UUU)UU	ApoB @ 4182	115
12	(AAAUGUC)CAGCGUACCCUGUU	ApoB @ 12548	116
13	(CCCUGGA)CACCGCUGGAACUUUU	ApoB @ 279	117
14	(AAGUUCC)AAUAACUUUUCCAUUU	ApoB @ 9161	118
15	(AU)GGAAAAGGCAAG(UCCAGGG)UU	ApoB @ 9968	119
16	CCC(U)GGACACCGCUGG(AACUUU)UU	ApoB @ 278	120
17	(AAA)GUUCCAAUAACUU(UU)CC(AU)UU	ApoB @ 9161	121
18	(AUGGAAA)AUGGCAAG(UCCAGGG)UU	ApoB @ 9968	122
19	UCAGGGCCGCUCUGUAUUUUU	ApoB @ 6427	133
20	AAAUACAUUUCUGGAAGAGUU	ApoB @ 8144	134
21	CUCUUCCAAAAAGCCCUGAUU	ApoB @ 12831	135
22	AAAUACAUUUCUGGAAGAGuu&CUCUUCCAAAAAGCCCUGAuu&UCAGGGCCGCUCUGUAUUUuu	Linker construct for cleavage after annealing. "&" = PC Spacer, or linkage phosphoramidite	136

To make synthetic MV-siRNA bivalent complexes from the sequences in Table 10 above, the individual oligos can be combined and annealed as follows. 1) MV-siRNA_268/9950/1703; Anneal sequences 1 & 2, and then 3. 2) MV-siRNA_448/2288/6609; Anneal sequences 4 & 5, and then 6. 3) MV-siRNA_469/458/12263; Anneal sequences 7 & 8, and then 9. 4) MV-siRNA_520/4182/12548; Anneal sequences 10 & 11, and then 12. 5) MV-siRNA_279/9161/9986; Anneal sequences 13 & 14, and then 15. 6) MV-siRNA_278/9161/9986; Anneal sequences 16 & 17, and then 18. 7) MV-siRNA_6427/8144/12831; Anneal sequences 19 & 20, and then 21. 7b) MV-siRNA_6427/8144/12831; Anneal strand 22, then cleave linkage phosphate with ammonium hydroxide. 7b) MV-siRNA_6427/8144/12831; Anneal strand 22, then cleave PC Spacer with UV light in the 300-350 nm spectral range.

In certain embodiments, multivalent-siRNA that are designed with potent primary and secondary strands can employ wobble, spacer, or abasic base types (examples are indicated by (*) in Table 11 below) to complete target compliments, or blunt ended DNA to deactivate the strand from silencing any target. In some embodiments, UNA, linker phosphoramidites, rSpacer, 5-nitroindole can act as effective abasic bases in place of mismatched nucleotides. If desired, the use of abasic bases can result in weakened Tm, and/or pyrimidines surrounding an abasic site can utilize 2'-fluoro bases to increase Tm by about 2 degrees for every 2'-fluoro base.

Table 11: Exemplary MV-siRNA Targeted to ApoB

No	Sequence	Target Gene	SEQ ID NO
23	UGAAUCGAGUUGCAUCUUUUU	ApoB @ 223	123
24	AAAGAUGCUGCUCAUCACAUU	ApoB @ 883	124
25	UGUGAUGACACUCGAUUCAUU	ApoB @ 10116 (G/A pairs)	125
26	U*UGAU*ACACUCGAUUCAUU	ApoB @ 10116 (* rSPACER base)	126
27	TGTGATGACACTCGATTCA	null @ 10116	127
28	CAGCUUGAGUUCGUACCUGUU	ApoB @ 483	128
29	CAGGUACAGAGAACUCCAAUU	ApoB @ 11596	129
30	UUGGAGUCUGACCAAGCUGUU	ApoB @ 2454	130
31	UUGGAGUCUGAC*AAGCU*UU	ApoB @ 2454 (* abasic base)	131
32	TTGGAGTCTGACCAAGCTG	null @ 2454	132
33	AACCCACUUUCAAAUUUCCUU	ApoB @ 9244	137
34	GGAAAUUGAGAAUUCUCCAUU	ApoB @ 1958	138
35	UGGAGAAUCUCAGUGGGUUUU	ApoB @ 8005	139
36	rUrGrGfA-fArArUrCrUrCrA-fUrGrGrG-fUrUrU	ApoB @ 8005	140
37	GAUGAUGAAACAGUGGGUUUU	ApoB @ 10439	141
38	AACCCACUUUCAAAUUUCCUU	ApoB @ 9244	137
39	GGAAAUUGGAGACAUCAUCUU	ApoB @ 2284	142

No	Sequence	Target Gene	SEQ ID NO
40	-rGfAfAfArUrUrGrGrArGrArCfA-rCfArUrCrUrU	ApoB @ 2284	143
41	GCAAACUCUUCAGAGUUCUUU	ApoB @ 452	144
42	AGAACUCCAAGGGUGGGAUUU	ApoB @ 11588	145
43	AUCCCACUUUCAAGUUUGCUU	ApoB @ 9244	146
44	fA-rCrCrCrArCrUrUrUrCrAfA-fUrUrU-rC	ApoB @ 9244	147

To make synthetic MV-siRNA bivalent complexes from the sequences in Table 11 above, the individual oligos can be combined and annealed as follows. 7a) MV-siRNA_223/883/10116); Anneal sequences 23, 24, and 25. 7b) MV-siRNA_223/883/10116*); Anneal sequences 23, 24, and 26. 7c) MV-siRNA_223/883/null); Anneal sequences 23, 24, and 27. 8a) MV-siRNA_483/11596/2454); Anneal sequences 28, 29, and 30. 8b) MV-siRNA_483/11596/2454*); Anneal sequences 28, 29, and 31. 8c) MV-siRNA_483/11596/null); Anneal sequences 28, 29, and 32. 9) MV-siRNA_9244/1958/8005); Anneal sequences 33, 34, and 35. 9b) MV-siRNA_9244/1958/8005); Anneal sequences 33, 34, and 36. 10) MV-siRNA_10439/9244/2284); Anneal sequences 37, 38, and 39. 10b)MV-siRNA_10439/9244/2284); Anneal sequences 37, 38, and 40. 11)MV-siRNA_452/11588/9244); Anneal sequences 41, 42, and 43. 11b) MV-siRNA_ 452/11588/9244); Anneal sequences 41, 42, and 44.

As exemplified in Table 12 below, multivalent siRNA can be targeted against human ApoB. Bivalent MV-siRNA can function with various tolerances to structure and target complementarity of each strand

Table 12: Exemplary Multivalent-siRNA Targeted to Human ApoB

No	Sequence	ApoB Gene site	SEQ ID NO
1	CUUCAUCACUGAGGCCUCUUU	1192	148
2	AGAGGCCAAGCUCUGCAUUUU	5140	149
3	AAUGCAGAUGAAGAUGAAGAA	10229	150

No	Sequence	ApoB Gene site	SEQ ID NO:
4	UUCAGCCUGCAUGUUGGCUUU	2724	151
5	AGCCAACUAUACUUGGAUCUU	13294	152
6	GAUCCAAAAGCAGGCUGAAGA	4960	153
7	CCCUCAUCUGAGAAUCUGGUU	8927	154
8	CCAGAUUCAUAAACCAAGUUU	9044	155
9	ACUUGGUGGCCCAUGAGGGUU	3440	156
10	UCAAGAAUUCCUUCAAGCCUU	9595	157
11	GGCUUGAAGCGAUCACACUUU	758	158
12	AGUGUGAACGUAUUCUUGAUU	4367	159
13	UUGCAGUUGAUCCUGGUGGUU	344	160
14	CCACCAGGUAGGUGACCACUU	1354	161
15	GUGGUCAGGAGAACUGCAAUU	2483	162
16	CCUCCAGCUCAACCUUGCAUU	358	163
17	UGCAAGGUCUCAAAAAAUGUU	6341	164
18	CAUUUUUGAUCUCUGGAGGUU	4043	165
19	CAGGAUGUAAGUAGGUUCAUU	570	166
20	UGAACCUUAGCAACAGUGUUU	5687	167
21	ACACUGUGCCCACAUCCUGUU	9109	168
22	GGCUUGAAGCGAUCACACUUU	758	169
23	AGUGUGAACGUAUUCUUGUUU	4367	170
24	ACAAGAAUUCCUUCAAGCCUU	9595	171

No	Sequence	ApoB Gene site	SEQ ID NO:
25	UGAAGAGAUUAGCUCUCUGUU	1153	172
26	CAGAGAGGCCAAGCUCUGCUU	5143	173
27	GCAGAGCUGGCUCUCUUCAUU	10304	174
28	CUCAGUAACCAGCUUAUUGUU	1170	175
29	CAAUAAGAUUUAUAACAAAUU	7084	176
30	UUUGUUAUCUUAUACUGAGUU	9650	177
31	GAACCAAGGCUUGUAAAGUUU	1258	178
32	ACUUUACAAAAGCAACAAUUU	6286	179
33	AUUGUUGUUAAAUUGGUUCUU	6078	180
34	CAGGUAGGUGACCACAUCUUU	1350	181
35	AGAUGUGACUGCUUCAUCAUU	1203	182
36	UGAUGAACUGCGCUACCUGUU	8486	183
37	CCAGUCGCUUAUCUCCCGGUU	1786	184
38	CCGGGAGCAAUGACUCCAGUU	2678	185
39	CUGGAGUCAUGGCGACUGGUU	2486	186
40	UGGAAGAGAAACAGAUUUGUU	2046	187
41	CAAAUCUUUAAUCAGCUUCUU	2403	188
42	GAAGCUGCCUCUUCUUCCAUU	12299	189
43	AUCCAAAGGCAGUGAGGGUUU	2152	190
44	ACCCUCAACUCAGUUUUGAUU	12242	191
45	UCAAAACCGGAAUUUGGAUUU	3316	192
46	UAGAGACACCAUCAGGAACUU	2302	193

No	Sequence	ApoB Gene site	SEQ ID NO:
47	GUUCCUGGAGAGUCUUCAAUU	1102	194
48	UUGAAGAAUUAGGUCUCUAUU	1153	195
49	GCUCAUGUUUAUCAUCUUUUU	2350	196
50	AAAGAUGCUGAACUUAAAGUU	7622	197
51	CUUUAAGGGCAACAUGAGCUU	2863	198
52	GGAGCAAUGACUCCAGAUGUU	2675	199
53	CAUCUGGGGGAUCCCCUGCUU	2544	200
54	GCAGGGGAGGUGUUGCUCCUU	912	201
55	UCACAAACUCCACAGACACUU	2761	202
56	GUGUCUGCUUUAUAGCUUGUU	5672	203
57	CAAGCUAAAGGAUUUGUGAUU	9683	204
58	GCAGCUUGACUGGUCUCUUUU	2914	205
59	AAGAGACUCUGAACUGCCCUU	4588	206
60	GGGCAGUGAUGGAAGCUGCUU	8494	207
61	CAGGACUGCCUGUUCUCAAUU	2996	208
62	UUGAGAACUUCUAAUUUGGUU	8522	209
63	CCAAAUUUGAAAAGUCCUGUU	9855	210
64	UGUAGGCCUCAGUUCCAGCUU	3132	211
65	GCUGGAAUUCUGGUAUGUGUU	8335	212
66	CACAUACCGAAUGCCUACAUU	9926	213
67	GACUUCACUGGACAAGGUCUU	3300	214
68	GACCUUGAAGUUGAAAAUGUU	5301	215

No	Sequence	ApoB Gene site	SEQ ID NO:
69	CAUUUUCUGCACUGAAGUCUU	11983	216
70	AAGCAGUUUGGCAGGCGACUU	3549	217
71	GUCGCCUUGUGAGCACCACUU	5039	218
72	GUGGUGCCACUGACUGCUUUU	12521	219
73	CAGAUGAGUCCAUUUGGAGUU	3568	220
74	CUCCAAACAGUGCCAUGCCUU	9142	221
75	GGCAUGGAGCCUUCAUCUGUU	3256	222
76	CACAGACUUGAAGUGGAGGUU	4086	223
77	CCUCCACUGAGCAGCUUGAUU	2924	224
78	UCAAGCUUCAAAGUCUGUGUU	974	225
79	AUGGCAGAUGGAAUCCCACUU	4102	226
80	GUGGGAUCACCUCCGUUUUUU	2971	227
81	AAAACGGUUUCUCUGCCAUUU	12836	228
82	UGAUACAACUUGGGAAUGGUU	4148	229
83	CCAUUCCCUAUGUCAGCAUUU	2971	230
84	AUGCUGACAAAUUGUAUCAUU	12836	231

To make synthetic MV-siRNA bivalent complexes from the sequences in Table 12 above, the individual oligos can be combined and annealed as follows. MV-siRNA; Anneal sequences 1, 2, and 3. MV-siRNA; Anneal sequences 4, 5, and 6. MV-siRNA; Anneal sequences 7, 8, and 9. MV-siRNA; Anneal sequences 10, 11, and 12. MV-siRNA; Anneal sequences 13, 14, and 15. MV-siRNA; Anneal sequences 16, 17, and 18. MV-siRNA; Anneal sequences 19, 20, and 21. MV-siRNA; Anneal sequences 22, 23, and 24. MV-siRNA; Anneal sequences 25, 26,

and 27. MV-siRNA; Anneal sequences 28, 29, and 30. MV-siRNA; Anneal sequences 31, 32, and 33. MV-siRNA; Anneal sequences 34, 35, and 36. MV-siRNA; Anneal sequences 37, 38, and 39. MV-siRNA; Anneal sequences 40, 41, and 42. MV-siRNA; Anneal sequences 43, 44, and 45. MV-siRNA; Anneal sequences 46, 47, and 48. MV-siRNA; Anneal sequences 49, 50, and 51. MV-siRNA; Anneal sequences 52, 53, and 54. MV-siRNA; Anneal sequences 55, 56, and 57. MV-siRNA; Anneal sequences 58, 59, and 60. MV-siRNA; Anneal sequences 61, 62, and 63. MV-siRNA; Anneal sequences 64, 65 and 66. MV-siRNA; Anneal sequences 67, 68, and 69. MV-siRNA; Anneal sequences 70, 71, and 72. MV-siRNA; Anneal sequences 73, 74, and 75. MV-siRNA; Anneal sequences 76, 77, and 78. MV-siRNA; Anneal sequences 79, 80, and 81. MV-siRNA; Anneal sequences 82, 83, and 84.

MV-siRNA directed to ApoB can be used to treat or manage a wide variety of diseases or conditions associated with the expression of that target protein, as described herein and known in the art.

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CLAIMS:

1. A polynucleotide complex of at least three separate polynucleotides, comprising

(a) a first polynucleotide comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region;

(b) a second polynucleotide comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and

(c) a third polynucleotide comprising a null region or a target-specific region that is complementary to a third target specific, a 5' region, and a 3' region,

wherein each of the target-specific regions of the first, second, and third polynucleotides are complementary to a different target sequence,

wherein the 5' region of the first polynucleotide is complementary to the 3' region of the third polynucleotide, wherein the 3' region of the first polynucleotide is complementary to the 5' region of the second polynucleotide, and wherein the 3' region of the second polynucleotide is complementary to the 5' region of the third polynucleotide,

and wherein the three separate polynucleotides hybridize via their complementary 3' and 5' regions to form a polynucleotide complex with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

2. The polynucleotide complex of claim 1, wherein the first, second, and/or third polynucleotide comprises about 15-30 nucleotides.

3. The polynucleotide complex of claim 1, wherein the first, second, and/or third polynucleotide comprises about 17-25 nucleotides.

4. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises about 5-10 nucleotide pairs.

5. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises about 7-8 nucleotide pairs.

6. The polynucleotide complex of claim 1, wherein each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA.

7. The polynucleotide complex of claim 1, wherein at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

8. The polynucleotide complex of claim 1, wherein all or a portion of the 5' and/or 3' region of each polynucleotide is also complementary to the target sequence for that polynucleotide.

9. The polynucleotide complex of claim 1, wherein one or more of the self-complementary regions comprises a 3' overhang.

10. A self-hybridizing polynucleotide molecule, comprising

(a) a first nucleotide sequence comprising a target-specific region that is complementary to a first target sequence, a 5' region, and a 3' region,

(b) a second nucleotide sequence comprising a target-specific region that is complementary to a second target sequence, a 5' region, and a 3' region; and

(c) a third nucleotide sequence comprising a null region or a target-specific region that is complementary to a third target sequence, a 5' region, and a 3' region,

wherein the target-specific regions of each of the first, second, and third nucleotide sequences are complementary to a different target sequence,

wherein the 5' region of the first nucleotide sequence is complementary to the 3' region of the third nucleotide sequence, wherein the 3'

region of the first nucleotide sequence is complementary to the 5' region of the second nucleotide sequence, and wherein the 3' region of the second nucleotide sequence is complementary to the 5' region of the third nucleotide sequence,

and wherein each of the 5' regions hybridizes to their complementary 3' regions to form a self-hybridizing polynucleotide molecule with a first, second, and third single-stranded region, and a first, second, and third self-complementary region.

11. The self-hybridizing polynucleotide molecule of claim 10, wherein the first, second, or third nucleotide sequence comprises about 15-60 nucleotides.

12. The self-hybridizing polynucleotide molecule of claim 10, wherein the target-specific regions each comprise about 15-30 nucleotides.

13. The self-hybridizing polynucleotide molecule of claim 10, wherein one or more of the self-complementary regions comprises about 10-54 nucleotides.

14. The self-hybridizing polynucleotide molecule of claim 10, wherein one or more of the self-complementary regions comprises a 3' overhang.

15. The self-hybridizing polynucleotide molecule of claim 10, wherein one or more of the self-complementary regions forms a stem-loop structure.

16. The self-hybridizing polynucleotide molecule of claim 10, wherein one or more of the self-complementary regions comprises a proximal box of dinucleotides AG/UU that is outside of the target specific region

17. The self-hybridizing polynucleotide molecule of claim 10, wherein one or more of the self-complementary regions comprises a distal box of 4 nucleotides that is outside of the target-specific region, wherein the third nucleotide of the distal box is not a G.

18. The self-hybridizing polynucleotide molecule of claim 10, wherein each of said first, second, and third target sequences are present in the same gene, cDNA, mRNA, or microRNA.

19. The self-hybridizing polynucleotide molecule of claim 10, wherein at least two of said first, second, and third target sequences are present in different genes, cDNAs, mRNAs, or microRNAs.

20. A vector that encodes a self-hybridizing polynucleotide molecule according to claim 10.

21. A method of reducing expression of a gene, comprising introducing a polynucleotide complex of any one of claims 1-9, a polynucleotide molecule of any one of claims 10-19, or a vector of claim 20 into a cell.

22. The method of claim 21, wherein said method is practiced *in vitro*.

23. The method of claim 21, wherein said method is practiced *in vivo*.

24. A polynucleotide complex of any one of claims 1-9, a polynucleotide molecule of any one of claims 10-19, a vector of claim 20, or a method of any one of claims 21-23, wherein each of two or more of the first, second, and/or third target-specific regions is complementary to a different target region in an mRNA transcript of an HIV gene.

25. A polynucleotide complex of any one of claims 1-9, a polynucleotide molecule of any one of claims 10-19, a vector of claim 20, or a method of any one of claims 21-23, wherein each of two or more of the first, second, and/or third target-specific regions is complementary to a different target region in an mRNA transcript of a human apolipoprotein B (ApoB) gene.

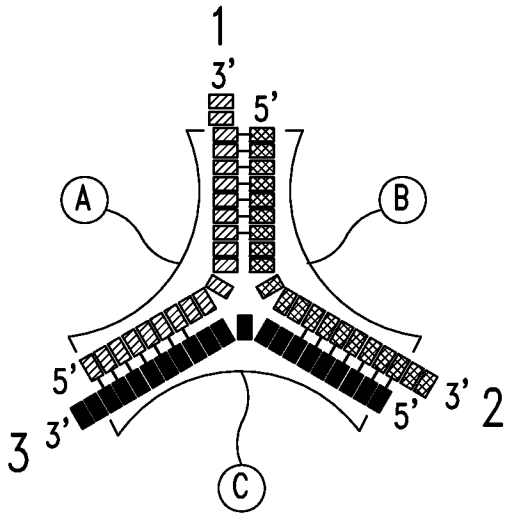

FIG. 1

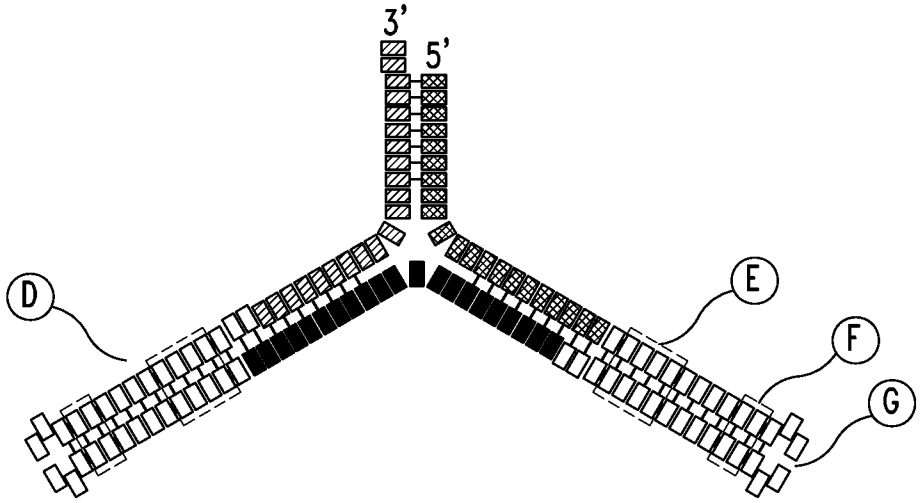

FIG. 2

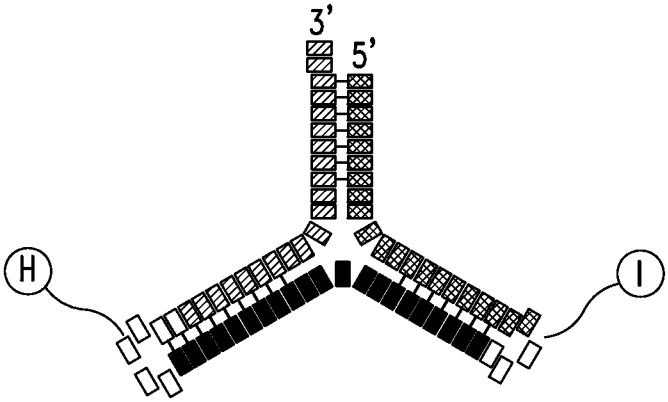

FIG. 3

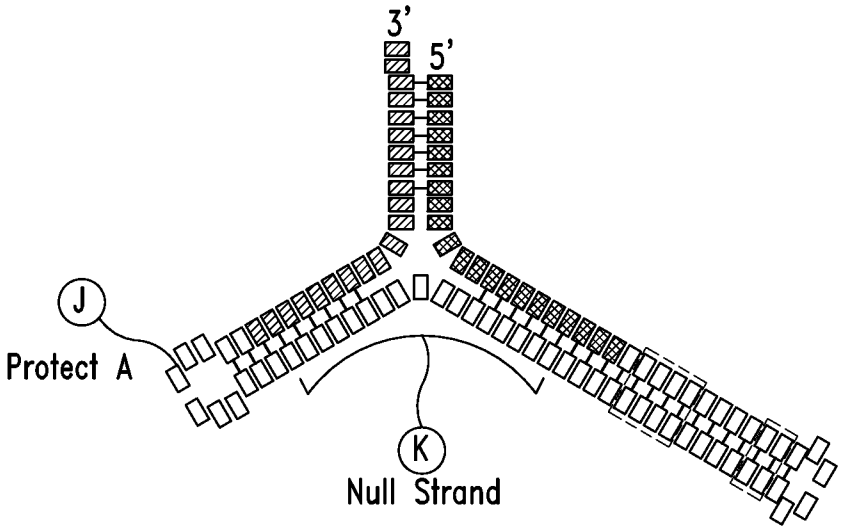

FIG. 4

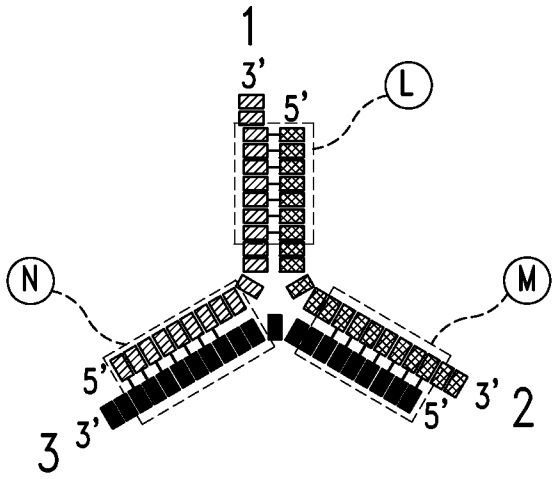

FIG. 5

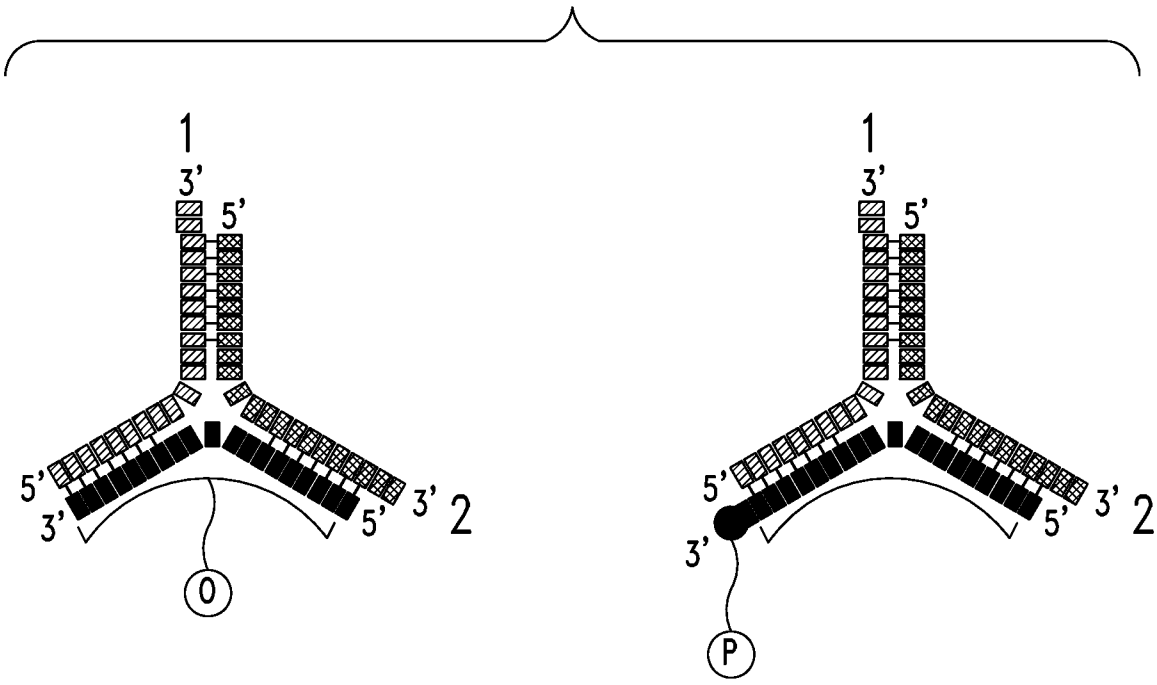

FIG. 6

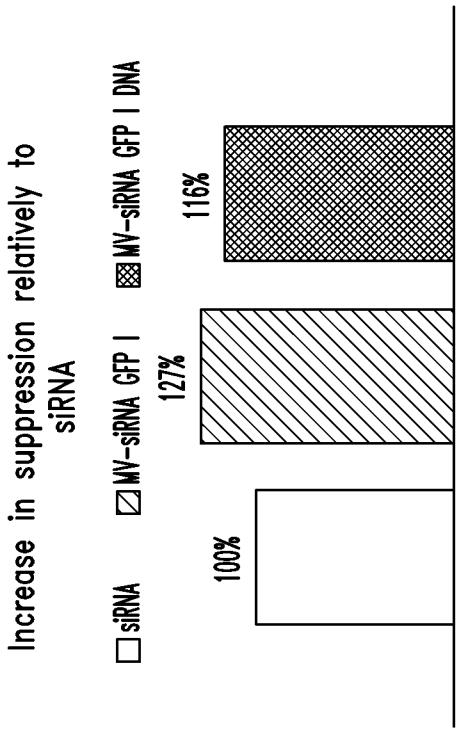
Increase in suppression relatively to siRNA
siRNA
MV-siRNA GFP I
MV-siRNA GFP I DNA
100%
127%
116%

FIG. 7B

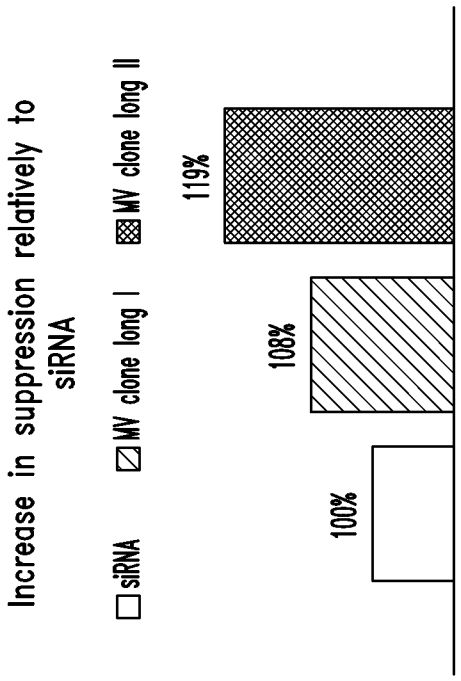
Increase in suppression relatively to siRNA
siRNA
MV clone long I
MV clone long II
100%
108%
119%

FIG. 7A

GFP Targeting
AUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGGUGCCCAUCCUGGUCGAGCUGGACGGCGACGUAA
ACGGCCACAAGUUCAGCGUGUCCGGCGAGGGCGAGGGCGaUGCCaCCUACGGCAAGCUGACCCUGAAGUU
CAUCUGCACCACCGGCAAGCUGCCCGUGCCCUGGCCCACCCUCGUGACCACCCUGACCUACGGCGUGCAG
UGCUUCAGCCGCUACCCCGACCACAUGAAGCAGCACGACUUCUUCAAGUCCGCCAUGCCCGAAGGCUACG
UCCAGGAGCGCACCAUCUUCUUCAAGGACGACGGCAACUACAAGACCCGCGCCGAGGUGAAGUUCGAGGG
CGACACCCUGGUGAACCGCAUCGAGCUGAAGGGCAUCGACUUCAAGGAGGACGGCAACAUCCUGGGGCAC
AAGCUGGAGUACAACUACAACAGCCACAACGUCUAUAUCAUGGCCGACAAGCAGAAGAACGGCAUCAAGG
UGAACUUCAAGAUCCGCCACAACAUCGAGGACGGCAGCGUGCAGCUCGCCGACCACUACCAGCAGAACAC
CCCCAUCGGCGACGGCCCCGUGCUGCUGCCCGACAACCACUACCUGAGCACCCAGUCCGCCCUGAGCAAA
GACCCCAACGAGAAGCGCGAUCACAUGGUCCUGCUGGAGUUCGUGACCGCCGCCGGGAUCACUCUCGGCA
UGGACGAGCUGUACAAGUAAA

FIG. 8A

GFP Targeting
AUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGGUGCCCAUCCUGGUCGAGCUGGACGGCGACGUAA
ACGGCCACAAGUUCAGCGUGUCCGGCGAGGGCGAGGGCGaUGCCaCCUACGGCAAGCUGACCCUGAAGUU
CAUCUGCACCACCGGCAAGCUGCCCGUGCCCUGGCCCACCCUCGUGACCACCCUGACCUACGGCGUGCAG
UGCUUCAGCCGCUACCCCGACCACAUGAAGCAGCACGACUUCUUCAAGUCCGCCAUGCCCGAAGGCUACG
UCCAGGAGCGCACCAUCUUCUUCAAGGACGACGGCAACUACAAGACCCGCGCCGAGGUGAAGUUCGAGGG
CGACACCCUGGUGAACCGCAUCGAGCUGAAGGGCAUCGACUUCAAGGAGGACGGCAACAUCCUGGGGCAC
AAGCUGGAGUACAACUACAACAGCCACAACGUCUAUAUCAUGGCCGACAAGCAGAAGAACGGCAUCAAGG
UGAACUUCAAGAUCCGCCACAACAUCGAGGACGGCAGCGUGCAGCUCGCCGACCACUACCAGCAGAACAC
CCCCAUCGGCGACGGCCCCGUGCUGCUGCCCCGACAACCACUACCUGAGCACCCAGUCCGCCCUGAGCAAA
GACCCCAACGAGAAGCGCGAUCACAUGGUCCUGCUGGAGUUCGUGACCGCCGCCGGGAUCACUCUCGGCA
UGGACGAGCUGUACAAGUAAA

FIG. 8B

GFP Targeting

AUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGGUGCCCAUCCUGGUCGAGCUGGACGGCGACGUAA
ACGGCCACAAGUUCAGCGUGUCCGGCGAGGGCGAGGGCGAUGCCACCUACGGCAAGCUGACCCUGAAGUU
CAUCUGCACCACCGGCAAGCUGCCCGUGCCCUGGCCCACCCUCGUGACCACCUGACCUACGGCGUGCAG
UGCUUCAGCCGCUACCCCGACCACAUGAAGCAGCACGACUUCUUCAAGUCCGCCAUGCCCGAAGGCUACG
UCCAGGAGCGCACCAUCUUCUUCAAGGACGACGGCAACUACAAGACCCGCGCCGAGGUGAAGUUCGAGGG
CGACACCCUGGUGAACCGCAUCGAGCUGAAGGGCAUCGACUUCAAGGAGGACGGCAACAUCCUGGGGCAC
AAGCUGGAGUACAACUACAACAGCCACAACGUCUAUAUCAUGGCCGACAAGCAGAAGAACGGCAUCAAGG
UGAACUUCAAGAUCCGCCACAACAUCGAGGACGGCAGCGUGCAGCUCGCCGACCACUACCAGCAGAACAC
CCCCAUCGGCGACGGCCCCGUGCUGCCCGACAACCACUACCUGAGCACCCAGUCCGCCCUGAGCAAA
GACCCCAACGAGAAGCGCGAUCACAUGGUCCUGCUggAGUUCGUGACCGCCGCGGGAUCACUCUCGGCA
UGGACGAGCUGUACAAGUAAA

FIG. 8C

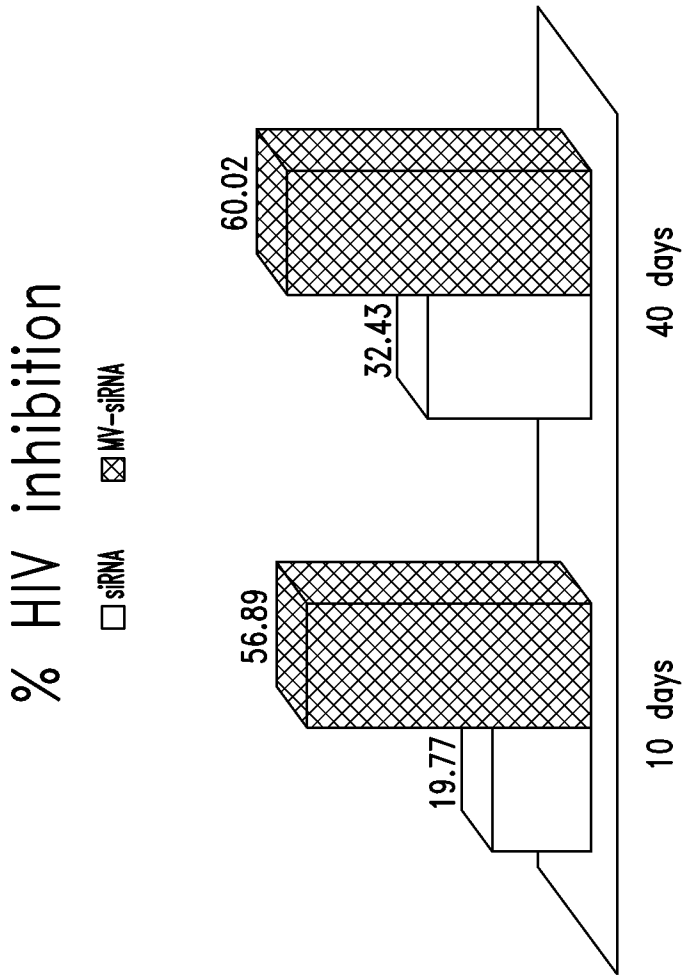
% HIV inhibition
siRNA
MV-siRNA
60.02
32.43
56.89
19.77
40 days
10 days

FIG. 9

EXEMPLARY HIV GENOME:

GUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCUCUGGCUAACUAGGGAACCCACUGCUUAAGCCUCAAUAAAG
CUUGCCUUGAGUGCUUCAAGUAGUGUGUGCCCGUCUGUUGUGUGACUCUGGUAACUAGAGAUCCCUCAGACCCUUUUA
GUCAGUGUGGAAAAUCUCUAGCAGUGGCGCCCGAACAGGGACAUGAAAGCGAAAGGGAAACCAGAGGAGCUCUCUCGA
CGCAGGACUCGGCUUGCUGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACUGGUGAGUACGCCAAAAAUUUUGACU
AGCGGAGGCUAGAAGGAGAGAGAUGGGUGCGAGAGCGUCAGUAUUAAGCGGGGGAAAAUUAGAUCGAUGGGAAAAAAU
UCGGUUAAGGCCAGGGGGAAAGAAAAAAUAUAAAUUAAAACAUAUAGUAUGGGCAAGCAGGGAGCUAGAACGAUUCGC
AGUUAAUCCUGGCCUGUUAGAAACAUCAGAAGGCUGUAGACAAAUACUGGGACAGCUACAACCAUCCCUUCAGACAGG
AUCAGAAGAACGUAGAUCAUUAUAUAAUACAGUAGCAACCCUCUAUUGUGUGCAUCAAAGGAUAGAGAUAAAAGACAC
CAAGGAAGCUUUAGACAAGAUAGAGGAAGAGCAAAACAAAAGUAAGAAAAAAGCACAGCAAGCAGCAGCUGACACAGG
ACACAGCAGCCAGGUCAGCCAAAAUUACCCUAUAGUGCAGAACAUCCAGGGGCAAAUGGUACAUCAGGCCAUAUCACC
UAGAACUUUAAAUGCAUGGGUAAAAGUAGUAGAAGAGAAGGCUUUCAGCCCAGAAGUGAUACCCAUGUUUUCAGCAUU
AUCAGAAGGAGCCACCCCACAAGAUUUAAACACCAUGCUAAACACAGUGGGGGGGACAUCAAGCAGCCAUGCAAAUGUU
AAAAGAGACCAUCAAUGAGGAAGCUGCAGAAUGGGAUAGAGUGCAUCCAGUGCAUGCAGGGCCUAUUGCACCAGGCCA
GAUGAGAGAACCAAGGGGAAGUGACAUAGCAGGAACUACUAGUACCCUUCAGGAACAAAUAGGAUGGAUGACACAUAA
UCCACCUAUCCCAGUAGGAGAAAUCUAUAAAAGAUGGAUAAUCCUGGGAUUAAAUAAAAUAGUAAGAAUGUAUAGCCC
UACCAGCAUUCUGGACAUAAGACAAGGACCAAAGGAACCCUUUAGAGACUAUGUAGACCGAUUCUAUAAAACUCUAAG
AGCCGAGCAAGCUUCACAAGAGGUAAAAAAUUGGAUGACAGAAACCUUGUUGGUCCAAAAUGCGAACCCAGAUUGUAA
GACUAUUUUAAAAGCAUUGGGACCAGGAGCGACACUAGAAGAAAUGAUGACAGCAUGUCAGGGAGUGGGGGGACCCGG
CCAUAAAGCAAGAGUUUUGGCUGAAGCAAUGAGCCAAGUAACAAAUCCAGCUACCAUAAUGAUACAGAAAGGCAAUUU
UAGGAACCAAAGAAAGACUGUUAAGUGUUUCAAUUGUGGCAAAGAAGGGCACAUAGCCAAAAAUUGCAGGGCCCCUAG
GAAAAAGGGCUGUUGGAAAUGUGGAAAGGAAGGACACCAAAUGAAAGAUUGUACUGAGAGACAGGCUAAUUUUUUAGG
GAAGAUCUGGCCUUCCCACAAGGGAAGGCCAGGGAAUUUUCUUCAGAGCAGACCAGAGCCAACAGCCCCACCAGAAGA
GAGCUUCAGGUUUGGGGAAGAGACAACAACUCCCCUCUCAGAAGCAGGAGCCGAUAGACAAGGAACUGUAUCCUUUAGC
UUCCCUCAGAUCACUCUUUGGCAGCGACCCCUCGUCACAAUAAAGAUAGGGGGGCAAUUAAAGGAAGCUCUAUUAGAU
ACAGGAGCAGAUGAUACAGUAUUAGAAGAAAUGAAUUUGCCAGGAAGAUGGAAACCAAAAAUGAUAGGGGGAAUUGGA
GGUUUUAUCAAAGUAAGACAGUAUGAUCAGAUACUCAUAGAAAUCUGCGGACAUAAAGCUAUAGGUACAGUAUUAGUA
GGACCUACACCUGUCAACAUAAUUGGAAGAAAUCUGUUGACUCAGAUUGGCUGCACUUUAAAUUUUCCCAUUAGUCCU
AUUGAGACUGUACCAGUAAAAUUAAAGCCAGGAAUGGAUGGCCCAAAAGUUAAACAAUGGCCAUUGACAGAAGAAAAA
AUAAAAGCAUUAGUAGAAAUUUGUACAGAAAUGGAAAAGGAAGGAAAAAUUUCAAAAAUUGGGCCUGAAAAUCCAUAC
AAUACUCCAGUAUUUGCCAUAAAGAAAAAAGACAGUACUAAAUGGAGAAAAUUAGUAGAUUUCAGAGAACUUAAUAAG
AGAACUCAAGAUUUCUGGGAAGUUCAAUUAGGAAUACCACAUCCUGCAGGGUUAAAACAGAAAAAAUCAGUAACAGUA
CUGGAUGUGGGCGAUGCAUAUUUUUCAGUUCCCUUAGAUAAAGACUUCAGGAAGUAUACUGCAUUUACCAUACCUAGU

FIG. 10A

AUAAACAAUGAGACACCAGGGAUUAGAUCAGUACAAUGUGCUUCCACAGGGAUCGGAAAGGAUCACCAGCAAUAUUC
CAGUGUAGCAUGACAAAAAAUCUUUAGAGCCUUUUAGAAAACAAAAUCCAGACAUUAGUCAUCUAUCAAUACAUGGAUGAU
UUGUAUGUAGGAUCUGACUUAGAAAUAGGGCAGCAUAGAACUGAGACAACAUCUGUUGAGGGUGG
GGAUUUACCACACCAGACAAAAAAACAUCAGAAAGAACCUCCAUUCCUUUGGAUGGGUUAUGAACUCCAUCCUGAUAAA
UGGACAGUACAGCCUAUAGUGCUGCCAGAAAAGGACAGCUGGACUGUCAAUGACAUACAGAAAUUAGUGGGAAAAUUG
AAUUGGGCAAGUCAGAUUUAUGCAGGGAUUAAAGUAAGGCAAUUAUGUAAACUUCUUAGGGAACCAAAGCACUAACA
GAAGUAGUACCACCACUAACAGAAGAGCAGAGCUAGAAACAGGGAGAUUCUAAAAGAACCGGUACAUGGA
GUGUAUUAUGACCCAUCAAAAGACUUAAUAGCAGAAAUACAGAAGGCAAGGGCCAAGGCCAUGGACAUAUUCAAAUUUAU
CAAGAGCCAUUUAAAAAAUCUGAAAAACAGGAAAGUAUGCAAGAAUGAAGGUGCCCCACACUAAUGAUGUGAAACAAUUA
ACAGAGGCAGUACAAAAAAAUAGCCACAGAAAGCAUAGUAAAUGGGGAAAGACUCCUAAAUUUAAAUUACCCAUACAA
AAGGAAACAUGGGAAGCAUGGUGGACAGAGUAUUGGCAAGCCACCUGGAUUCCUGAGUGGAGUUUUGUCAAUACCCCU
CCCUUAGUGAAGUUAUGGUACCAGUUAGAGAAAGAACCCAUAAAUAGAGCAGAAACUUUCUAUGUAGAUGGGCGCAGCC
AAUAGGGAAAACUAAAAUUAGGCAGGAAAAGCAGGAUAUGUAACUGACAGAGGAAGACAAAAAGUUGUCCCCCUAACGGACACA
ACAAAUCAGAAGACUGAGUUACAAGCAAUUCAUCUAGCUUUGCAGGAUUCGGAUUAGAAACAUAGUGACAGAC
UCACAAUAUGCAUUGGGAAUCAUUCAAGCACAACCAGAUAAGAGUGAAUCAGAGUUAGUCAGUCAAAUAAUAGAGCAG
UUAAUAAAAAAGGAAAAAAGUCUACCUGGCAUGGACCAGCACACAAAGGAAUUGGAGGAAAUGAACAAGUAGAUAAA
UUGGUCAGUGCUGGAAUCAGGAAAGUACUAUUUUAGAUGGAAUAGAUUAAGGCCCAAGAAGAACAUGAGAAAUAUCAC
AGUAAUUGGAGAGCAAUGGCUAGUGAUUUUAACCUACCACCUGUAGUAGCAAAAGAAAUAGUAGCCAGCUGUGAUAAA
UGUCAGCUAAAAGGGAAGCCAUGCAUGGACAAGUAGACUGUAGCCCAGGAAUAUGGCAGCUAGAUUGUACACAUUUA
GAAGGAAAAGUUAUCUUGGUAGCAGUCAUGUAGCCAGUGGAUAUAUAGAAGCAGAAGUAAUUCCAGCAGACAGGG
CAAGAAACAGCAUACUUCCUCUUAAAAUUAGCCAGGAAGAUGGCCAGUAAAAACAGUACAUACAGACAAUGGCAGCAAU
UUCACCAGUACUACAGUUAAAGGCCGCCUGUUGGUGGGGAUCAAGCAGGAAUUUGGCAUUCCCUACAAUCCCCAA
AGUCAAGCGAGUAAUAGAAUCUAUGAAUAAAGAUAAUUAUAGGACAGGAUCAGGCUGAACAUCUU
AAGACAGCAGUACAAAUGGCAGUAUUCAUCCACAAUUUUAAAAGACAAAAGGGCGGGUACAGUGCAGGGAA
AGAAUAGUAGACAUAAUAGCAACAGACAUACAAACUAAAGAAUUACAAAAUUACAAAAUUCAAAAUUUUCGG
GUUUAUUACAGGGACACAGCAGAUCCAGUUUGGAAAGGACCAGCAAAGCUCCUCUGGAAGGUGAAGGGCAGUACUA
AUACAAGAUAAUAGUGACAUAAAAAGUAGUGCCAAGAAGCAAAGAUCAUCAGGGAUUAUGGAAAACAGAUGGCA
GGUGAUGAUUGUGUGGCAAGUAGACAGGAUGAGGAUUAACACACAUGGAAAAGAUUAGUAAAACACCAUAUGUAUUUC
AAGGAAAGCUAAGGACUCGUUUUAUAGACACAUCACUAUGAAAGUACUAAUCCAAAAAUAGUACAGUACACAUCCC
ACUAGGGGAUGCUAAAUUAGUAAUAACAACAUAUUGGGGUCUGCAUACAGGAGAAAGAGACUGGGCAUUUGGGUCAGGG

FIG. 10B

AGUCUCCAUAGAAUGGAGGAAAAAGAGAUAUAGCACACAAGUAGACCUGACCUAGCAGACCAACUAAUUCAUCUGCA
CUAUUUUGAUUGUUUUUCAGAAUCUGCUAUAAGAAAUACCAUAUUAGGACGUAUAGUUAGUCCUAGGUGUGAAUAUCA
AGCAGGACAUAAACAAGGUAGGAUCUCUACAGCAUUAAUAAAAACCAAAAACAGAUAAAAGCCACC
UUUGCCUAGUGUUAGGAAACUGACAGAGGACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCAUAC
AAUGAAUGGACACUAGAGCUUUUAGAGGAACUUAAGAGUGAAGCUGUUAGACAUUUUCCUAGGAUAUGGCUCCAUAAC
UUAGGACAACAUAUCUAUGAAACUUACGGGCAGGAGUGGAAGCCAUAAUAAGAAUUCUGCAACAACTUG
CUGUUUAUCCAUUUCAGAAUUGGGUCGACAUAGCAGAAUAGGCGUUACUCGACAGAGGAGAGCAAGAAAUGGAGCC
AGUAGAUCCUAGACUAGAGCCCUGGAAGCAUCCAGGAAGUCAGCCUAAAAACUGCUGUACCAAUUGCUAUUGUAAAAA
GUGUUGCUUUCAUUGCCAAGUUUGUUCAUAACAAAAGCCUUAGGCAUCUCCUAUGGCAGGAAGAAGCGGAGACAGCG
ACGAAGACCUCCUCCAAGGCAGUCAGACUCAUCAAGUUUCUCUAUCAAAGCAGUAAGUAUAAUCAUGUAAUGCAACCUAU
ACAAAUAGCAAUAGUAGUAGCAUUAGUAGCAAUACAAUAGCUGUGUGCUCCAUAGUAAUCAUACAGAAAUAUAG
GAAAAUAUUAAGACAAAGAAAAUAGACAGGUUAAUUGAUAGACUAAUAGAAAGAGCAGAAGACAGUGGCAAUGAGAG
UGAAGGAGAAAUAUAUCAGCACUUGUGGAGAUGGGGGUGGAGAUGGGGCACCAUGCUCCUUGGGAUGUUGAUGAUCUGUA
GUGCUACAGAAAAAUUGUGGGUCACAGUCUAUUAUGGGGUACCUGUGUGGAAGGAAGCAACCACACUCAUUUUGUG
CAUCAGAUGCUAAAGCAUAUGAUACAGAGGUACAUAAUGUUUGGGCCCACACAUGCCCUGUGUACCCACAGACCCCAACC
CACAAGAAGUAGUAUUGGUAAAUGUGACAGAAAAUUUUAACAUGUGGAAAAAUGACAUGGUAGAACAGAUGCAUGAGG
AUAUAAUCAGUUUAUGGGAUCAAAGCCUAAAGCCAUGUGUAAAUAACCCCACUCUGUGUUAGUUUAAAGUGCACUG
AUUUGAAGAAUGAUACUAAUACCAAUAGUAGCGGCGAGAAUGAUAAUGGAGAAGAGAUAAAAAACUGCUGUCUU
UCAAUAUCAGCACAAGCAUAAGAGGUAAGGUGCAGAAAGAAUAUGCAUUUUUUAUAAACUUGAUAUAAUACCAAUAG
AUAAUGAUACUACCAGCUAUAUACGUUGACAAGUUGUCAGUCAUUACACAGGCCUGUCCAAAGGUAUCCCUUUG
AGCCAAUUCCCAUACAUUAUUGUGCCCCGGCUGCUUUUGCGAUUCUAAAAUGUAAUAAGACGUUCAAUGGAACAG
GACCAUGUACAAAUGUCAGCACAGUACAAUGGAAUUAGGCCAGUAGUAUCAACUCAACUGCUGUUAAAUG
GCAGUCUAGCAGAAGAGGUAGUAAUUAGAUCUGUCAAUUUCACGGACAAUGCUAAAACCAUAAUAGUACAGCUGA
ACACAUCUGUAGAAAUUAUGUACAAGACCCAACAACAAUACAAGAAAAAAAAUCCGUAUCCAGAGGGGACCAGGGA
GAGCAUUUGUUACAAUAGGAAAAUAUGAGACAAGCACAUUGUAACAUUAGUGAGAGCAAAAUGGAAUGCCA
CUUUAAACAGAUAGCUAGCAAUUAAGAGAACAAUUAAUAAACAAUAAUCUUUAAGCAAUCCUCAGGAG
GGGACCCAGAAAUUCGUAACGCACAGUUUUAAUUGUCGAGGGAAUUUUUCUACUGUAAUUCAACACAACUGUUUAAUA
GUACUUGGUUUAAUAGUACUUGGAGUACUGAAGGGUCAAAUAACACUGAAGGAAGUGACACAAUCACACUCCCAUGCA
GAAUAAAACAAUUUAUAAACAUGUGGCAGGAAGUAGUAAUGGCCCCCUCCCAUCAGCGGACAAAUUUAGAU
GUUCAUCAAAUAUUACACAGGCUGCUAAUGACGCUGACGGUACAGGCCAGACAAUUAUUGUCUGGUAUAG
GAGGAGGAGAUGAGGGACAAUUGGAAGUGAAUUAUAAAUAUAAGUGUAAAAAUUGAACCAUUAGGAGUAG
CACCCACCAAGGCAAAGAGAAGAAAAAAGAGCAGUGGAAUAGGAGCUUUGUUCCUUGGUCGUUCU
UGGGAGCAGCAGGAAGCACUAUGGCGCAGCGUCAAUGACGCUGACGGUACAGGCCAGACAAUUAUUGUCUGGUAUAG
UGCAGCAGCAGAACAAUUUGCUGAGGGCUAUUGAGGCGCAACAGCAUCUGUUGCAACUCACAGUCUGGGGCAUCAAGC
AGCUCCAGGCAAGAAUCCUGUGGUGGAAAGAUACCUAAAGGAUCAACAGCUCCUGGGGAUUUGGGGUUGCUCUGGAA

FIG. 10C

AACUCAUUUGCACCACUGCUGUGCCUUGGAAUGCUAGUUGGAGUAAUAAAUCUCUGCAACAGAUUUUGGAAUCACACGA
CCUGGAUCGAGUCGGACAGAGAAAUUAACAAUUACACAAGCUUAAUACACUCCUUAAUUGAACAUCGCAAAACCAGC
AAGAAAACAAUCAACAAGAAUUAUUCGAAUUAGAUAAAUGGGCAAGUUUGUGGAAUUGGUUUAAACAUAAACAAAUUGGC
UGUGGUAUAUAAAAUUAUUCAUAAAUGAUAGUAGGAGGCUUGGUAGGUUUAAGAAUAGUUUUUUGCUGUACUUUUCUGUAG
UCAAUACAGUUAGGCAGGGAUAUUCACCAUUAUCGUUUCAGACCCACCUCCCAAUCCCCGAGGCGACCCGACAGGCCCG
AAGGAAUAGAAGAAGAAGGUGGAGAGAGACAGACACAGAUCCAUUCGAUUAGUGAACGGAUCCUUAGCACUUAUCU
GGGACGAUCUGUCGGAGCCUGUGCCUUCAGCUACCACCGCUUGAGAGACUUACUCUUGAUUGUAACGAGGAUUGUGG
AACUUCUCGGGACGCACGGGGUGCGGAAGCCCUCAAAUAUUGGUGCAAUCUCCUACAAUAUUGCAGUCAGGACUAAAGA
AUAGUGCUGUUAGCUUGCUCAAUGCCACAGCUAUAGCAGUAGCUGAGGGCACACAGAUAGGUUAUAGAAGUACUACAAG
AAGCUUAUAGAGCUAUUCGCCACAUACCUAGAAGAAUAGGACAGGGCUUGGAAAGGAUUUUUGCUAUAAGAUGGGUGGC
AAGUGGUCAAAAAGUAGUGGUUGCGAUGGCCCUGCUGUAAGGGAAAGAAUGAGACGAGCCAGCAGCAGAUGGGG
GUGGGAGCAGCAUCUCGAGACCUAGAAAAACAUGGAGCAAUCACAAGUAGCAACACAGCAGGCUAAACAAUGCUGCUUGU
GCCUGGCUAGAAGCACAAGAGGAGGAAGGUGGGUUUUCCAGUCACACCUCAGGUACCUUUAAGACCAAUGACUUAC
AAGGCAGCUGUAGAUCUUAGGCCACUUUUUAAAAGAAAAGGGGGACUGGAAGGGCUAATTUCACUCCCAACGAAGACAA
GAUAUCCUUGAUCUGUGGAUCUACCACACAAGGCCUACUUCCCCUGAUUGGCCAGAACCUACACACCAGGGACCAGGGAUC
AGAUAUCCACUGACCUUUGGAUGGCGCUACAAGCUAGUUGAGCCAGAGAAGUUAGAAGAAGGCCAACACAAAGGA
GAGAACACCCAGCUUGUUACACCCUGUGAGGCCUGCAUGGAAUGGAUGACCCCGGAGAGAGAAGUGCUUAGAGUGGAGGUUU
GACAGCCGCCUAGCAUUUCAUCACGUGGCCCGAGAGCUGCAUCCCGGAGUACUUCAAGAACUGCUGAUAUCGAGCCUUGC
UACAAGGGACUUUCCGCUGGGGACUUUCCAGGGAGGCGUGGCCUGGGCGGACUGGGAGUGGCGAGCCCUCAGAUCC
UGCAUAUAAGCAGCUGCUUUUUGCCUGUACUGGGUCUCUCUUGGUUAGACCAGAUCUGAGCCUCUGGAGCUUCGCUA
ACUAGGGAACCCACUGCUUAAGCCUCAAUAAAGCUUGCCUUGAGUGCUUCAAGUAGUGUGUGCCCGUCUGUUGUGUGA
CUCUGGUAACUAGAGAUCCCUCAGACCCUUUUAGUCAGUGUGGAAAAUCUCUAGCAGU

FIG. 10D

>gi|9629357:5771-8341 Human immunodeficiency virus 1, complete genome (ENV gene)
ATGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGGTGGAGATGGGGCACCATGCTCCTTGGGATGTTGATGATCTGTAG
TGCTACAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAGGAAGCAACCACTCTATTTTGTGCATCAGATG
CTAAAGCATATGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAACCCACACAAGAAGTAGTATTG
GTAAATGTGCACAGAAAATTTTAACATGTGGAAAAATGACATGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGGATCAAAG
CCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTAGTTTAAGTGCACTGATTGGAAGAATGATACTAATACCAATAGTAGTA
GCGGGAGAATGATAATGGAGAAAGGAGAGATAAAAAACTGCTCTTTCAATATCAGCACAAGCATAAGAGGTAAGGTGCAGAAAGAA
TATGCATTTTTTATAAACTTGATAATAATACCAATAGATACTACCAGCTATAAGTTGACAAGTTGTAACACCTCAGTCAT
TACACAGGCCTGTCCAAAGGTATCCTTTGAGCCAATTCCCATACATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAATGTAATA
ATAAGACGTTCAATGGAACAGGACCATGTACAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCAGTAGTATCAACTCAA
CTGCTGTTAAATGGCAGTCTAGCAGAAGAGGTAGTAATTAGATCTGTCAATTTCACGGACAATGCTAAAACCATAATAGTACA
GCTGAACACATCTGTTAGAAATTATTGTACAAGACCCAACAACAATACAAGAAAAAGAATCCGTATCCAGACAGGGACCAGGAGAG
CATTTGTTACAATAGCAAAAATAGGAAATATGAGACAAGCACATTGTAACATTAGTAGAGCAAAATGGAATAACACTTTAAAACAG
ATAGCTAGCAAATTAAGAGAACAATTTGGAAATAAAACAATAATCTTTAAGCAATCCTCAGGAGGGGACCCAGAAATTGTAAC
GCACAGTTTTATTGTGGAGGGGAATTTTTCTACTGTAATTCAACACAACTGTTTTTAATAGTACTTGGTTTAATAGTACTTGGAGTA
CTGAAGGGTCAAATAACACTGAAGGAAGTGACACAATCACCCTCCCATGCAGAATTATAAAACAAATTTATAAAATCATGTGGCAGAAAGTA
GGAAAAGCAATGTATGCCCCTCCCATCAGTGGACAAATTATTAGATGTTTCATCAAATATTACAGGGCTGcuATTAACAAGACATGGTGC
TAATAGCAACAATGAGTCCGAGATCTTCAGACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAG
TAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGA
GCTTTGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCCTCAATGACGCTGACGGTACAGGCCAGACAATTATT
GTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCA
AGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTC
ATTTGCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATCACACGACCTGGATGGAGTG
GGACAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGAAT
TATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTTTAACATAACAAATTGGCTGTGGTATATAAAATTATTCATAATGATA
GTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTTCTATAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGTT
TCAGACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCA
TTCGATTAGTGAACGGATCCTTGGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTTA
CTCTTGATTGTAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAATCTCCTACAGTATTG
GAGTCAGGAACTAAAGAATAGTGCTGTTAGCTTGCTCAATGCCACAGCCATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAG
TAGTACAAGGAGCTTGTAGAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTTTGCTATAA

FIG. 11

>gi|9629357:336-1838 Human immunodeficiency virus 1, complete genome (GAG gene)
ATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATA
TAAATTAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTA
GACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCTAT
TGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGC
ACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCAGCCAAAATTACCCTATAGTGCAGAACATCCAGGGGCAAATGGTAC
ATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTGATACCCATGTTT
TCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTT
AAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTGCATCCAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGAGAG
AACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTA
GGAGAAATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCATTCTGGACATAAGACA
AGGACCAAAGGAACCCTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAATT
GGATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCGGCTACACTAGAA
GAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTGAAGCAATGAGCCAAGTAACAAATTC
AGCTACCATAATGATGCAGAGAGGCAATTTTAGGAACCAAAGAAAGATTGTTAAGTGTTTCAATTGTGGCAAAGAAGGGCACACAG
CCAGAAATTGCAGGGCCCCTAGGAAAAAGGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAAGATTGTACTGAGAGACAG
GCTAATTTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAGCAGACCAGAGCCAACAGCCCCAC
AGAAGAGAGCTTCAGGTCTGGGGTAGAGACAACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTATCCTTTAACTT
CCCTCAGGTCACTCTTTGGCAACGACCCCTCGTCACAATAA

FIG. 12A

>(gi|9629357:5377-5591, 7925-7970) Human immunodeficiency virus 1, complete genome
(TAT gene)
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAA
GTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAG
CTCATCAGAACAGTCAGACTCATCAAGCTTCTCTATCAAAGCAACCCACCTCCCAACCCCGAGGGGACCCGACAGGCCCGAAGGAA
TAG

FIG. 12B

>NM_009693 (MM ApoB)
UACCUGCCUGAGCUCCGCCUCCGAAGACCCUGUAGAGCAAGCAGCAGGGGCUAGGCCCGUGGCCAGGCCACAGCCAGGAAGCCACC
CCACCAUCCAUCCGCCAUGGGCCCACGAAAGCCUGCCCUGCGGACGCCGUUACUGCUGCUGUUCCUGCUACUGUUCUUGGACACCA
GCGUCUGGGCUCAAGAUGAAGUCCUGGAAAACUUAAGCUUCAGCUGUCCAAAAGAUGCAACUCGAUUCAAGCACCUCCGAAAGUAC
GUGUACAACUAUGAAGCUGAAAGUUCCAGCGGUGUCCAGGGCACAGCUGACUCCAGAAGCGCCACCAAGAUCAACUGUAAGGUAGA
GCUGGAGGUCCCCCAAAUCUGUGGUUUCAUCAUGAGGACCAACCAGUGUACCCUUAAAGAGGUGUAUGGCUUCAACCCUGAGGGCA
AGGCCUUGAUGAAGAAAACCAAGAACUCUGAAGAGUUUGCAGCUGCCAUGUCCAGGUACGAACUCAAGCUGGCCAUUCCUGAAGGG
AAACAAAUUGUUCUUUACCCUGACAAGGAUGAACCUAAAUAUAUCCUGAACAUCAAGAGGGGCAUCAUCUCUGCUCUUCUGGUUCC
CCCAGAGACAGAAGAGGACCAACAAGAGUUGUUCCUGGAUACCGUGUAUGGAAACUGCUCAACUCAGGUUACCGUGAAUUCAAGAA
AGGGAACCGUACCAACAGAAAUGUCCACAGAGAGAAACCUGCAGCAAUGUGACGGCUUCCAGCCCAUCAGUACAAGUGUCAGCCCU
CUCGCUCUCAUCAAAGGCCUGGUCCACCCCUUGUCAACUCUUAUCAGCAGCAGCCAAACUUGCCAGUACACCCUGGAUCCUAAGAG
GAAGCAUGUGUCUGAAGCUGUCUGUGAUGAGCAGCAUCUUUUCCUGCCUUUCUCCUACAAGAAUAAGUAUGGGAUCAUGACACGUG
UUACACAGAAACUGAGUCUUGAAGACACACCUAAGAUCAACAGUCGCUUCUUCAGUGAAGGUACCAACCGGAUGGGUCUGGCCUUU
GAGAGCACCAAGUCCACGUCAUCCCCAAAGCAGGCUGAUGCUGUUUUGAAGACCCUUCAAGAACUGAAAAAAUUGUCCAUCUCAGA
GCAGAAUGCUCAGAGAGCAAAUCUCUUCAAUAAACUGGUUACUGAGCUGAGAGGCCUCACUGGUGAAGCAAUCACAUCCCUCUUGC
CACAGCUGAUUGAAGUGUCCAGCCCCAUCACUUUACAAGCCUUGGUUCAGUGUGGACAGCCACAGUGCUAUACUCACAUCCUCCAG
UGGCUGAAAACUGAGAAGGCUCACCCCCUCCUGGUUGACAUUGUCACCUACCUGAUGGCUCUGAUCCCAAAUCCCUCAACACAGAG
GCUGCAGGAAAUCUUUAAUACUGCCAAGGAGCAGCAGAGCCGAGCCACUCUGUAUGCACUGAGCCACGCAGUUAACAGCUAUUUUG
AUGUGGACCAUUCAAGGAGCCCAGUUCUGCAGGAUAUCGCUGGUUACCUGUUGAAACAGAUCGACAAUGAAUGCACGGGCAAUGAA
GACCACACCUUCUUGAUUCUGAGGGUCAUUGGAAAUAUGGGAAGAACCAUGGAACAAGUAAUGCCAGCCCUCAAGUCCUCAGUCCU
GAGCUGUGUACGAAGUACAAAACCAUCUCUGCUGAUUCAGAAAGCUGCUCUCCAGGCCCUGAGGAAGAUGGAACUGGAAGAUGAGG
UCCGGACGAUCCUUUUUGAUACAUUUGUAAAUGGUGUCGCUCCCGUGGAGAAGAGACUGGCUGCCUAUCUCUUGCUGAUGAAGAAC
CCUUCCUCAUCAGAUAUUAACAAAAUUGCCCAACUUCUCCAAUGGGAACAGAGUGAGCAGGUGAAGAACUUCGUGGCAUCUCACAU
UGCCAACAUCUUGAACUCGGAAGAACUGUAUGUCCAAGAUCUGAAAGUUUUGAUCAAAAAUGCUCUGGAGAAUUCUCAAUUUCCAA
CGAUCAUGGACUUCAGAAAAUUUUCCCGAAACUAUCAGAUUUCCAAAUCUGCUUCUCUCCCAAUGUUCGACCCAGUCUCAGUCAAA
AUAGAAGGGAAUCUUAUAUUUGAUCCAAGCAGUUAUCUUCCCAGAGAAAGCUUGCUGAAAACAACCCUCACAGUCUUUGGACUUGC
UUCACUUGAUCUCUUUGAGAUUGGUUUAGAAGGAAAAGGGUUUGAGCCAACACUAGAAGCUCUUUUUGGUAAGCAAGGAUUCUUCC
CAGACAGUGUCAACAAGGCUUUGUAUUGGGUCAAUGGCCGAGUUCCAGAUGGUGUCUCCAAGGUCUUGGUGGACCACUUUGGCUAU
ACUACAGAUGGCAAGCAUGAACAGGACAUGGUGAAUGGAAUCAUGCCCAUUGUGGACAAGUUGAUCAAAGAUCUGAAAUCUAAAGA
AAUUCCUGAAGCCAGGGCCUAUCUCCGCAUCCUAGGAAAAGAGCUAAGCUUUGUCAGACUCCAAGACCUCCAAGUCCUGGGGAAGC
UGUUGCUGAGUGGUGCACAAACUUUGCAGGGAAUCCCCCAGAUGGUUGUACAGGCCAUCAGAGAAGGGUCAAAGAAUGACUUGUUU
CUCCACUACAUCUUCAUGGACAAUGCCUUUGAGCUCCCCACUGGAGCAGGGUUACAGCUGCAAGUGUCCUCGUCUGGAGUCUUCAC
CCCCGGGAUCAAGGCUGGUGUAAGACUGGAAUUAGCCAACAUACAGGCAGAGCUAGUGGCAAAGCCCUCUGUGUCCUUGGAGUUUG
UGACAAAUAUGGGCAUCAUCAUCCCAGACUUCGCUAAGAGCAGUGUCCAGAUGAACACCAACUUCUUCCACGAGUCAGGCCUGGAG
GCGCGAGUGGCCCUGAAGGCUGGGCAGCUGAAGGUCAUCAUUCCUUCUCCAAAGAGGCCAGUCAAGCUGUUCAGUGGCAGCAACAC
ACUGCAUCUGGUCUCUACCACCAAAACAGAAGUGAUCCCACCUCUGGUUGAGAACAGGCAGUCCUGGUCAACUUGCAAGCCUCUCU
UCACUGGAAUGAACUACUGUACCACAGGAGCUUACUCCAACGCCAGCUCCACGGAGUCUGCCUCUUACUACCCACUGACAGGGGAC
ACAAGGUAUGAGCUGGAGCUGAGGCCCACGGGAGAAGUGGAGCAGUAUUCUGCCACUGCAACCUAUGAACUCCUAAAAGAGGACAA

FIG. 13A

GUCUUUGGUUGACACAUUGAAGUUCCUAGUUCAAGCAGAAGGAGUGCAGCAGUCUGAAGCUACUGUUCUCAAAUAUAAUCGGA
GAAGCAGGACCUUAUCUAGUGAAGUCCUAAUUCCAGGGUUUGAUGUUCAACUUCGGGACAAUACUAAGAGUUAAUGAUGAAUCUGCU
AAGGACAAAAACACUUACAAACUCAUCCUGGACAUUCAGAACAAGAAAAUCACUGAGGUCUCUCGUGGGCCACUUGAGUUAUGA
UAAAAAGGGAGAUGGCAAGAUCAAAGGUGUUCCAUACCACGUUUGCAAGCAGAAGCCAGGAGUGAGGUCCCACACCCACUGGU
CCUCCACCAAACUGCUCUUCCAAAUGGACUCAUCUGCUACAGCUUACGCUCAACACAAUUUCCAAGAGAGUGACAUGGCGUUACGAU
AAUGAGAUAAUAGAAUUUGAUUUGGAACACGGGAACCAAUGUGGAUACCAAAAAAGUGGCCUCCAAUUUCCCUGUGAUCUUUCCCA
UUAUCCUAGAAUGUUGCAUGAGUAUGGCCAAUGGUCUCCUGGAUCACAGAGUCCCUCAAACAGAUGUGACUUUUCGGGACAUUGGUU
CCAAAUUAAUUGUUGCAACAAACACAUGGCUUCAGAUGGCAACCAGGGUCUUCCUACCCCAAACUCUCUACAGGAUCACCUCAAU
AGCCUCUCAGAGUUGAACCUCCUGAAAAUGGGACUGUCUGACUUCCAUAUUCCAGACAACCUCUUCCUAAAGACUGAUGGCAGAGU
CAAAUACACAAUGAACAGGAACAAAAUAAACAUUUGACAUCCCUUUGCCUUUGGGUGCAAGUCUUCAAAAGACCUCAAGAUGCCAG
AGAGUGUGAGGACACCAGCCCUCAACUUCAAGUCUGUGGGAUUCCAUCUCGAGAGGUCCCCACUUUUACAAUC
CCCAAGACACAUCAGCUUCAAGUGCCUCUCUUGGGUGUUCUAGACCUUUCCACAAAUUUGUACAACUGGUCAGC
CUCCUACACUGGUGUGCAACACCAGCAGACCACUUCAGGCUUCAGUACCGCAUGAAGACUGACUCUGUGGUUGACCUGU
UUUCCUACAGUGUGCAAGGAUCUGGAGAAACAACAUAUGACACAGCAAGAACACAUUUACAUGUCCUGUGAUGGAUCUCCUACACCAU
AAAUUUCUAGACUCACAAAAUUCAAAGUCAGCCACGUAGAAAAAUUUGGAAACAGCCCAGUCUCAAAAGGUUACUACAUUUGAAAC
AUCUAGUGCCUUGGGACCACAGAUGUCUGCUACUGUUCACCUAGACUCAAAAAAGAAACAACAUCUAUACGUCAAAGAUAUCAAGG
UUGAUGGACAGUUCAGAGCUUCUUCAUUUUAUGCCUCAAGGCAAAUAUGGCCUGUCUUGUGAGAGAUGUUUACAACUGGCCAGCUG
AGCGGCGAAUCCAACAUGAGAUUUAACUCCACCUACUUCCAGGGCACCAACCAGAUCGUGGGAAUGUACCAGGAUGGGAGCCCUGUC
CAUCACCUCCACUUCUGACCUGCAAGAUGGCAUAUUCAAGAACACAGCUUCCUUGAAAUAUGAAAACUAUGAGCUGACUCUGAAAU

FIG. 13B

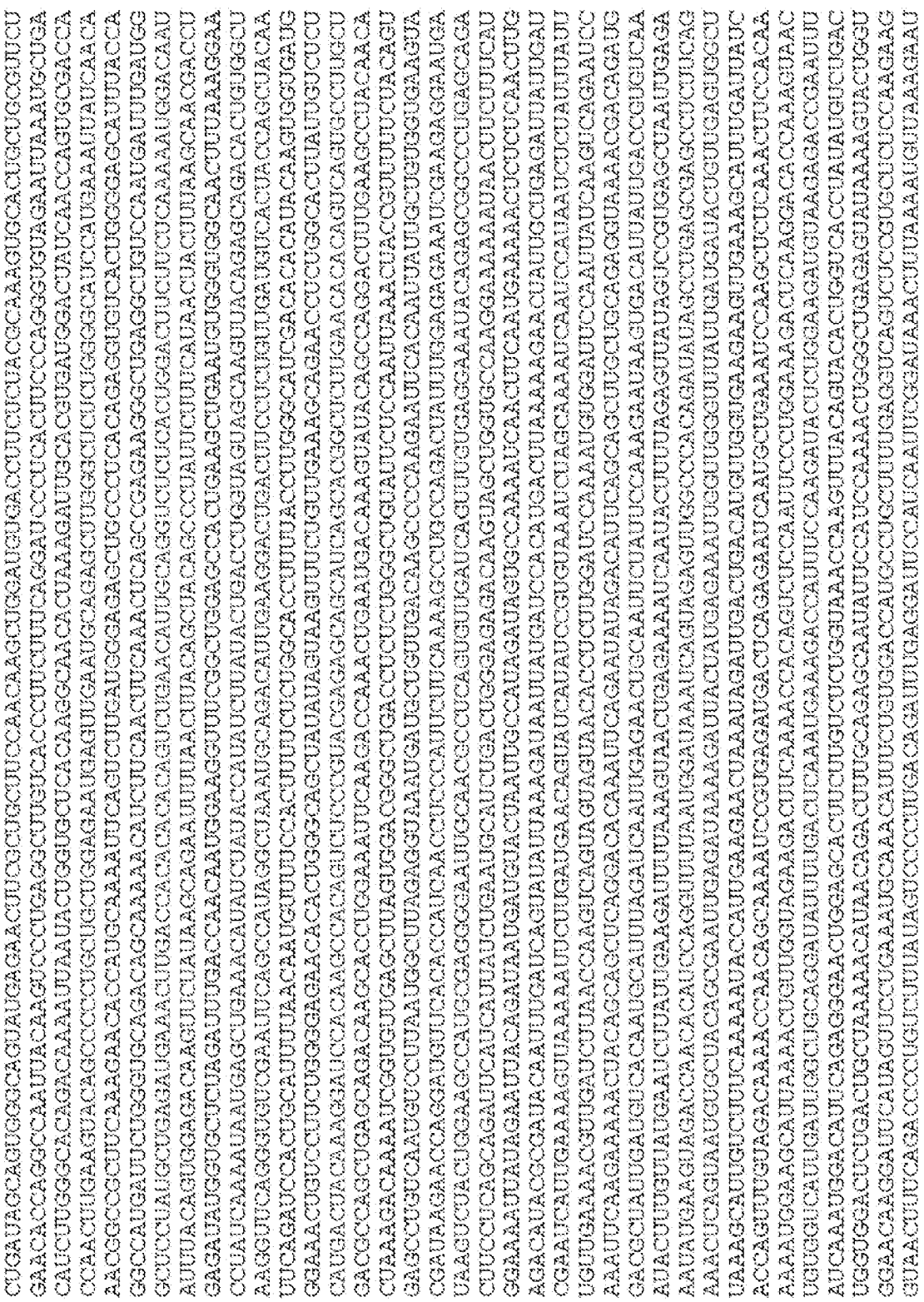

FIG. 13C

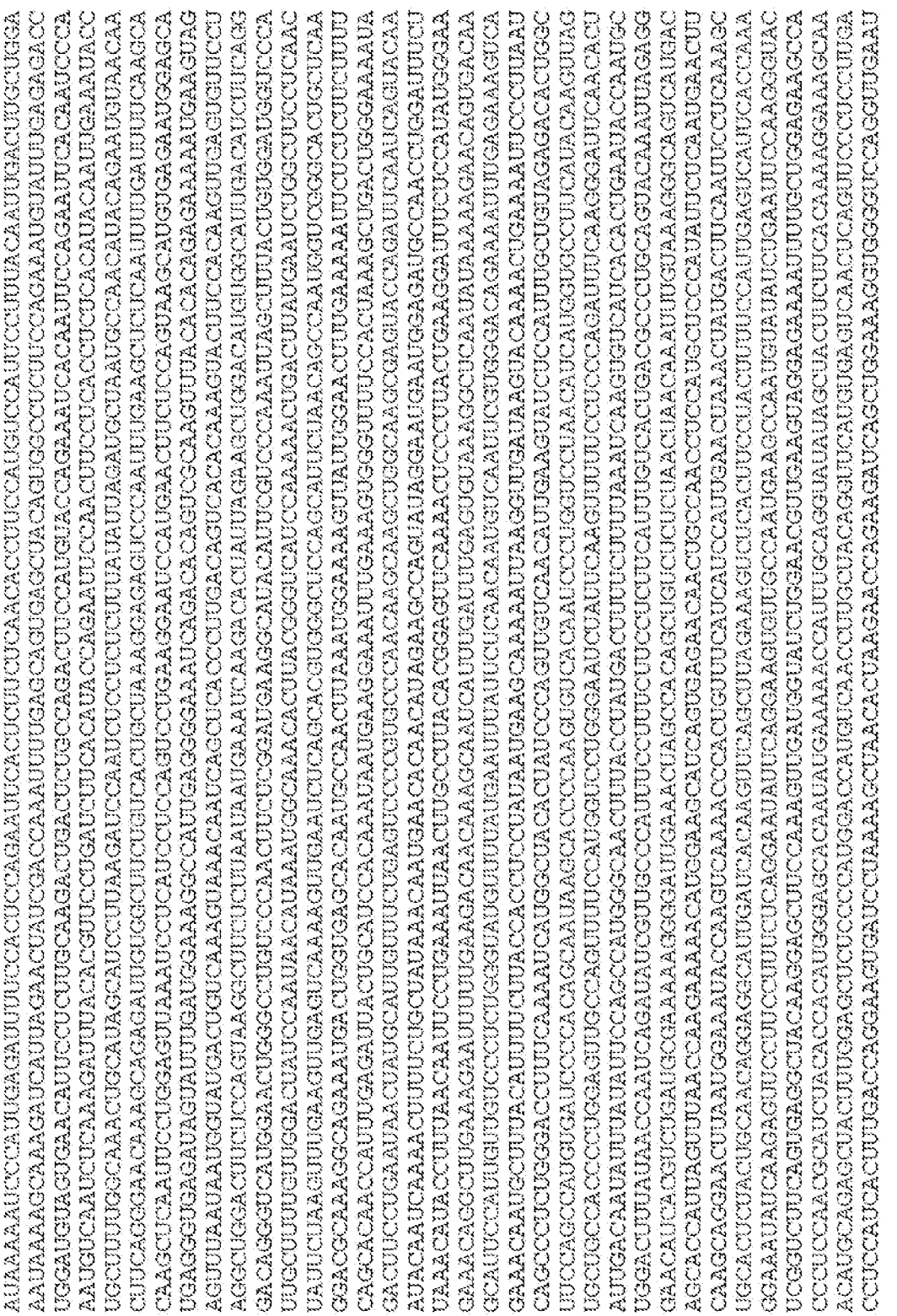

FIG. 13D

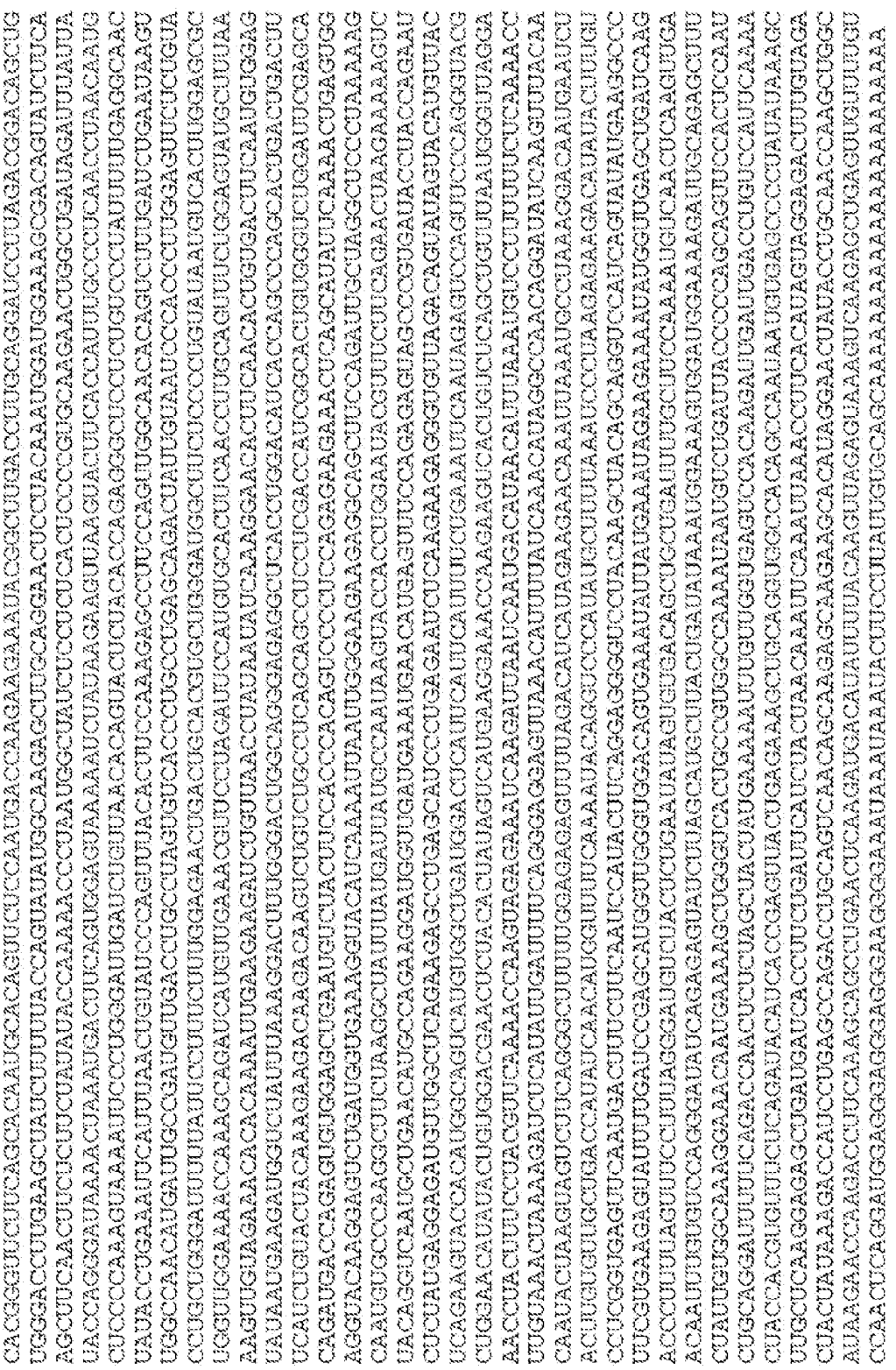

FIG. 13E

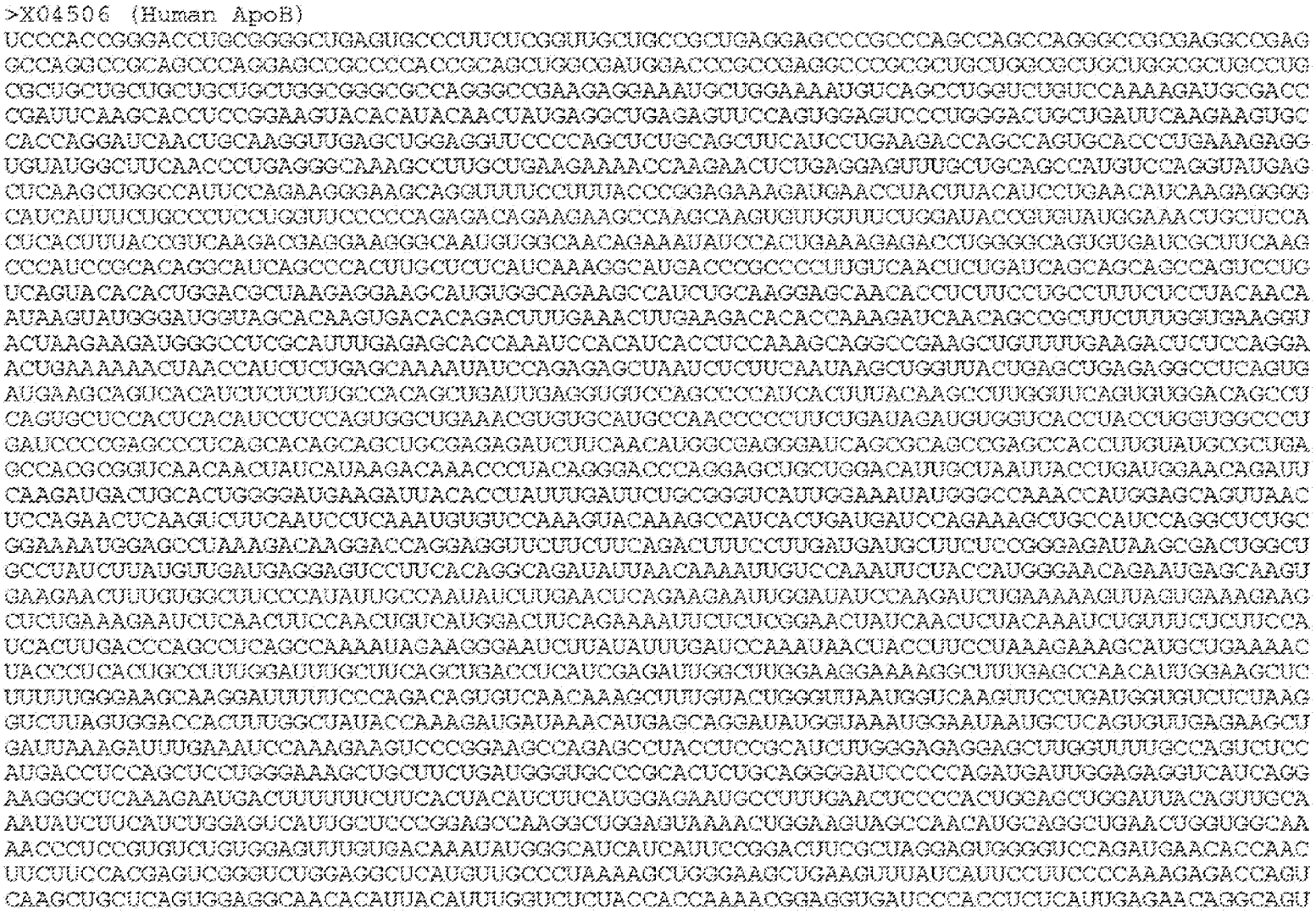
>X04506 (Human ApoB)
UCCCACCGGGACCUGCGGGGCUGAGUGCCCUUCUCGGUUGCUGCCGCUGAGGAGCCCGCCCAGCCAGCCAGGGCCGCGAGGCCGAG
GCCAGGCCGCAGCCCAGGAGCCGCCCCACCGCAGCUGGCGAUGGACCCGCCGAGGCCCGCGCUGCUGGCGCUGCUGGCGCUGCCUG
CGCUGCUGCUGCUGCUGCUGGCGGGCGCCAGGGCCGAAGAGGAAAUGCUGGAAAAUGUCAGCCUGGUCUGUCCAAAAGAUGCGACC
CGAUUCAAGCACCUCCGGAAGUACACAUACAACUAUGAGGCUGAGAGUUCCAGUGGAGUCCCUGGGACUGCUGAUUCAAGAAGUGC
CACCAGGAUCAACUGCAAGGUUGAGCUGGAGGUUCCCCAGCUCUGCAGCUUCAUCCUGAAGACCAGCCAGUGCACCCUGAAAGAGG
UGUAUGGCUUCAACCCUGAGGGCAAAGCCUUGCUGAAGAAAACCAAGAACUCUGAGGAGUUUGCUGCAGCCAUGUCCAGGUAUGAG
CUCAAGCUGGCCAUUCCAGAAGGGAAGCAGGUUUUCCUUUACCCGGAGAAAGAUGAACCUACUUACAUCCUGAACAUCAAGAGGGG
CAUCAUUUCUGCCCUCCUGGUUCCCCCAGAGACAGAAGAAGCCAAGCAAGUGUUGUUUCUGGAUACCGUGUAUGGAAACUGCUCCA
CUCACUUUACCGUCAAGACGAGGAAGGGCAAUGUGGCAACAGAAAUAUCCACUGAAAGAGACCUGGGGCAGUGUGAUCGCUUCAAG
CCCAUCCGCACAGGCAUCAGCCCACUUGCUCUCAUCAAAGGCAUGACCCGCCCCUUGUCAACUCUGAUCAGCAGCAGCCAGUCCUG
UCAGUACACACUGGACGCUAAGAGGAAGCAUGUGGCAGAAGCCAUCUGCAAGGAGCAACACCUCUUCCUGCCUUUCUCCUACAACA
AUAAGUAUGGGAUGGUAGCACAAGUGACACAGACUUUGAAACUUGAAGACACACCAAAGAUCAACAGCCGCUUCUUUGGUGAAGGU
ACUAAGAAGAUGGGCCUCGCAUUUGAGAGCACCAAAUCCACAUCACCUCCAAAGCAGGCCGAAGCUGUUUUGAAGACUCUCCAGGA
ACUGAAAAAACUAACCAUCUCUGAGCAAAAUAUCCAGAGAGCUAAUCUCUUCAAUAAGCUGGUUACUGAGCUGAGAGGCCUCAGUG
AUGAAGCAGUCACAUCUCUCUUGCCACAGCUGAUUGAGGUGUCCAGCCCCAUCACUUUACAAGCCUUGGUUCAGUGUGGACAGCCU
CAGUGCUCCACUCACAUCCUCCAGUGGCUGAAACGUGUGCAUGCCAACCCCCUUCUGAUAGAUGUGGUCACCUACCUGGUGGCCCU
GAUCCCCGAGCCCCUCAGCACAGCAGCUGCGAGAGAUCUUCAACAUGGCGAGGGAUCAGCGCAGCCGAGCCACCUUGUAUGCGCUGA
GCCACGCGGUCAACAACUAUCAUAAGACAAACCCUACAGGGACCCAGGAGCUGCUGGACAUUGCUAAUUACCUGAUGGAACAGAUU
CAAGAUGACUGCACUGGGGAUGAAGAUUACACCUAUUUGAUUCUGCGGGUCAUUGGAAAUAUGGGCCAAACCAUGGAGCAGUUAAC
UCCAGAACUCAAGUCUUCAAUCCUCAAAUGUGUCCAAAGUACAAAGCCAUCACUGAUGAUCCAGAAAGCUGCCAUCCAGGCUCUGC
GGAAAAUGGAGCCUAAAGACAAGGACCAGGAGGUUCUUCUUCAGACUUUCCUUGAUGAUGCUUCUCCGGGAGAUAAGCGACUGGCU
GCCUAUCUUAUGUUGAUGAGGAGUCCUUCACAGGCAGAUAUUAACAAAAUUGUCCAAAUUCUACCAUGGGAACAGAAUGAGCAAGU
GAAGAACUUUGUGGCUUCCCAUAUUGCCAAUAUCUUGAACUCAGAAGAAUUGGAUAUCCAAGAUCUGAAAAACUUAGUGAAAGAAG
CUCUGAAAGAAUCUCAACUUCCAACUGUCAUGGACUUCAGAAAAUUCUCUCGGAACUAUCAACUCUACAAAUCUGUUUCUCUUCCA
UCACUUGACCCAGCCUCAGCCAAAAUAGAAGGGAAUCUUAUAUUUGAUCCAAAUAACUACCUUCCUAAAGAAAGCAUGCUGAAAAC
UACCCUCACUGCCUUUGGAUUUGCUUCAGCUGACCUCAUCGAGAUUGGCUUGGAAGGAAAAGGCUUUGAGCCAACAUUGGAAGCUC
UUUUUGGGAAGCAAGGAUUUUUCCCAGACAGUGUCAACAAAGCUUUGUACUGGGUUAAUGGUCAAGUUCCUGAUGGUGUCUCUAAG
GUCUUAGUGGACCACUUUGGCUAUACCAAAGAUGAUAAACAUGAGCAGGAUAUGGUAAAUGGAAUAAUGCUCAGUGUUGAGAAGCU
GAUUAAAGAUUUGAAAUCCAAAGAAGUCCCGGAAGCCAGAGCCUACCUCCGCAUCUUGGGAGAGGAGCUUGGUUUUGCCAGUCUCC
AUGACCUCCAGCUCCUGGGAAAGCUGCUUCUGAUGGGUGCCCGCACUCUGCAGGGGAUCCCCCAGAUGAUUGGAGAGGUCAUCAGG
AAGGGCUCAAAGAAUGACUUUUUUCUUCACUACAUCUUCAUGGAGAAUGCCUUUGAACUCCCCCACUGGAGCUGGAUUACAGUUGCA
AAUAUCUUCAUCUGGAGUCAUUGCUCCCGGAGCCAAGGCUGGAGUAAAACUGGAAGUAGCCAACAUGCAGGCUGAACUGGUGGCAA
AACCCUCCGUGUCUGUGGAGUUUGUGACAAAUAUGGGCAUCAUCAUUCCGGACUUCGCUAGGAGUGGGGUCCAGAUGAACACCAAC
UUCUUCCACGAGUCGGGUCUGGAGGCUCAUGUUGCCCUAAAAGCUGGGAAGCUGAAGUUUAUCAUUCCUUCCCCAAAGAGACCAGU
CAAGCUGCUCAGUGGAGGCAACACAUUACAUUUGGUCUCUACCACCAAAACGGAGGUGAUCCCACCUCUCAUUGAGAACAGGCAGU

FIG. 14A

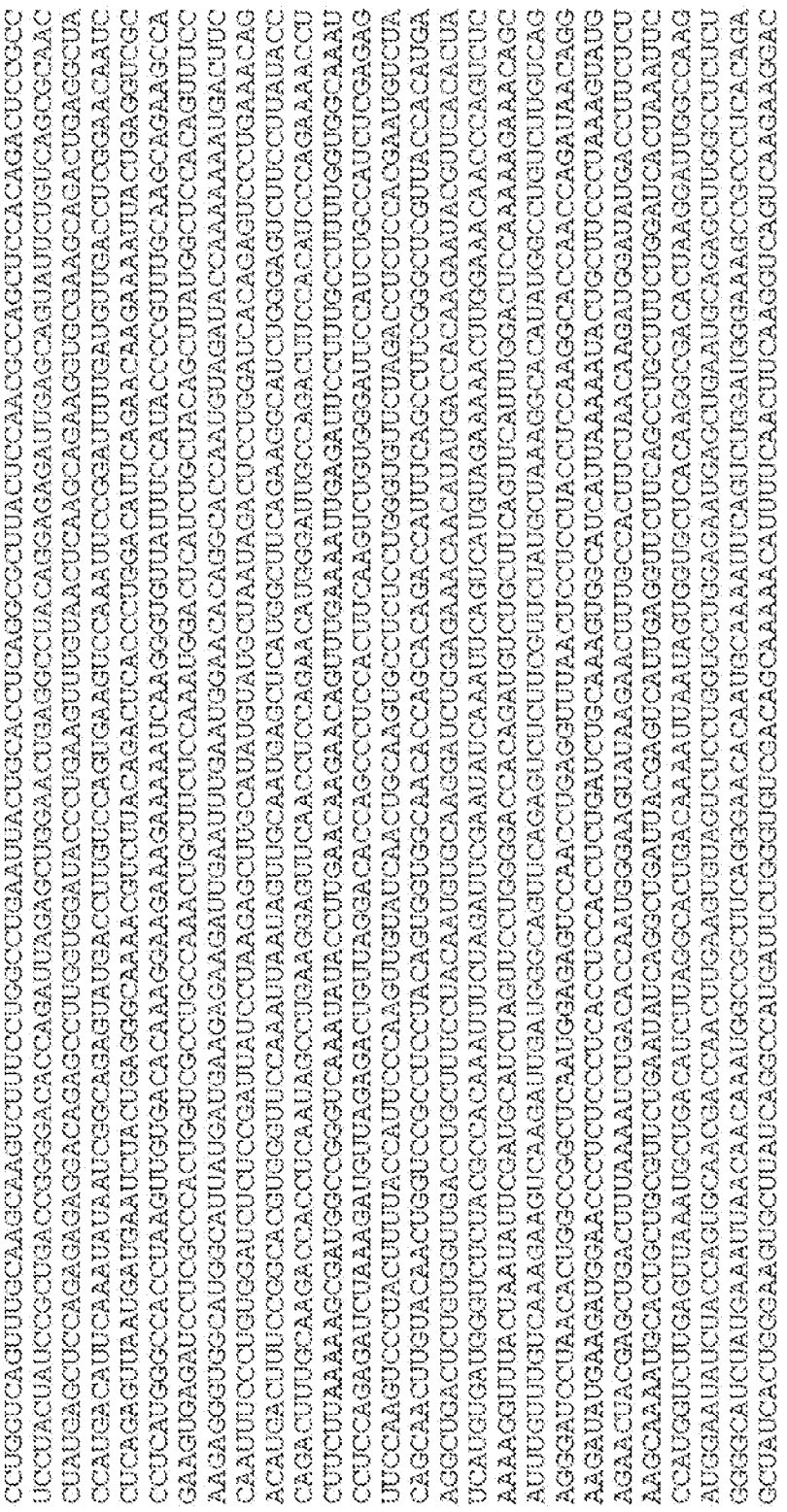

FIG. 14B

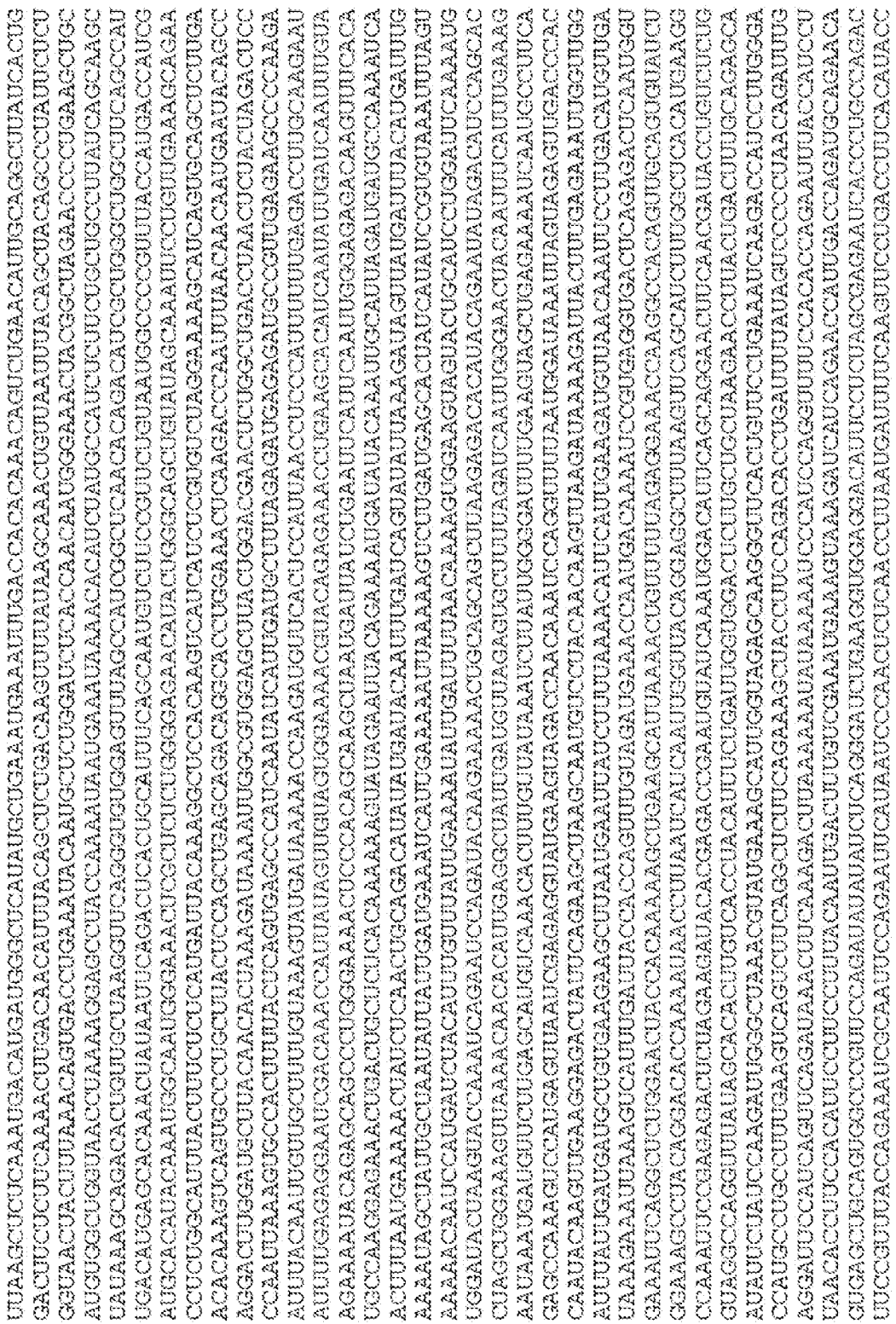

FIG. 14C

AGAAUUCCAGCUUCCCCACACAUCUCACAAUUGAAGUACCUACUUUUGGCAAGCUAUACAGUAUUCUGAAAAUCCAAUCCUCC
UUUUCACAUUAGAUGCCAAAUGCUGACAUAGGGAAUGGAACCACCUCAGCAAACGAAGCAGGUAUCGGCAGCUUCCAUCACUGCCAAA
GGAGAGUCCAAAUUAGAAGUUCUCAAUUUUGAUUUUCAAGCAAAUGCACAACUCUCAAACCCUAAGAUUAAUCCCGCUGGCUCUGAA
GGAGUCAGUGAAGUUCUCCAGCAAGUACCUGAGCAUGGCAGUGAAAUGCUGUUUUUUGGAAAUGCUAUUGAGGGAAAAU
CAAACACAGUGGCAAGUUUACACACAGAAAAAAAUACACUGGAGCUUAGUAAUGGAGUGAUUGUCAAGAUAAACAAUCAGCUUACC
CUGGAUAGCAACACUAAAUACUUCCACAAAUUGAACAUCCCCAAACUGGACUUCUCUAGUCAGGCUGACCUGGCGCAACGAGAUCAA
GACACUGUUGAAAAGCUGGCCACAUAGCAUGGAAAAGGGUCAUGGAAAUGGGCCUGCCCCAGAUUCUCAGAUGAGG
GAACACAUGAAUCACAAAUUAGUUUCACCAUAGAAGGACCCCUCACUUCCUUUGGACUGUCCAAUAAGAUCAAUAGCAAACACCUA
AGAGUAAACCAAAAACUUGGCUUUAUGAAUCUGGCUCCCUCAACUUUUCUAAACUUGAAAUUCAAUCACAAGUCGAUUCCCAGCAUGU
GGGCCACAGUGUUCUAACUGCUAAAGGCAUGGCAAGAGGCAGAGUUUACUGGGAGGCAUGAUGCUCUACCUUACACA
AUGGAAAGGUUAUUCGAACUUUGAAAAAUCUUCUUUCUCAGCCCAGCCAUUUGAGAUCACGGCAUCCCACAAACAAUCAAGGG
AAUUUGAAAGUUUCGUUUUCCAUUAACGUUAACAGGGAAGAUAGACUUCCUGAAUAACUAUGCACUGUUUCUGAGUUCCCAGUGCCCA
GCAAGCAAGUUGGCAAGUAAGUGCUAGUCAAUCAGUAUAAGUACAACCAAAAUUUCUCUGCUGGAAACAACGAGAACAUUAUGG
AGGCCCAUUGAGGAAUAAAUGGAGAAGCAAAUCUGGAUUUCUUAAAACAUUCCUUAACAAUUCCUGAAUGCGUCUACCUUACACA
AUAAUCACAACUCCUCCACUGAAGAUUUCUCUUUUCUUCAGCCCAGCCAUUUGAGAUCACGGCAUCCCACAAACAAUCAAGGG
UGAUUUAAGUGUAAAAGCUCAGUAUAAGAAAAAACACAGGCAUUCCAUUCACACAAAUCCUUUGGCUUGCUGAGUUAUCA
GUCAGAGCAUCAAAUCCUUUGACAGGCAUUUUUGAAAAAAACAGAAACAAUGCAUUAGAUUUUGUCACCAAAUCCUAUAAUGAAACA
AAAAUUAAGUUUGAUAAGUACAAAGCUGAAAAAAAUCUCACGACGAGCUCCCCCAGGAACCUUUCAAAUUCCUGAUUCCAGU
UGUCAAUGUUGAAGUGUCUCCAUUCACCAUAGAGAUGUCGGCAUUCGGCAUUCCCAAAAGCAGUCAGCAUGCCUAGUUCU
CCAUCCUAGGUUCUCGACGUCCGUGUGCCUUCAUAACACAUAAUCCUGCCAUCAUUAGAGCUGCCAGUCCCUAGAAAU
CUCAAGCUUUCUCUCCACAUUUCAAGGAAUUGUGUACCAUAAGCCAUAUUUUAUAUUCCUGCCAUGGGCAAUAUUACCUAUGAUUU
CUCCUUUAAAUCAAGUGUCAUCACACUGAAUACCAAUGCUGCUGAACUUUUUAACCAGUCAGAUAUUGUUGCUCAUCUCCUUCUCAU
CUUCAUCUGUCAUGACUGCAGUACAAAUUAGAGGGCACCACAAGAUUGACAAGAAAAAGGGGAUUAGCCACAGCU
CUGUCUCUGAGCAACAAAUUUGUGAGGGUAGUCAUAACAGUACUGAGCUUAACCACGAAAAAUAUGGAAAUACCAAGUGGCAAA
AACCACAAAAGCCGAAAUUCCAAUUUUGAGAAUGAAUUUCAAGCAAGAACUUAAUGGAAAUACCUACUGUCUCUUU
CCUCCAUGGAAUUUAAGUAUGAUUUCAAUGCUGUACUCUACCGCUAAAGGAGCAGUUGACCACAAGCUUGGAA
AGCCUCACCUCUUACUUUUCCAUGAGUCAUGUCAAGGAUGUCGGUUCUUUCUCGGGAAUAUUUCAGGAACUUAU
UGCUAGUGAGGCCAACACUUACUUGAAUUCCAAGAGCACACGGUCUUCAGUGAAGCUGCAGGGCACUUCCAAAUUGAUGAUAUCU
GGAACCUUGAAGUAAAAGAAAAUUUUGCUGGAGAAGCCACACUCCAACGCAUAUAUUCCCUCUGGGAGCACAGUACGAAAAACCAC
UUACAGCUAGAGGGCCUCUUUUCACCAACGGAGAACAUACAAGCAAAGCCUCCUCCCAUGGCAAAUGUCAGCUU
UGUUCAGGUCCAUGCAAGUCAGCCCAGUUCCUUCCAUGAUUUCCCUGACCUUGGCCCUGAAGUCGAUUUCAAAGA
ACCAGAAGAUCAGAUGGAAAAUGAAGUCCGAUUCAUUCUGGGUCUUUUCCAGAGCCAGGUCGAGCUUUCCAAUGACUUAGGAAAAG
GCACACCUUGACAUUGCAGGAAGACCUUAGAAGGUUCCUCAAAAAUAUCAUCCUACCAGUCUAUGACAAGAGCUUAUG
GGAUUUCCUAAAGCUGGAUGUAACCACAGCAUUGUGGUAGACAGCAUCUUCGUUGUUUCAACUGCCUUUGUGCUACACCAAAAACC
CCAAUGGCUAUUCAUUCUCCAUCCCUGUAAAAAGUUUUUGGCUGAUAAAUUCAUUACUCUCCUGGGCUGAAACUAAAUGAUCUAAAUUCA
GUUCUUGUCAUGCCUACGUUCCAUGUCCCAUUUACAGAUCUUCAGGUUCCAUCGUGCAAACUUGACUUCAGAGAAAUACAAAUCUA
UAAGAAGCUGAGAACUUCAUUGCCCUCAACCUACCAACACUCCCCGAGCGUAAAAUUCCCUGAAGUUGAUGUGUUAACAAAAU

FIG. 14D

AUUCUCAACCAGAAGACUCCUUUUUGAGAUAACCGUGCCUGAAUCUCAGUGUAACUGUGUCCCAGUUCACGCUUKCCA
AAAAGUGUUUUCAGAUGGCAUUKGCUGCUUUGGAUCUAAAUGCCAGUAGCCAACAGAUCGCAGACUUUGAGUUGCCCCACCAUCAUCGU
GCCUGAGCAGACCAUUGAGAUUCCCUCCAUUAAGUUCUCUGUACCUGGAAUUGUCAUCCUUCUUCAAGCACUGACUGACUGCAC
GCUUUGAGGUAGACUCUCCCCGUGUAUAAUGCCACUUGGAGUGCCAGUUUGAAAAACAAAGCAGAUUAUGUUGAAACAGUCCUGGAU
UCCACAUGCAGCUCAACCGUACAGUUCCUAGAAUAUGAACUAAAUGUUUUGGGAACACACACAAAAUCGAAGAUGGUACGUUAGCCUC
UAAGACUAAAGGAACACUUGCACACCGUGACUUCAGUGCAGAAUAUGAAGAGAUGGCAAAUUUGAAGGACUUCAGGAAUGGGAAG
GAAAAGCGCACCUCAAUAUCAAAAGCCCAGCGUUCACCCGAUCUCCAUGCGCUACCAGAAAGACAAGAAAGCAUCUCCACCUCA
GCAGCCUCCCCAGCCGUAGGCACCGUGGGCAUGAUAUGGAUGACCACUUUUCUAAAUGGAACUUCUACUACAGCCCUCA
GUCCUCUCCAGAUAAAAAACUCACCAUAUUCAAAACUGAGUUGAGGGUCCGGGAAUCUGAUGAGGAAACUCAGAUCAAAGUUAAUU
GGGAAGAGAGGCAGCUUCUGGCUUGCUAACCUCUCUGAAAGACAACGUGCCCAAGGCCACAGGGUCCCUUUAUGAUUAUGUCAAC
AAGUACCACUGGGAACACACACAGGGCUCACCCUGAGAGAAGUGUCUUCAAAGCUGAGAAGAAAUCUGCAGAACAAUGCUGAGUGGU
UUAUCAAGGGGCCAUUAGGCAAAUUGAUCGACGUGAGGUUCCAGAAAGCAGCCAGUGGCACCACUGGGACCUACCAAGAGU
GGAAGGACAAGGCCCAGAAUCUGUACCAGGAACUGUUGACUCAGGAAGCCCAAGCCAGUUUCCAGAUAACGUGUUU
GAUGGCUUGGUACGAGUUACUCAAAAAUUCCAUAUGAAAGUCAAGCAUCUGAUUGACUCACUCAUUGAUUUCUGAACUUCCCCAG
AUUCCAGUUUCCGGGAAACCUGGGAUAUCACUAGGGAGGAACUUUGCACUAUGUUCAUAAGGGAGGUAGGGACGGUACUGUCCC
AGGUAUAUUCGAAAGUCCAUAAUGGUUCAGAAAUACUGUUUUCCUAUUUCCAAGACCUAGUGAUUACACUUCCUUCGAGUUAAGG
AAACAUAAACUAAUAGAUGUAAUCUCGAUGUAUAGGGAACUGUUGAAAGAUUUAUCAAAAGAAGCCCAAGAGGUAUUUAAAGCCAU
UCAGUCUCUCUCAAGACCACACAGAGGUGCUACGUAAUCUUCAGGACCUUUUACAAUUCAUUUUCCAACUAAUAGAAGAUAACACAUUAAAC
AGCUGAAAGAUGAAAUUUACUUAUCUUAUUAAUUAUAUCCAAGAUGAGAUCAACACAAUCUCUUCAAUGAUUAUAUCCCAUAUGGUU
UUUAAAUUGUUGAAAGAAAACCUAUGCCUUAAUCUUCAUAAGUUCAAUGAAUUUAUUCAAAACGAGCUUCAGGAAGCUUCUCAAGA
GUUACAGCAGAUCCAUCAAUACAUUAUGGAAGAAUAUUUUGAUCCAAGUAUAGUUGGCUGGACAGUGAAAUAUUAUG
AACUUGAAGAAAAGAUAGUCAGCUGAUCAAGAACCUGUUAGUUGCUCUUAAGGACUUCCAUUCUGAAUAUAUUGUCAGUGCCUCU
AACUUUACUUCCCAACUCUCAAGUCAAGUUGAGCACAGAAAUAUUCUGCACAGAAAUAUUCAGGAUACCUUACCGAUCCAGA
UGGAAAAGGGAAAGAGAAGAUUGCAGAGCUUUCUGCCACUGCUCAGGAAAUAUAAUUAAAAGCCAGGCGAAGAAAAUAA
UUUCUGAUUACCACCAGCAGUUUAGAUAAACUGCAAGAUUUUUUCAGACCAUCUCUCUGAUUACUAUGAAAAAUUUUAUUGCUGAA
UCCAAAAGAUUGAUUGACCUGUCCAUUCAAAACUACCACACAUUUCUGAUAUACAUCACGGAGUUACUGAAAAAGCUGCAAUCAAC
CACAGUCAUGAACCCCUACAUGAAGCUUGCUCCAGGAGAACUUUACUAUCAUCCUCUAAUUUUUAAAAGAAAUCUUCAUUUAUUUCU
UCUUUUCCAAUUGAACUUUCACAUAGCACAGAAAAAAUUCAAACUGCCUAUAUUGAUAAAACCAUACAGUGAGCCAGCCUUGCAGU
AGGCAGUAGACUAUAAGCAGAAGCACAUAUGACCUGCACCAAGCUGGCACCAGGGCUCCGAAGGUCUCUGAACUCAGAA
GGAUGGCAUUUUUUGCAAGUUAAAGAAAAUCAGGAUCUGAGUUAUUUUGCUAAACUUGUGGGGAGGAGGAACAAAUAAAAUGGAGUCU
UUAUUGUGUAUCAUA

FIG. 14E