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(54) **Title**: MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF

Figure 9A

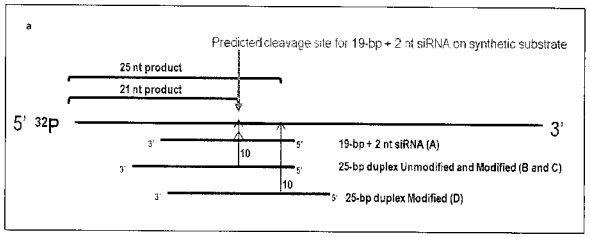
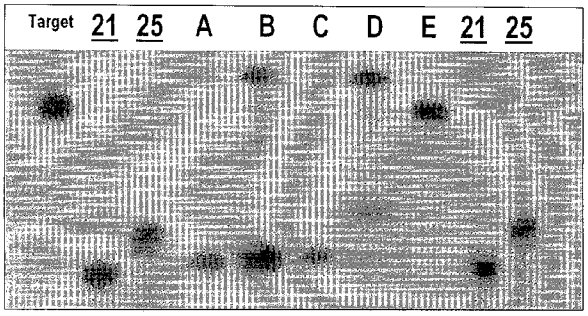


Figure 9B



(57) **Abstract**: The invention relates to improved RNAi constructs and uses thereof. The construct has a double stranded region of 19-49 nucleotides, preferably 25, 26, or 27 nucleotides, and preferably blunt-ended. The construct has selective minimal modifications to confer an optimal balance of biological activity, toxicity, stability, and target gene specificity. For example, the sense strand may be modified (e.g., one or both ends of the sense strand is/are modified by four 2'-O-methyl groups), such that the construct is not cleaved by Dicer or other RNAse III, and the entire length of the antisense strand is loaded into RISC. In addition, the antisense strand may also be modified by 2'-O-methyl group at the 2nd 5'-end nucleotide to greatly reduce off-target silencing. The constructs of the invention largely avoids the interferon response and sequence- independent apoptosis in mammalian cells, exhibits better serum stability, and enhanced target specificity.

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MODIFIED RNAI POLYNUCLEOTIDES AND USES THEREOF

Background of the Invention

Complementary oligonucleotide sequences are promising therapeutic agents and useful research tools in elucidating gene functions. However, prior art oligonucleotide molecules suffer from several problems that may impede their clinical development, and frequently make it difficult to achieve intended efficient inhibition of gene expression (including protein synthesis) using such compositions.

For example, classic siRNAs have limitations and drawbacks that may result in those agents being only moderately useful as human therapeutics. Specifically, classic siRNA is double-stranded. For each molecule, two strands need to be synthesized and paired up. Classic siRNA is made from naturally occurring ribonucleotides and is vulnerable to nucleases and spontaneous hydrolysis. The strands of classic siRNA are paired to each other except for an overhang of one strand at each end, and are about 19 to 23 nucleotides long. This configuration limits the variety and activity of the compound. For example, longer oligonucleotides can have higher binding activity to target RNA, which often correlates with higher activity. The overhangs of classic siRNA cause instability (because single strands are more nuclease resistant than double strands in most cases) and degradation, and may be the cause of the molecules "sticking" to each other or other nucleotides.

In addition, it is widely believed that double stranded RNAs longer than 21-mer are cleaved by Dicer or Dicer-like RNAse III in mammalian cells, resulting in classic siRNA products. One strand of the Dicer-cleavage products is then loaded onto the RISC complex, and guides the loaded RISC complex to effect RNA interference (RNAi). However, since Dicer is not sequence specific, the Dicer-cleavage products of unmodified long dsRNA is a heterogeneous mixture of 21-mers, each may have different biological activity and/or pharmacological property. In addition, each 21-mer may have a distinct off-target effect (*e.g.*, inhibiting the function of an unintended target due to, for example, spurious sequence homology between the Dicer cleavage product and target mRNAs). In other words, the active drug (*e.g.*, the 21-mers) may be multiple species with relatively unpredictable target specificities, biological activities and/or pharmacological properties. Also, Dicer product is shorter than the parent, which leads to a lower affinity guide strand.

Other problems include the susceptibility of the siRNAs to non-specific nuclease degradation when applied to biological systems. Therefore, it would be of great benefit to

improve upon the prior art oligonucleotides by designing improved oligonucleotides that either are free of or have reduced degree of the above-mentioned problems.

Summary of the Invention

One aspect of the invention relates to a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand includes a 2'-modified ribose sugar at the 2nd nucleotide from the 5'-end of the antisense strand, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

Another aspect of the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand comprises, at the 3'-end of the antisense strand, (i) at least four consecutive 2'-modified ribose sugars with non-hydrolyzable internucleotide linkages, (ii) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 2'-modified ribose sugars, preferably 2'-O-methyl modified ribose sugars, or, (iii) a protective group, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

Another aspect of the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and said sense strand comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

Another aspect of the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein four consecutive 2'-O-methyl nucleotides are present at each of said 5'- and 3'-ends of said sense strand, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand: (a) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages; or, (b) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

Another aspect of the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein the sense strand comprises 12 and 10 consecutive 2'-O-methyl nucleotides at the 5'-end and the 3'-end, respectively, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand: (a) is unmodified; (b) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages; or, (c) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

In certain embodiments, the antisense strand directs the uniform cleavage of the target gene mRNA at a single site between the 10th and 11th nucleotides from the 5'-end of the antisense strand.

In certain embodiments, the sense strand of the dsRNA is cleavable by RISC at a single site between the 10th and the 11th nucleotides from the 3'-end of the sense strand.

In certain embodiments, the dsRNA construct is blunt-ended.

In certain embodiments, the 5'-end 12 nucleotides and the 3'-end 10 nucleotides of the sense strand are 2'-modified ribose sugars.

In certain embodiments, each end of the sense strand comprises a continuous stretch of 2'-modified ribose sugars.

In certain embodiments, each end of the sense strand comprises a continuous stretch of

four 2'-modified ribose sugars.

In certain embodiments, the antisense strand comprises discontinuous 2'-modified ribose sugars, wherein the 10th and 11th antisense nucleotides are not modified.

In certain embodiments, the antisense strand comprises 2'-modified ribose sugars for each 2, 3, 4, 5, 6, 7, 8, or 9 nucleotides.

In certain embodiments, the most 5'-end 2'-modified ribose sugar on the antisense strand is the 2nd nucleotide.

In certain embodiments, the dsRNA construct is: 12-35 nucleotides in length; 25-30 nucleotides in length; 25, 26, 27, 28, 29, or 30 nucleotides in length; >22 nucleotides in length; >25 nucleotides in length; or 31-49 nucleotides in length.

In certain embodiments, each end of the sense strand comprises, independently, 4-16 2'-modified ribose sugars and/or non-hydrolyzable internucleotide linkages.

In certain embodiments, each end of the sense strand comprises a symmetrical or an asymmetrical number of 2'-modified ribose sugars.

In certain embodiments, the 2'-modified ribose sugars are 2'-O-alkyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy nucleotides, 2'-H (deoxyribonucleotides), or combination thereof.

In certain embodiments, the 2'-O-alkyl nucleotides are 2'-O-methyl nucleotides.

In certain embodiments, the 2'-O-alkyl nucleotides are 2'-O-allyl nucleotides.

In certain embodiments, the antisense strand comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end of the antisense strand and no other modified nucleotides.

In certain embodiments, the dsRNA has enhanced target specificity or reduced off-target silencing compared to a similar construct without the 2'-modification at said position(s).

In certain embodiments, the antisense strand comprises at least four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages.

In certain embodiments, the sense strand of the dsRNA comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand.

In certain embodiments, the dsRNA has improved stability in serum and/or cerebral spinal fluid compared to an unmodified dsRNA having the same sequence.

In certain embodiments, the last 2nd-8th nucleotides at the 3'-end of the sense strand mismatch their corresponding antisense strand nucleotides.

In certain embodiments, the dsRNA does not induce interferon response in primary cells.

In certain embodiments, either end of the sense strand and/or the 3'-end of the antisense strand is blocked by a protective group.

In certain embodiments, the protective group is an inverted nucleotide, an inverted abasic moiety, or an amino-end modified nucleotide.

In certain embodiments, the inverted nucleotide comprises an inverted deoxynucleotide.

In certain embodiments, the inverted abasic moiety comprises an inverted deoxyabasic moiety.

In certain embodiments, the inverted deoxyabasic moiety is a 3',3'-linked or 5',5'-linked deoxyabasic moiety.

In certain embodiments, alternating nucleotides on the ends of the sense and/or antisense strands comprise 2'-modified ribose sugars, and wherein each of the 2'-modified ribose sugars faces an unmodified nucleotide on the opposite strand.

In certain embodiments, the first 2'-modified antisense nucleotide is the most 5'-end antisense nucleotide or the 2nd nucleotide from the 5'-end of the antisense strand.

In certain embodiments, the target gene is SOD1, PPIB, RIP140, PCSK9, TNFα, AP2 (adipocyte lipid-binding protein), or MAP4K4.

In certain embodiments, the sense strand nucleotides between the 2'- modified ribose nucleotides are 2'-F modified.

In certain embodiments, the sense strand nucleotides between the 2'- modified ribose nucleotides are purine nucleotides, optionally having 2'-F modification and/or phosphorothioate linkage.

In certain embodiments, the sense strand nucleotides between the 2'- modified ribose nucleotides form one or more bulges of 1-5 nucleotides each.

In certain embodiments, each continuous stretch of 2'-modified ribose nucleotides independently starts from the terminal nucleotide, the second nucleotide from the terminal nucleotide, or the third nucleotide from the terminal nucleotide.

In certain embodiments, between 50-100% of the pyrimidine nucleotides of the antisense strand are, independently, 2'-F modified or 2'-O-methyl-modified.

In certain embodiments, the 5'-end of the antisense strand is phosphorylated.

In a related aspect, the invention also provides a construct for inhibiting expression of a target gene, wherein the construct is identical to any of the subject dsRNA, except for a single nick on the sense strand. For example, the nick may occupy the opposite position of the nucleotide about 10 bases from the 5' end of the antisense strand. The nick may also occupy the

opposite position of a nucleotide about 5-15 bases from the 5' end of the antisense strand. In certain embodiments, the ΔG of each duplex region is less than about -13 kcal/mole.

Another aspect of the invention provides a vector expressing at least one strand of the subject dsRNA (*e.g.*, unmodified sense strand, *etc.*).

Another aspect of the invention provides a cell comprising the subject vector, or the subject dsRNA.

In certain embodiments, the cell is a mammalian cell in culture.

In certain embodiments, the cell is a human cell.

Another aspect of the invention provides a composition comprising the dsRNA of any of claims 1-5, and a pharmaceutically acceptable carrier or diluent.

Another aspect of the invention provides a method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with the subject dsRNA construct.

In certain embodiments, the mammalian cell is in culture.

In certain embodiments, the mammalian cell is a human cell.

In certain embodiments, the mammalian cell is contacted in the presence of a delivery reagent.

In certain embodiments, the delivery reagent is a lipid.

In certain embodiments, the lipid is a cationic lipid.

In certain embodiments, the delivery reagent is a liposome.

Another aspect of the invention provides a method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with a vector expressing at least one strand of the subject dsRNA construct.

Another aspect of the invention provides a method for improving the gene silencing effect of a small interference RNA (siRNA), comprising modifying the sense and/or antisense nucleotides of the siRNA to become the subject dsRNA construct.

Another aspect of the invention provides a method for evaluating *in vivo* delivery of an siRNA construct to a target site, comprising co-delivering the siRNA construct with any of the subject dsRNA construct that targets PPIB, and assaying the inhibition of PPIB function at the target site, wherein successful inhibition of the PPIB function at the target site is indicative of successful *in vivo* delivery of the siRNA construct to the target site.

Another aspect of the invention relates to any of the dsRNA constructs disclosed herein, such as those disclosed in the tables and/or those against SOD1 or other specific target genes.

It is further contemplated that, any embodiments recited herein and elsewhere in the specification can be combined with any other embodiments where applicable.

Brief Description of the Drawings

Figure 1 shows certain modified RNAi constructs of the invention (Alternate RNAi Compounds for simplicity).

Figure 2 illustrates SOD1 expression 24 hours post-transfection with R1 siRNA or Alternate RNAi Compounds.

Figure 3 indicates Alternate RNAi Compounds sequences with activity in reducing SOD1 mRNA levels.

Figure 4 shows that certain exemplary Alternate RNAi Compounds (*e.g.*, 25-mer with blunt ends, with four 2'OMe on each end of the sense strand) for selected target sites are effective against mouse and human SOD1.

Figure 5A illustrates a dose response analysis for SOD1 Alternate RNAi Compounds and identification of active duplexes with EC_{50} values of <50 pM. Figure 5B shows dose response analysis for SOD1 Alternate RNAi Compounds in human HEK293 cells, and identification of active duplexes with EC_{50} values of about 50 pM. Figure 5C shows dose response analysis for SOD1 Alternate RNAi Compounds in murine NIH3T3 cells, and identification of active duplexes with EC_{50} values of about 50 pM.

Figure 6A shows the expression of PPIB (cyclophilin B), a ubiquitous protein, after transfection with siRNA or Alternate RNAi Compound. Figure 6B shows dose response analysis for PPIB Alternate RNAi Compounds in human HEK293 cells, and identification of active duplexes with EC_{50} values of about 25 or 72 pM for 25-mer and 27-mer, respectively. Figure 6C shows dose response analysis for PPIB Alternate RNAi Compounds in murine NIH3T3 cells, and identification of active duplexes with EC_{50} values of about 200 or about 500 pM for 25-mer and 27-mer, respectively.

Figure 7 demonstrate that the Alternate RNAi Compound 25-mers with sense strand modifications are not cleaved by the Dicer enzyme.

Figure 8A demonstrates that the 25-mer duplexes are found to be associated with the RNAi silencing Ago2 complex. Figure 8B shows a Northern blot of the guide strand of SOD1 targeting RNA duplexes. Figure 8C shows expression of SOD1 after transfection of 293 cells expressing c-myc Ago2.

Figures 9A and 9B indicate the uniform cleavage point at 10 nt from the 5'-end of the

antisense strand for non-Dicer processed duplexes longer that 21 nt. A synthetic substrate was chemically synthesized to correspond to a 50 nt region of the SOD1 gene containing the target sequence for the RNA duplexes tested. Figure 9A is a schematic drawing of the synthetic substrate and predicted cleavage position and products. In Figure 9B, RNA duplexes targeting SOD1 were transfected into 293 cells expressing c-myc Ago2 as described in methods. The cells were harvested, lysed, c-myc Ago2 was immunoprecipitated, and reconstituted in buffer. The immunoprecipitates were incubated with a 50 nt ^{32}-P-labeled synthetic substrate for 2 h at 30 °C as described in methods. After 2 h incubations, samples were loaded onto a denaturing, polyacrylamide gel along with size markers (shown underlined). Sample letters correspond to the following duplexes shown in the schematic in panel A. A = unmodified 19-bp + 2 nt siRNA, B = unmodified 25-bp duplex, C = 25-bp duplex with 4/4 2'OMe, D = 25-bp duplex with 4/4 2'OMe, E = Luciferase Ctrl duplex.

Figure 10A indicates two exemplary sequences, ID Nos. 10015 and 10023, that were used for extensive chemical modification analysis.

Figure 10B lists each modification chemistry ID for sense strand (001, 002, 003, *etc.*) and antisense strand (011, 012, 013, *etc.*), indicating for each ID a detailed overview of modifications on each nucleotide position for all 25-mers designed and/or tested.

Figure 11 illustrates the varying degree of SOD1 mRNA level reduction that is dependent on the type of modifications on the sense and antisense strands.

Figures 12A-12C show relative activity of duplexes for inhibiting SOD1 expression as a function of varying the 2'OMe positions on only the sense strand. Each duplex is based on the sequence of ID No. 10015 or 10023.

Figure 13A-13D show relative activity of duplexes for inhibiting SOD1 expression as a function of varying the 2'OMe positions on the sense strand in combination with various antisense chemistries indicated (011, 013, 042, *etc.* See Figure 11B).

Figures 14A and 14B demonstrate the improvement of duplex stability in serum and/cerebral spinal fluid as a result of modifications on each duplex as indicated.

Figure 15A demonstrates that Dicer is NOT required for RNAi activity with duplexes greater than 21 bp. The bars represent the mRNA levels of SOD1 48 h after transfection as measured by bDNA hybridization assay. SOD1 mRNA reduction data for the three duplexes are shown; 19-bp + 2 nt siRNA (solid white bar), unmodified (0/0) 25-bp duplex (solid black bar), and "4/4" 2'OMe 25-bp duplex (striped bar). Figure 15B shows the sequences used in this study.

Figure 16A shows that, similar to the observation of Figure 15A, Dicer is not required for RNAi activity with duplexes of 27 bp. The bars represent the mRNA levels of PPIB 48 h after transfection as measured by bDNA hybridization assay. The solid white bars are a 19-bp + 2 nt siRNA, the dotted bars are an unmodified 25-bp duplex, the solid black bars are a 25-bp duplex with 2'OMe chemistry, and the striped bars are a 27-bp duplex with 2'OMe chemistry. Sequences are shown in Figure 16B.

Figure 17 shows that 2'-O-Me modifications inhibit and prevent Dicer enzyme processing of >21nt RNA duplexes into siRNA. Figure 17A is a schematic of chemical modifications applied to an RNA duplex that targets SOD1. The R in the duplexes represents a normal or unmodified RNA nucleotide having no 2' position modification. The M represents an O-methyl modification to the 2' position of the RNA nucleotide. Most nucleotide modifications are placed on the passenger strand unless otherwise noted. Figure 17B is a TBE-polyacrylamide gel of RNA duplexes after overnight incubation with recombinant human dicer enzyme as described herein. Lane M designates a siRNA marker, sizes (top to bottom): 25-bp, 21-bp, 17-bp. Figure 17C shows quantification of processing of 25-bp RNA duplex with "2/2" 2'-O-Me chemistry. Density of the partially processed bands was quantified using LabWorks software. Figure 17D is a TBE-polyacrylamide gel of RNA duplexes that have different combinations of (4) 2'-O-Me on each end of the duplex after overnight incubation with recombinant human Dicer enzyme as described herein. Figure 17E shows similar results obtained with 2'-O-Me modified 25-bp duplexes targeting a different sequence in the SOD1 gene.

Table 1 provides Alternative RNAi Compound sequences to SOD1 and PPIB. Full Sequence Name is represented by gene name-start site-length-Alternate RNAi Compound ID Number. "P" represents 5' phosphate, while "m" represents 2'O-methyl base modification. "F" represents 2'Fluoro base modification. "*" denotes phosphothioate backbone linkage while "." represents a normal RNA backbone linkage.

Table 2 provides additional Alternative RNAi Compound sequences to SOD1. The notations used in Table 1 applies to Table 2.

Table 3 is a list of RNA duplexes. Single stranded RNA or duplexed RNA was chemically synthesized and annealed as described in methods. "G" = Guanine, "U" = Uracil, "C" = Cytosine, "A" = Adenine, "m" = 2'OMethyl base modified, "." = normal RNA backbone linkage. Polarity is shown as PS = Passenger or Sense strand and GS = Guide or antisense strand. Numbers in parentheses correspond to internal sequence database number for duplex tracking.

Table 4 is a list of RNA duplexes in certain figures above. Single stranded RNA or duplexed RNA was chemically synthesized and annealed as described in methods. "G" = Guanine, "U" = Uracil, "C" = Cytosine, "A" = Adenine, "m" = 2'OMethyl base modified, "." = normal RNA backbone linkage. Polarity is shown as PS = Passenger or Sense strand and GS = Guide or antisense strand. Numbers in parentheses correspond to internal sequence database number for duplex tracking.

Table 5 is a list of RNA size markers and synthetic substrates used in cleavage assays.

Detailed Description of the Invention

I. *Overview*

The invention is partly based on the surprising discoveries that certain longer dsRNAs (*e.g.*, those with a double-stranded region of more than 21 base pairs) are not cleaved by Dicer or other Dicer-like RNAse III when the sense strands are modified (*e.g.*, at both ends of the sense strand by, for example, 2'-O-methyl groups), and that the antisense strands of such dsRNAs can be loaded into the RISC complex, with the 5'-ends of the antisense strands aligning with the 5'-end of a 21-mer (*i.e.*, the Dicer cleavage product). While the art taught that RNAi compound activity was increased (compared to siRNA) if the compound formed a substrate for Dicer, Applicants have in fact found extremely active RNAi compounds that are not Dicer substrates. The invention is also partly based on the discovery that a RISC complex loaded with such a longer antisense (guide) strand will cleave the target mRNA at a single position corresponding to the position between the 10th and 11th nucleotides from the 5'-end of the antisense (guide) sequence.

A direct implication of Applicants' discoveries is that the antisense strand of such a longer dsRNA becomes the *single* species of active RNAi reagent, thus facilitating the development of RNAi reagents or therapeutics with higher target specificity, and better-defined biological activity and/or pharmacological property.

Furthermore, with the knowledge that longer dsRNA can be engineered to resist Dicer cleavage, and the knowledge that the Dicer-resistant antisense strand can be loaded onto the RISC complex at defined location to create a single species of active RNAi reagent, one can engineer additional features or modifications into the sense and/or antisense strands to improve the property of the RNAi reagent or therapeutics. In particular, the positioning of modifications in the guide strand relative to the 5' end can now be defined, and this positioning is critical to defining the specificity and activity of such modified RNAi compounds.

Using an exemplary target gene superoxide dismutase 1 (SOD1), Applicants have designed and tested numerous sense and antisense modifications and combinations thereof, and have identified multiple specific modifications that render the long dsRNA Dicer-resistant, yet providing effective target gene silencing and/or a host of other associated benefits.

Thus in one aspect, the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

In certain embodiments, the antisense strand is unmodified. In other embodiments, the antisense strand includes a 2'-modified ribose sugar at the 2nd nucleotide from the 5'-end of the antisense strand.

As used herein, "2'-modified ribose sugar" includes those ribose sugars that do not have a 2'-OH group. For example, the 2'-modified ribose sugar may be 2'-O-alkyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy nucleotides, 2'-H (deoxyribonucleotides), or combination thereof.

In certain embodiments, the dsRNA of the invention with the above-referenced antisense modification exhibits significantly (*e.g.*, at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or more) less "off-target" gene silencing when compared to similar constructs without the specified antisense modification, thus greatly improving the overall specificity of the RNAi reagent or therapeutics.

As used herein, "off-target" gene silencing refers to unintended gene silencing due to, for example, spurious sequence homology between the antisense (guide) sequence and the unintended target mRNA sequence.

In another aspect, the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand comprises, at the 3'-end of the

antisense strand, (i) at least four consecutive 2'-modified ribose sugars with non-hydrolyzable internucleotide linkages, (ii) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 2'-modified ribose sugars, preferably 2'-O-methyl modified ribose sugars, or, (iii) a protective group, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

According to this aspect of the invention, certain antisense modifications further increase nuclease stability, and/or lower interferon induction, without significantly decrease RNAi activity (or no decrease in RNAi activity at all).

In another aspect, the invention provides a double-stranded RNA (dsRNA) construct of 19-49 nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and said sense strand comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

According to this aspect of the invention, certain mismatches at the 3'-end of the sense strand allow more efficient loading of the antisense strand into the RISC complex, thus leading to more potent RNAi activity. Preferred such sense strand mismatches include: a mismatch at the second to the last nucleotide of the sense strand (which base pairs with the second nucleotide of the Dicer-resistant antisense strand in the RISC complex); and mismatches at the most 3'-end 9 nucleotides, except for the most 3'-end nucleotide, of the sense strand.

It is contemplated that different features of the invention, such as the different sense and/or antisense strand modifications, may be combined except when indicated otherwise, in order to create RNAi constructs with multiple advantages or features over the conventional siRNA constructs.

For example, for all applicable aspects of the invention, the antisense strand may comprise a 2'-modified ribose sugar, such as 2'-O-methyl modified nucleotide, at the 2nd nucleotide on the 5'-end of the antisense strand and, preferably no other modified nucleotides. The dsRNA having such modification may have enhanced target specificity or reduced off-target silencing compared to a similar construct without the 2'-O-methyl modification at said position.

For all applicable aspects of the invention, the antisense strand may comprise at least four consecutive 2'-modified ribose sugars, such as 2'-O-methyl modified, 3'-end nucleotides with non-hydrolyzable internucleotide linkages, such as phosphothioate linkages.

For all applicable aspects of the invention, the dsRNA may be cleaved by RISC at a single site between the 10^{th} and 11^{th} nucleotides of the 3'-end of the sense strand.

For all applicable aspects of the invention, the 5'-end 12 nucleotides and the 3'-end 10 nucleotides of a 25-mer may be 2'-modified ribose sugars. The number of the modified bases may be adjusted depending on the overall length of the construct. For example, for a 27-mer, the 5'-end 12-14 nucleotides and the 3'-end 10-12 nucleotides may be 2'-modified nucleotides, *etc.*

For all applicable aspects of the invention, the dsRNA may comprise a mismatch nucleotide at the 2^{nd} nucleotide from the 3'-end of the sense strand.

Certain combinations of specific antisense and sense strand modifications may even result in unexpected advantages, as partly manifested by enhanced ability to inhibit target gene expression, enhanced serum stability, and/or increased target specificity, *etc.*

Thus, in another aspect, the invention provides double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein four consecutive 2'-O-methyl nucleotides are present at each of said 5'- and 3'-ends of said sense strand, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand: (a) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages; or, (b) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

In another aspect, the invention provides a double-stranded RNA (dsRNA) construct of 12-49 (preferably 19-49) nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising: (1) a sense strand having a 5'-end and a 3'-end, wherein the sense strand comprises 12 and 10 consecutive 2'-O-methyl nucleotides at the 5'-end and the 3'-end, respectively, and, (2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand: (a) is unmodified; (b) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate

linkages; or, (c) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides, wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

In certain embodiments, the antisense strand of the subject dsRNA directs the uniform cleavage of the target gene transcript at a single site between the 10^{th} and 11^{th} nucleotides from the 5'-end of the antisense strand.

In certain embodiments, the sense strand of the dsRNA is cleavable by RISC at a single site between the 10th and the 11th nucleotides from the 3'-end of the sense strand.

According to this embodiment of the invention, certain sense strand sequences may be cleaved by the RISC complex loaded with the Dicer-resistant guide sequence, at the position where an equivalent mRNA is cleaved. While not wishing to be bound by any particular theory, this is partly because the sense strand share the same or similar sequence as the target mRNA. Therefore, the subject dsRNA constructs include those with a sense strand that can be cleaved between the 10th and 11th 3'-end nucleotides.

The constructs of the invention may have different lengths. In certain embodiments, the preferred lengths of the construct are 12-35, or 12-49, preferably 19-49 nucleotides in length. In certain embodiments, the length of the construct is greater than or equal to 22 nucleotides in length. In certain embodiments, the length of the construct is greater than or equal to 25 nucleotides in length. In certain embodiments, the length of the construct is 26, 27, 28, 29, 30, or 31-49 nucleotides in length. Other lengths are also possible, so long as the lower length limit is the minimal length for a Dicer substrate, and the upper limit is a length that generally will not trigger PKR response in a target cell. In certain embodiments, modifications may alter that upper limit such that longer lengths (such as 50, 60, 70, 80, 90, 100 bp) are tolerable.

In certain embodiments, the dsRNA construct is blunt-ended. In other embodiments, 5'- and/or 3'-end overhangs of 1-4 nucleotides may be present on one or both strands.

For a 25-mer construct, each end of the sense strand may comprise, independently, 4-16 2'-modified nucleotides and/or non-hydrolyzable linkages (*e.g.*, phosphothioate linkages). The number of the modified bases may be adjusted depending on the overall length of the construct. For example, for a 27-mer, each end of the sense strand may comprise, independently, 4-18 2'-modified nucleotides and/or phosphothioate linkages, *etc.*

In certain embodiments, the 5'-end 12 nucleotides and the 3'-end 10 nucleotides of the sense strand are 2'-modified ribose sugars.

In certain embodiments, each end of the sense strand comprises a continuous stretch of 2'-modified ribose sugars, although each end may have the same number or different numbers of 2'-modified ribose sugars.

In certain embodiments, each end of the sense strand comprises a continuous stretch of four 2'-modified ribose sugars.

In certain embodiments, the antisense strand comprises discontinuous 2'-modified ribose sugars, wherein the 10th and 11th antisense nucleotides are not modified. For example, the antisense strand may comprises 2'-modified ribose sugars for each 2, 3, 4, 5, 6, 7, 8, or 9 nucleotides. The most 5'-end 2'-modified ribose sugar on the antisense strand may be the 2nd nucleotide, or the first nucleotide.

In certain embodiments, the 2'-modified nucleotides are 2'-O-alkyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy nucleotides, 2'-H (deoxyribonucleotide), or combination thereof.

In certain embodiments, the 2'-modified nucleotides are pyrimidine nucleotides (*e.g.*, C /U).

For example, the 2'-O-alkyl nucleotides may be 2'-O-methyl nucleotides, or 2'-O-allyl nucleotides.

In certain embodiments, the antisense strand comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end of the antisense strand and no other modified nucleotides.

In certain embodiments, the modified dsRNA may have improved stability in serum and/or cerebral spinal fluid compared to an unmodified dsRNA having the same sequence.

In certain embodiments, the dsRNA has enhanced target specificity or reduced off-target silencing compared to a similar construct without the 2'-modification at said position(s).

In certain embodiments, the antisense strand comprises at least four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages.

In certain embodiments, the sense strand of the dsRNA comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand.

In certain embodiments, the last 2nd-8th nucleotides at the 3'-end of the sense strand mismatch their corresponding antisense strand nucleotides.

In certain embodiments, the dsRNA does not induce interferon response in primary cells, such as mammalian primary cells, including primary cells from human, mouse and other rodents, and other non-human mammals.

In certain embodiments, the dsRNA may also be used to inhibit expression of a target gene in an invertebrate organism.

In certain embodiments, the 10th and 11th antisense nucleotides from the 5'-end are not modified.

To further increase the stability of the subject constructs *in vivo*, either end of the sense strand and/or the 3'-end of the antisense strand may be blocked by protective group(s). For example, protective groups such as inverted nucleotides, inverted abasic moieties, or amino-end modified nucleotides may be used. Inverted nucleotides may comprise an inverted deoxynucleotide. Inverted abasic moieties may comprise an inverted deoxyabasic moiety, such as a 3',3'-linked or 5',5'-linked deoxyabasic moiety.

In certain embodiments, alternating nucleotides on the ends of the sense and antisense strands are modified by 2'-O-alkyl modification, and wherein each of the 2'-O-modified nucleotides faces an unmodified nucleotide on the opposite strand. In a preferred embodiment, the first 2'-O-modified antisense nucleotide (with this pattern of modification) is the most 5'-end antisense nucleotide.

In certain embodiments, alternating nucleotides on the ends of the sense and/or antisense strands comprise 2'-modified ribose sugars, and wherein each of the 2'-modified ribose sugars faces an unmodified nucleotide on the opposite strand. In certain embodiment, the first 2'-modified antisense nucleotide is the most 5'-end antisense nucleotide or the 2nd nucleotide from the 5'-end of the antisense strand.

In certain embodiments, RNAi constructs having the same or similar structures of any of the Alternate RNAi Compounds described herein, but differing by not being resistant to Dicer cleavage, are also desirable, so long as they show high activity against their respective intended target (*e.g.*, mRNA).

In certain embodiments, the subject double-stranded RNA may be chemically cross-linked at one or more points, or linked by a nucleotide loop structure at one or both ends (*e.g.*, a single-stranded hairpin structure or a circular structure). In one embodiment, the chemical cross-link or the loop of the hairpin structure is at the 3'-end of the antisense strand (*e.g.*, linking the 3'-end of the antisense strand to the 5'-end of the sense strand). In another embodiment, the chemical cross-link or the loop of the hairpin structure is at the 5'-end of the antisense strand (*e.g.*, linking the 3'-end of the sense strand to the 5'-end of the antisense strand. In these embodiments, other structural features of the cross-linked or looped constructs, such as 5'-end and 3'-end modifications on the sense strand and/or the other modifications on the antisense

strand, are essentially the same as those for the dsRNA described herein.

Double-stranded and/or duplex oligonucleotide constructs of the invention are capable of inhibiting the synthesis of any target protein encoded by target gene(s). The invention includes methods to inhibit expression of a target gene either in a cell *in vitro*, or *in vivo*. The target gene can be endogenous or exogenous (*e.g.*, introduced into a cell by a virus or using recombinant DNA technology) to a cell. Such methods may include introduction of RNA into a cell in an amount sufficient to inhibit expression of the target gene, where the RNA is a double-stranded duplex. By way of example, such an RNA molecule may have a first strand having a ribonucleotide sequence that corresponds to a nucleotide sequence of the target gene, and a second strand having a ribonucleotide sequence that is complementary to the nucleotide sequence of the target gene, in which the first and the second strands are separate complementary strands, and they hybridize to each other to form said double-stranded molecule, such that the duplex composition inhibits expression of the target gene. Exemplary (but non-limiting) target genes that are used extensively in this application to illustrate the general principles of the invention include SOD1, PPIB, RIP140, PCSK9, TNFα, AP2 (adipocyte lipid-binding protein), or MAP4K4, just to name a few.

While not wishing to be bound by any particular theory, the presence of 2'-O-methyl block regions on the "sense" strand of the RNAi entity (such as the regions at the ends of the sense strand) may result in significant increase in stability, specificity and minimization of immune response, compared to constructs without such modifications. In some cases, it may be even more beneficial to further increase RNAi duplex stability by applying modifications to the antisense strand. In particular, some of preferred chemical modification patterns might comprise antisense strand containing majority of C and Us modified by 2'-O-methyl, or C and Us modified by 2'F. In some cases, the antisense strand might be modified with the mixture of several chemical modifications.

In certain embodiments, heavily modified antisense strand might not be good substrates for kinases. Thus chemical phosphorylation of the antisense strand might be used to alleviate this problem. In addition, some preferred sequences might contain only purine nucleotides (*i.e.*, A and Gs) in the unmodified region of the sense strand or/and 2'-F or phosphorothiate (PS) modifications. In some cases, additional introduction of PS modifications in combination with 2'-O-methyl and 2'F might be preferred.

In some cases, modification of both the sense and antisense strands is preferred to achieve maximal stabilization of the constructs. Since heavily modified duplexes sometimes are

poor substrates for RISC assembly, in some preferred embodiments, presence of single or multiple bulges (*e.g.*, about 1-5 nucleotides in size) might be necessary or at least helpful to enhance RISC entry and efficacy.

In some other embodiments, heavily modified duplexes may contain a single nick in the sense strand. The preferred nick position may be 10 bp on the opposing strand from the 5' end of the antisense strand. In some embodiments, the nick can be placed within 5 bases from the above-referenced preferred position (*i.e.*, 10 bp on the opposing strand from the 5' end of the antisense strand). In some embodiments, additional chemical modifications providing duplex stabilization might be applied to the subject constructs. In some embodiments, the sequences may be selected to have certain thermodynamic characteristics, *e.g.*, ΔG of each duplex region being < -13kcal/mol.

Therefore, in certain embodiments, the constructs of the invention may contain certain modifications on either the sense / passenger strand or the antisense / guide strand, or both, and confer certain advantages to the construct.

For example, in certain embodiments, the sense strand nucleotides between the 2'-modified ribose nucleotides (*e.g.*, the middle portion or stretch of the sense strand) are 2'-F modified. Alternatively or in addition, the same portion might contain purine nucleotides only, and optionally having 2'-F modification and/or phosphorothioate linkages for some or all of the nucleotides. Alternatively or in addition, the same part of the sense strand may form one or more bulges, such as bulges of about 1-5 nucleotides each.

As used herein, the continuous stretch of 2'-modified ribose nucleotides does not need to start from the 5'-end or 3'-end nucleotides, although such stretches preferably start from the end nucleotides. Thus, in certain embodiments, the continuous stretch of 2'-modified ribose nucleotides independently starts from the terminal nucleotide, the second nucleotide from the terminal nucleotide, or the third nucleotide from the terminal nucleotide, etc.

In certain embodiments, between about 50-100% of the pyrimidine nucleotides of the antisense strand are, independently, 2'-F modified or 2'-O-methyl-modified.

In certain embodiments, the 5'-end of the antisense strand may be phosphorylated.

In certain related embodiments, the invention also provides an RNA construct for inhibiting expression of a target gene, wherein the construct is identical to any of the dsRNA described above, except for a single nick on the sense strand. Thus in those embodiments, the RNA construct in fact contains three polynucleotides forming, via hybridization, a double-stranded structure. The antisense strand is a single-stranded polynucleotide, while the two other

polynucleotides both hybridize to the antisense polynucleotide, and forms a "sense strand" that corresponds to any of the other dsRNA constructs described herein.

The location of the nick may vary in this embodiments. For example, the nick may occupy the opposite position of the nucleotide about 10 bases from the 5' end of the antisense strand. Alternatively, the nick may be within 5 nucleotides from this position (*e.g.*, the nick occupies the opposite position of a nucleotide about 5-15 bases from the 5' end of the antisense strand. The sequences of the double-stranded regions in this type of construct may also be selected such that the ΔG of each duplex region is less than about -13 kcal/mole.

The invention also relates to vectors expressing at least one strand of the subject dsRNA constructs, and cells comprising such vectors or the subject dsRNA constructs. The cell may be a mammalian cell in culture, such as a human cell.

The invention further relates to compositions comprising the subject dsRNA constructs, and a pharmaceutically acceptable carrier or diluent.

Another aspect of the invention provides a method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with the any of the subject dsRNA constructs.

The method may be carried out *in vitro* or *in vivo*, in, for example, mammalian cells in culture, such as a human cell in culture.

The target cells (*e.g.*, mammalian cell) may be contacted in the presence of a delivery reagent, such as a lipid (*e.g.*, a cationic lipid) or a liposome.

Another aspect of the invention provides a method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with a vector expressing at least one strand of any of the subject dsRNA constructs.

Another aspect of the invention provides a method for improving the gene silencing effect of a small interference RNA (siRNA), comprising modifying the sense and/or antisense nucleotides of the siRNA to become any of the subject dsRNA constructs.

Another aspect of the invention provides a method for evaluating *in vivo* delivery of an siRNA construct to a target site, comprising co-delivering the siRNA construct with any of the subject dsRNA constructs designed to target PPIB, a ubiquitous gene universally expressed in almost all tissues, and assaying the inhibition of PPIB function at the target site, wherein successful inhibition of the PPIB function at the target site is indicative of successful *in vivo* delivery of the siRNA construct to the target site.

More detailed aspects of the invention are described in the sections below.

II. *Duplex Structure*

Duplex Characteristics

Double-stranded oligonucleotides of the invention may be formed by two separate complementary nucleic acid strands. Duplex formation can occur either inside or outside the cell containing the target gene.

As used herein, the term "double-stranded" includes one or more nucleic acid molecules comprising a region of the molecule in which at least a portion of the nucleomonomers are complementary and hydrogen bond to form a duplex.

As used herein, the term "duplex" includes the region of the double-stranded nucleic acid molecule(s) that is (are) hydrogen bonded to a complementary sequence. Double-stranded oligonucleotides of the invention may comprise a nucleotide sequence that is sense to a target gene and a complementary sequence that is antisense to the target gene. The sense and antisense nucleotide sequences correspond to the target gene sequence, *e.g.*, are identical or are sufficiently identical to effect target gene inhibition (*e.g.*, are about at least about 98% identical, 96% identical, 94%, 90% identical, 85% identical, or 80% identical) to the target gene sequence.

In certain embodiments, the double-stranded oligonucleotide of the invention is double-stranded over its entire length, *i.e.*, with no overhanging single-stranded sequence at either end of the molecule, *i.e.*, is blunt-ended. In other embodiments, the individual nucleic acid molecules can be of different lengths. In other words, a double-stranded oligonucleotide of the invention is not double-stranded over its entire length. For instance, when two separate nucleic acid molecules are used, one of the molecules, *e.g.*, the first molecule comprising an antisense sequence, can be longer than the second molecule hybridizing thereto (leaving a portion of the molecule single-stranded). Likewise, when a single nucleic acid molecule is used a portion of the molecule at either end can remain single-stranded.

In one embodiment, a double-stranded oligonucleotide of the invention contains mismatches and/or loops or bulges, but is double-stranded over at least about 70% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 80% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 90%-95% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 96%-98% of the length of the oligonucleotide. In certain embodiments, the double-stranded oligonucleotide of

the invention contains at least or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mismatches.

Modifications

The nucleotides of the invention may be modified at various locations, including the sugar moiety, the phosphodiester linkage, and/or the base.

Sugar moieties include natural, unmodified sugars, *e.g.*, monosaccharide (such as pentose, *e.g.*, ribose, deoxyribose), modified sugars and sugar analogs. In general, possible modifications of nucleomonomers, particularly of a sugar moiety, include, for example, replacement of one or more of the hydroxyl groups with a halogen, a heteroatom, an aliphatic group, or the functionalization of the hydroxyl group as an ether, an amine, a thiol, or the like.

One particularly useful group of modified nucleomonomers are 2'-O-methyl nucleotides. Such 2'-O-methyl nucleotides may be referred to as "methylated," and the corresponding nucleotides may be made from unmethylated nucleotides followed by alkylation or directly from methylated nucleotide reagents. Modified nucleomonomers may be used in combination with unmodified nucleomonomers. For example, an oligonucleotide of the invention may contain both methylated and unmethylated nucleomonomers.

Some exemplary modified nucleomonomers include sugar- or backbone-modified ribonucleotides. Modified ribonucleotides may contain a non-naturally occurring base (instead of a naturally occurring base), such as uradines or cytidines modified at the 5'-position, *e.g.*, 5'-(2-amino)propyl uradine and 5'-bromo uradine; adenosines and guanosines modified at the 8-position, *e.g.*, 8-bromo guanosine; deaza nucleotides, *e.g.*, 7-deaza-adenosine; and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine. Also, sugar-modified ribonucleotides may have the 2'-OH group replaced by a H, alxoxy (or OR), R or alkyl, halogen, SH, SR, amino (such as NH_2, NHR, NR_2,), or CN group, wherein R is lower alkyl, alkenyl, or alkynyl.

Modified ribonucleotides may also have the phosphoester group connecting to adjacent ribonucleotides replaced by a modified group, *e.g.*, of phosphothioate group. More generally, the various nucleotide modifications may be combined.

In one embodiment, sense oligomers may have 2'-modifications on the ends (*e.g.*, 2 on each end, 3 on each end, and 4 on each end, *etc.*; as well as 1 on one end, 2 on one end, 3 on one end, and 4 on one end, *etc.*; and even unbalanced combinations such as 12 on one end and 10 on the other end, *etc.*). Likewise, the antisense strand may have 2'-modifications on the ends (1 on each end, 2 on each end, 3 on each end, and 4 on each end, and so on; as well as 1 on one end, 2

on one end, 3 on one end, and 4 on one end, and so on; and even unbalanced combinations such as 1 on one end and 2 on the other end, and so on). In preferred aspects, the 2′-modifications are 2'-O-methyl modifications in the sense RNA strand and/or the antisense strand.

According to the instant invention, the sense strand can tolerate many 2'-modifications (such as 2'-O-methyl modifications), so long as the central linkages are unmodified. As used herein, "central" is not limited to mean the geometric mid-point of the sense strand. Rather, it can include any location between the 5'-end portion and the 3'-end portion of the sense strand. The 5'-end portion and the 3'-end portion of the sense strand need not be symmetrical.

Thus, in certain embodiments, the sense strand is not completely modified (*i.e.*, at least one or more sense strand nucleotide(s) are unmodified). In certain embodiments, the unmodified sense strand nucleotides are in the central portion of the sense strand, or between the stretch of modified sense strand nucleotides on the 5'-end and the stretch of modified sense strand nucleotides on the 3'-end.

Also according to the instant invention, the sense strand tolerance for 2'-modification is not necessarily symmetrical. Rather, asymmetrical configurations may be desirable when using, for example, a sense strand of 25 or 26 nucleotides. 2'-mofications add nuclease stability, and reduce interferon induction, and are easier to synthesize. Thus it may be desirable to include more such 2'-modified ribose sugars (especially 2'-O-methyl modified) on the sense strand, so long as the teachings of the instant invention is followed to preserve RNAi activity.

In some embodiments of the present invention, the subject highly modified sense strands may be combined with either unmodified or lightly modified antisense strands to allow maximum guide strand activity.

To further maximize endo- and exo-nuclease resistance, in addition to the use of 2'-modified nucleomonomers in the ends, inter-nucleomonomer linkages other than phosphodiesters may be used. For example, such end blocks may be used alone or in conjunction with phosphothioate linkages between the 2'-O-methly linkages. Preferred 2'-modified nucleomonomers are 2'-modified end nucleotides.

Although the antisense strand may be substantially identical to at least a portion of the target gene (or genes), at least with respect to the base pairing properties, the sequence need not be perfectly identical to be useful, *e.g.*, to inhibit expression of a target gene's phenotype. Generally, higher homology can be used to compensate for the use of a shorter antisense gene. In some cases, the antisense strand generally will be substantially identical (although in antisense orientation) to the target gene.

One particular example of a composition of the invention has end-blocks on both ends of a sense oligonucleotide and only the 3'-end of an antisense oligonucleotide. Without wishing to be bound by theory, a 2'-O-modified sense strand may work less well than its unmodified version, possibly because it is not efficiently unwound. Thus, in certain embodiments, mismatches may be introduced into specific positions of the sense strand (modified 2'-O-methyl sense strand, or even unmodified sense strand) to facilitate easier loading into the RISC complex.

In some embodiments, the length of the sense strand can be 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 nucleotides. Similarly, the length of the antisense strand can be 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 nucleotides. Further, when a double-stranded nucleic acid molecule is formed from such sense and antisense molecules, the resulting duplex may have blunt ends or overhangs of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 nucleotides on one end or independently on each end. Further, double stranded nucleic acid molecules of the invention may be composed of a sense strand and an antisense strand wherein these strands are of lengths described above, and are of the same or different lengths, but share only 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides of sequence complementarity. By way of illustration, in a situation where the sense strand is 20 nucleotides in length and the antisense is 25 nucleotides in length and the two strands share only 15 nucleotides of sequence complementarity, a double stranded nucleic acid molecules may be formed with a 10 nucleotide overhang on one end and a 5 nucleotide overhang on the other end.

The use of 2'-O-methyl RNA may also be beneficially in circumstances in which it is desirable to minimize cellular stress responses. RNA having 2'-O-methyl nucleomonomers may not be recognized by cellular machinery that is thought to recognize unmodified RNA. The use of 2'-O-methylated or partially 2'-O-methylated RNA may avoid the interferon response to double-stranded nucleic acids, while maintaining target RNA inhibition. This may be useful, for example, for avoiding the interferon or other cellular stress responses, both in short RNAi (*e.g.*, siRNA) sequences that induce the interferon response, and in longer RNAi sequences that may induce the interferon response.

Overall, modified sugars may include D-ribose, 2'-O-alkyl (including 2'-O-methyl and 2'-O-ethyl), *i.e.*, 2'-alkoxy, 2'-amino, 2'-S-alkyl, 2'-halo (including 2'-fluoro), 2'-methoxyethoxy, 2'-allyloxy ($-OCH_2CH=CH_2$), 2'-propargyl, 2'-propyl, ethynyl, ethenyl, propenyl, and cyano and the like. In one embodiment, the sugar moiety can be a hexose and incorporated into an oligonucleotide as described (Augustyns, K., *et al.*, *Nucl. Acids. Res.*

18:4711 (1992)). Exemplary nucleomonomers can be found, *e.g.*, in U.S. Pat. No. 5,849,902, incorporated by reference herein.

The term "alkyl" includes saturated aliphatic groups, including straight-chain alkyl groups (*e.g.*, methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, *etc.*), branched-chain alkyl groups (isopropyl, tert-butyl, isobutyl, *etc.*), cycloalkyl (alicyclic) groups (cyclopropyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl), alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In certain embodiments, a straight chain or branched chain alkyl has 6 or fewer carbon atoms in its backbone (*e.g.*, C_1-C_6 for straight chain, C_3-C_6 for branched chain), and more preferably 4 or fewer. Likewise, preferred cycloalkyls have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C_1-C_6 includes alkyl groups containing 1 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkyl includes both "unsubstituted alkyls" and "substituted alkyls," the latter of which refers to alkyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety. Cycloalkyls can be further substituted, *e.g.*, with the substituents described above. An "alkylaryl" or an "arylalkyl" moiety is an alkyl substituted with an aryl (*e.g.*, phenylmethyl (benzyl)). The term "alkyl" also includes the side chains of natural and unnatural amino acids. The term "n-alkyl" means a straight chain (*i.e.*, unbranched) unsubstituted alkyl group.

The term "alkenyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but that contain at least one double bond. For example, the term "alkenyl" includes straight-chain alkenyl groups (*e.g.*, ethylenyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl, *etc.*), branched-chain alkenyl groups, cycloalkenyl (alicyclic) groups (cyclopropenyl, cyclopentenyl, cyclohexenyl, cycloheptenyl, cyclooctenyl), alkyl or alkenyl substituted cycloalkenyl groups, and cycloalkyl or

cycloalkenyl substituted alkenyl groups. In certain embodiments, a straight chain or branched chain alkenyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C_2-C_6 for straight chain, C_3-C_6 for branched chain). Likewise, cycloalkenyl groups may have from 3-8 carbon atoms in their ring structure, and more preferably have 5 or 6 carbons in the ring structure. The term C_2-C_6 includes alkenyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkenyl includes both "unsubstituted alkenyls" and "substituted alkenyls," the latter of which refers to alkenyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

The term "alkynyl" includes unsaturated aliphatic groups analogous in length and possible substitution to the alkyls described above, but which contain at least one triple bond. For example, the term "alkynyl" includes straight-chain alkynyl groups (*e.g.*, ethynyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, octynyl, nonynyl, decynyl, *etc.*), branched-chain alkynyl groups, and cycloalkyl or cycloalkenyl substituted alkynyl groups. In certain embodiments, a straight chain or branched chain alkynyl group has 6 or fewer carbon atoms in its backbone (*e.g.*, C_2-C_6 for straight chain, C_3-C_6 for branched chain). The term C_2-C_6 includes alkynyl groups containing 2 to 6 carbon atoms.

Moreover, unless otherwise specified, the term alkynyl includes both "unsubstituted alkynyls" and "substituted alkynyls," the latter of which refers to alkynyl moieties having independently selected substituents replacing a hydrogen on one or more carbons of the hydrocarbon backbone. Such substituents can include, for example, alkyl groups, alkynyl groups, halogens, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and

alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulfhydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfinyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moiety.

Unless the number of carbons is otherwise specified, "lower alkyl" as used herein means an alkyl group, as defined above, but having from one to five carbon atoms in its backbone structure. "Lower alkenyl" and "lower alkynyl" have chain lengths of, for example, 2-5 carbon atoms.

The term "alkoxy" includes substituted and unsubstituted alkyl, alkenyl, and alkynyl groups covalently linked to an oxygen atom. Examples of alkoxy groups include methoxy, ethoxy, isopropyloxy, propoxy, butoxy, and pentoxy groups. Examples of substituted alkoxy groups include halogenated alkoxy groups. The alkoxy groups can be substituted with independently selected groups such as alkenyl, alkynyl, halogen, hydroxyl, alkylcarbonyloxy, arylcarbonyloxy, alkoxycarbonyloxy, aryloxycarbonyloxy, carboxylate, alkylcarbonyl, arylcarbonyl, alkoxycarbonyl, aminocarbonyl, alkylaminocarbonyl, dialkylaminocarbonyl, alkylthiocarbonyl, alkoxyl, phosphate, phosphonato, phosphinato, cyano, amino (including alkyl amino, dialkylamino, arylamino, diarylamino, and alkylarylamino), acylamino (including alkylcarbonylamino, arylcarbonylamino, carbamoyl and ureido), amidino, imino, sulffiydryl, alkylthio, arylthio, thiocarboxylate, sulfates, alkylsulfmyl, sulfonato, sulfamoyl, sulfonamido, nitro, trifluoromethyl, cyano, azido, heterocyclyl, alkylaryl, or an aromatic or heteroaromatic moieties. Examples of halogen substituted alkoxy groups include, but are not limited to, fluoromethoxy, difluoromethoxy, trifluoromethoxy, chloromethoxy, dichloromethoxy, trichloromethoxy, *etc.*

The term "heteroatom" includes atoms of any element other than carbon or hydrogen. Preferred heteroatoms are nitrogen, oxygen, sulfur and phosphorus.

The term "hydroxy" or "hydroxyl" includes groups with an -OH or $-O^-$ (with an appropriate counterion).

The term "halogen" includes fluorine, bromine, chlorine, iodine, *etc.* The term "perhalogenated" generally refers to a moiety wherein all hydrogens are replaced by halogen atoms.

The term "substituted" includes independently selected substituents which can be placed on the moiety and which allow the molecule to perform its intended function. Examples of substituents include alkyl, alkenyl, alkynyl, aryl, $(CR'R'')_{0-3}NR'R''$, $(CR'R'')_{0-3}CN$, NO_2,

halogen, $(CR'R'')_{0-3}C(halogen)_3$, $(CR'R'')_{0-3}CH(halogen)_2$, $(CR'R'')_{0-3}CH_2(halogen)$, $(CR'R'')_{0-3}CONR'R''$, $(CR'R'')_{0-3}S(O)_{1-2}NR'R''$, $(CR'R'')_{0-3}CHO$, $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$, $(CR'R'')_{0-3}S(O)_{0-2}R'$, $(CR'R'')_{0-3}O(CR'R'')_{0-3}H$, $(CR'R'')_{0-3}COR'$, $(CR'R'')_{0-3}CO_2R'$, or $(CR'R'')_{0-3}OR'$ groups; wherein each R′ and R″ are each independently hydrogen, a C_1-C_5 alkyl, C_2-C_5 alkenyl, C_2-C_5 alkynyl, or aryl group, or R′ and R″ taken together are a benzylidene group or a —$(CH_2)_2O(CH_2)_2$- group.

The term "amine" or "amino" includes compounds or moieties in which a nitrogen atom is covalently bonded to at least one carbon or heteroatom. The term "alkyl amino" includes groups and compounds wherein the nitrogen is bound to at least one additional alkyl group. The term "dialkyl amino" includes groups wherein the nitrogen atom is bound to at least two additional alkyl groups.

The term "ether" includes compounds or moieties which contain an oxygen bonded to two different carbon atoms or heteroatoms. For example, the term includes "alkoxyalkyl," which refers to an alkyl, alkenyl, or alkynyl group covalently bonded to an oxygen atom which is covalently bonded to another alkyl group.

The term "base" includes the known purine and pyrimidine heterocyclic bases, deazapurines, and analogs (including heterocyclic substituted analogs, *e.g.*, aminoethyoxy phenoxazine), derivatives (*e.g.*, 1-alkyl-, 1-alkenyl-, heteroaromatic- and 1-alkynyl derivatives) and tautomers thereof. Examples of purines include adenine, guanine, inosine, diaminopurine, and xanthine and analogs (*e.g.*, 8-oxo-N^6-methyladenine or 7-diazaxanthine) and derivatives thereof. Pyrimidines include, for example, thymine, uracil, and cytosine, and their analogs (*e.g.*, 5-methylcytosine, 5-methyluracil, 5-(1-propynyl)uracil, 5-(1-propynyl)cytosine and 4,4-ethanocytosine). Other examples of suitable bases include non-purinyl and non-pyrimidinyl bases such as 2-aminopyridine and triazines.

In a preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are RNA nucleotides. In another preferred embodiment, the nucleomonomers of an oligonucleotide of the invention are modified RNA nucleotides. Thus, the oligunucleotides contain modified RNA nucleotides.

The term "nucleoside" includes bases which are covalently attached to a sugar moiety, preferably ribose or deoxyribose. Examples of preferred nucleosides include ribonucleosides and deoxyribonucleosides. Nucleosides also include bases linked to amino acids or amino acid analogs which may comprise free carboxyl groups, free amino groups, or protecting groups. Suitable protecting groups are well known in the art (see P. G. M. Wuts and T. W. Greene,

"Protective Groups in Organic Synthesis", 2nd Ed., Wiley-Interscience, New York, 1999).

The term "nucleotide" includes nucleosides which further comprise a phosphate group or a phosphate analog.

As used herein, the term "linkage" includes a naturally occurring, unmodified phosphodiester moiety (-O-(PO^{2-})-O-) that covalently couples adjacent nucleomonomers. As used herein, the term "substitute linkage" includes any analog or derivative of the native phosphodiester group that covalently couples adjacent nucleomonomers. Substitute linkages include phosphodiester analogs, *e.g.*, phosphorothioate, phosphorodithioate, and P-ethyoxyphosphodiester, P-ethoxyphosphodiester, P-alkyloxyphosphotriester, methylphosphonate, and nonphosphorus containing linkages, *e.g.*, acetals and amides. Such substitute linkages are known in the art (*e.g.*, Bjergarde *et al.* 1991. Nucleic Acids Res. 19:5843; Caruthers *et al.* 1991. Nucleosides Nucleotides. 10:47). In certain embodiments, non-hydrolizable linkages are preferred, such as phosphorothiate linkages.

In certain embodiments, oligonucleotides of the invention comprise 3′ and 5′ termini (except for circular oligonucleotides). In one embodiment, the 3′ and 5′ termini of an oligonucleotide can be substantially protected from nucleases *e.g.*, by modifying the 3′ or 5′ linkages (*e.g.*, U.S. Pat. No. 5,849,902 and WO 98/13526). For example, oligonucleotides can be made resistant by the inclusion of a "blocking group." The term "blocking group" as used herein refers to substituents (*e.g.*, other than OH groups) that can be attached to oligonucleotides or nucleomonomers, either as protecting groups or coupling groups for synthesis (*e.g.*, FITC, propyl (CH_2-CH_2-CH_3), glycol (-O-CH_2-CH_2-O-) phosphate (PO_3^{2-}), hydrogen phosphonate, or phosphoramidite). "Blocking groups" also include "end blocking groups" or "exonuclease blocking groups" which protect the 5′ and 3′ termini of the oligonucleotide, including modified nucleotides and non-nucleotide exonuclease resistant structures.

Exemplary end-blocking groups include cap structures (*e.g.*, a 7-methylguanosine cap), inverted nucleomonomers, *e.g.*, with 3′-3′ or 5′-5′ end inversions (see, *e.g.*, Ortiagao *et al.* 1992. *Antisense Res. Dev.* 2:129), methylphosphonate, phosphoramidite, non-nucleotide groups (*e.g.*, non-nucleotide linkers, amino linkers, conjugates) and the like. The 3′ terminal nucleomonomer can comprise a modified sugar moiety. The 3′ terminal nucleomonomer comprises a 3′-O that can optionally be substituted by a blocking group that prevents 3′-exonuclease degradation of the oligonucleotide. For example, the 3′-hydroxyl can be esterified to a nucleotide through a 3′→3′ internucleotide linkage. For example, the alkyloxy radical can be methoxy, ethoxy, or isopropoxy, and preferably, ethoxy. Optionally, the 3′→3′linked nucleotide at the 3′ terminus

can be linked by a substitute linkage. To reduce nuclease degradation, the 5′ most 3′→5′ linkage can be a modified linkage, *e.g.*, a phosphorothioate or a P-alkyloxyphosphotriester linkage. Preferably, the two 5′ most 3′→5′ linkages are modified linkages. Optionally, the 5′ terminal hydroxy moiety can be esterified with a phosphorus containing moiety, *e.g.*, phosphate, phosphorothioate, or P-ethoxyphosphate.

In one embodiment, the sense strand of an oligonucleotide comprises a 5′ group that allows for RNAi activity but which renders the sense strand inactive in terms of gene targeting. Preferably, such a 5′ modifying group is a phosphate group or a group larger than a phosphate group. Oligonucleotides of this type often exhibit increased specificity for a target gene in a cell that corresponds to the nucleotide sequence of the antisense strand. This is because the sense strand in such an oligonucleotide is often rendered incapable of mediating cleavage of any nucleotide sequence it might bind to non-specifically and thus will not inactivate any other genes in the cell. Thus, observed decrease in the expression of a gene within a cell transfected with such an oligonucleotide will often be attributed to the direct or indirect effect of the antisense strand. The term "specificity for a target gene," as used herein means the extent to which an effect of an oligonucleotide on a cell can be attributed directly or indirectly to the inhibition of expression of a target gene by an antisense nucleotide sequence present in said oligonucleotide.

Thus, according to another embodiment, the invention provides a method of increasing the specificity of an oligonucleotide for a target gene in a cell, wherein said oligonucleotide comprises a sense strand and an antisense strand, wherein both the sense strand and the antisense strand are capable of binding to corresponding nucleotide sequences if present in said cell, said method comprising the step of modifying the 5′ terminal hydroxy moiety of said sense strand with a phosphate group or a group larger than a phosphate group prior to contacting said oligonucleotide with said cell so as to render said sense strand incapable of mediating cleavage of any nucleotide sequence it might bind to non-specifically and thus will not inactivate any other genes in the cell.

Another way to increase target gene specificity, or to reduce off-target silencing effect, is to introduce a 2'-modification (such as the 2'-O methyl modification) at a position corresponding to the second 5'-end nucleotide of the Dicer-cleaved 21-mer. Applicants' discovery allows the positioning of this 2'-modification in the Dicer-resistant dsRNA, thus enabling one to design better siRNA constructs with less or no off-target silencing.

In one embodiment, a double-stranded oligonucleotide of the invention can comprise

(*i.e.*, be a duplex of) one nucleic acid molecule which is DNA and one nucleic acid molecule which is RNA. Antisense sequences of the invention can be "chimeric oligonucleotides" which comprise an RNA-like and a DNA-like region.

The language "RNase H activating region" includes a region of an oligonucleotide, *e.g.*, a chimeric oligonucleotide, that is capable of recruiting RNase H to cleave the target RNA strand to which the oligonucleotide binds. Typically, the RNase activating region contains a minimal core (of at least about 3-5, typically between about 3-12, more typically, between about 5-12, and more preferably between about 5-10 contiguous nucleomonomers) of DNA or DNA-like nucleomonomers. (See, *e.g.*, U.S. Pat. No. 5,849,902). Preferably, the RNase H activating region comprises about nine contiguous deoxyribose containing nucleomonomers.

The language "non-activating region" includes a region of an antisense sequence, *e.g.*, a chimeric oligonucleotide, that does not recruit or activate RNase H. Preferably, a non-activating region does not comprise phosphorothioate DNA. The oligonucleotides of the invention comprise at least one non-activating region. In one embodiment, the non-activating region can be stabilized against nucleases or can provide specificity for the target by being complementary to the target and forming hydrogen bonds with the target nucleic acid molecule, which is to be bound by the oligonucleotide.

In one embodiment, at least a portion of the contiguous polynucleotides are linked by a substitute linkage, *e.g.*, a phosphorothioate linkage.

In certain embodiments, most or all of the sense strand nucleotides (2'-modified or not) are linked by phosphorothioate linkages. Such constructs tend to have improved pharmacokinetics due to their higher affinity for serum proteins. The phosphothioate linkages in the sense strand generally do not interfere with guide strand activity, once the latter is loaded into RISC.

Antisense sequences of the present invention may include "morpholino oligonucleotides." Morpholino oligonucleotides are non-ionic and function by an RNase H-independent mechanism. Each of the 4 genetic bases (Adenine, Cytosine, Guanine, and Thymine/Uracil) of the morpholino oligonucleotides is linked to a 6-membered morpholine ring. Morpholino oligonucleotides are made by joining the 4 different subunit types by, *e.g.*, non-ionic phosphorodiamidate inter-subunit linkages. Morpholino oligonucleotides have many advantages including: complete resistance to nucleases (Antisense & Nucl. Acid Drug Dev. 1996. 6:267); predictable targeting (Biochemica Biophysica Acta. 1999. 1489:141); reliable activity in cells (Antisense & Nucl. Acid Drug Dev. 1997. 7:63); excellent sequence specificity

(Antisense & Nucl. Acid Drug Dev. 1997. 7:151); minimal non-antisense activity (Biochemica Biophysica Acta. 1999. 1489:141); and simple osmotic or scrape delivery (Antisense & Nucl. Acid Drug Dev. 1997. 7:291). Morpholino oligonucleotides are also preferred because of their non-toxicity at high doses. A discussion of the preparation of morpholino oligonucleotides can be found in Antisense & Nucl. Acid Drug Dev. 1997. 7:187.

III. *Synthesis*

Oligonucleotides of the invention can be synthesized by any method known in the art, *e.g.*, using enzymatic synthesis and/or chemical synthesis. The oligonucleotides can be synthesized *in vitro* (*e.g.*, using enzymatic synthesis and chemical synthesis) or *in vivo* (using recombinant DNA technology well known in the art).

In a preferred embodiment, chemical synthesis is used for modified polynucleotides. Chemical synthesis of linear oligonucleotides is well known in the art and can be achieved by solution or solid phase techniques. Preferably, synthesis is by solid phase methods. Oligonucleotides can be made by any of several different synthetic procedures including the phosphoramidite, phosphite triester, H-phosphonate, and phosphotriester methods, typically by automated synthesis methods.

Oligonucleotide synthesis protocols are well known in the art and can be found, *e.g.*, in U.S. Pat. No. 5,830,653; WO 98/13526; Stec *et al.* 1984. *J. Am. Chem. Soc.* 106:6077; Stec *et al.* 1985. *J. Org. Chem.* 50:3908; Stec *et al.* J. Chromatog. 1985. 326:263; LaPlanche *et al.* 1986. *Nucl. Acid. Res.* 1986. 14:9081; Fasman G. D., 1989. Practical Handbook of Biochemistry and Molecular Biology. 1989. CRC Press, Boca Raton, Fla.; Lamone. 1993. *Biochem. Soc. Trans.* 21:1; U.S. Pat. No. 5,013,830; U.S. Pat. No. 5,214,135; U.S. Pat. No. 5,525,719; Kawasaki *et al.* 1993. *J. Med. Chem.* 36:831; WO 92/03568; U.S. Pat. No. 5,276,019; and U.S. Pat. No. 5,264,423.

The synthesis method selected can depend on the length of the desired oligonucleotide and such choice is within the skill of the ordinary artisan. For example, the phosphoramidite and phosphite triester method can produce oligonucleotides having 175 or more nucleotides, while the H-phosphonate method works well for oligonucleotides of less than 100 nucleotides. If modified bases are incorporated into the oligonucleotide, and particularly if modified phosphodiester linkages are used, then the synthetic procedures are altered as needed according to known procedures. In this regard, Uhlmann *et al.* (1990, *Chemical Reviews* 90:543-584) provide references and outline procedures for making oligonucleotides with modified bases and

modified phosphodiester linkages. Other exemplary methods for making oligonucleotides are taught in Sonveaux. 1994. "Protecting Groups in Oligonucleotide Synthesis"; Agrawal. *Methods in Molecular Biology* 26:1. Exemplary synthesis methods are also taught in "Oligonucleotide Synthesis - A Practical Approach" (Gait, M. J. IRL Press at Oxford University Press. 1984). Moreover, linear oligonucleotides of defined sequence, including some sequences with modified nucleotides, are readily available from several commercial sources.

The oligonucleotides may be purified by polyacrylamide gel electrophoresis, or by any of a number of chromatographic methods, including gel chromatography and high pressure liquid chromatography. To confirm a nucleotide sequence, especially unmodified nucleotide sequences, oligonucleotides may be subjected to DNA sequencing by any of the known procedures, including Maxam and Gilbert sequencing, Sanger sequencing, capillary electrophoresis sequencing, the wandering spot sequencing procedure or by using selective chemical degradation of oligonucleotides bound to Hybond paper. Sequences of short oligonucleotides can also be analyzed by laser desorption mass spectroscopy or by fast atom bombardment (McNeal, *et al.*, 1982, *J. Am. Chem. Soc.* 104:976; Viari, *et al.*, 1987, *Biomed. Environ. Mass Spectrom.* 14:83; Grotjahn *et al.*, 1982, *Nuc. Acid Res.* 10:4671). Sequencing methods are also available for RNA oligonucleotides.

The quality of oligonucleotides synthesized can be verified by testing the oligonucleotide by capillary electrophoresis and denaturing strong anion HPLC (SAX-HPLC) using, *e.g.*, the method of Bergot and Egan. 1992. *J. Chrom.* 599:35.

Other exemplary synthesis techniques are well known in the art (see, *e.g.*, Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, Second Edition (1989); DNA Cloning, Volumes I and II (DN Glover Ed. 1985); Oligonucleotide Synthesis (M J Gait Ed, 1984; Nucleic Acid Hybridisation (B D Hames and S J Higgins eds. 1984); A Practical Guide to Molecular Cloning (1984); or the series, Methods in Enzymology (Academic Press, Inc.)).

In certain embodiments, the subject RNAi constructs or at least portions thereof are transcribed from expression vectors encoding the subject constructs. Any art recognized vectors may be use for this purpose. The transcribed RNAi constructs may be isolated and purified, before desired modifications (such as replacing an unmodified sense strand with a modified one, *etc.*) are carried out.

IV. *Delivery/Carrier*

Uptake of Oligonucleotides by Cells

Oligonucleotides and oligonucleotide compositions are contacted with (*i.e.*, brought into contact with, also referred to herein as administered or delivered to) and taken up by one or more cells or a cell lysate. The term "cells" includes prokaryotic and eukaryotic cells, preferably vertebrate cells, and, more preferably, mammalian cells. In a preferred embodiment, the oligonucleotide compositions of the invention are contacted with human cells.

Oligonucleotide compositions of the invention can be contacted with cells *in vitro*, *e.g.*, in a test tube or culture dish, (and may or may not be introduced into a subject) or *in vivo*, *e.g.*, in a subject such as a mammalian subject. Oligonucleotides are taken up by cells at a slow rate by endocytosis, but endocytosed oligonucleotides are generally sequestered and not available, *e.g.*, for hybridization to a target nucleic acid molecule. In one embodiment, cellular uptake can be facilitated by electroporation or calcium phosphate precipitation. However, these procedures are only useful for *in vitro* or ex vivo embodiments, are not convenient and, in some cases, are associated with cell toxicity.

In another embodiment, delivery of oligonucleotides into cells can be enhanced by suitable art recognized methods including calcium phosphate, DMSO, glycerol or dextran, electroporation, or by transfection, *e.g.*, using cationic, anionic, or neutral lipid compositions or liposomes using methods known in the art (see *e.g.*, WO 90/14074; WO 91/16024; WO 91/17424; U.S. Pat. No. 4,897,355; Bergan *et al.* 1993. *Nucleic Acids Research.* 21:3567). Enhanced delivery of oligonucleotides can also be mediated by the use of vectors (See *e.g.*, Shi, Y. 2003. Trends Genet 2003 Jan. 19:9; Reichhart J M *et al.* Genesis. 2002. 34(1-2):1604, Yu *et al.* 2002. Proc. Natl. Acad Sci. USA 99:6047; Sui *et al.* 2002. Proc. Natl. Acad Sci. USA 99:5515) viruses, polyamine or polycation conjugates using compounds such as polylysine, protamine, or Ni, N12-bis (ethyl) spermine (see, *e.g.*, Bartzatt, R. *et al.*1989. *Biotechnol. Appl. Biochem.* 11:133; Wagner E. *et al.* 1992. *Proc. Natl. Acad. Sci.* 88:4255).

The optimal protocol for uptake of oligonucleotides will depend upon a number of factors, the most crucial being the type of cells that are being used. Other factors that are important in uptake include, but are not limited to, the nature and concentration of the oligonucleotide, the confluence of the cells, the type of culture the cells are in (*e.g.*, a suspension culture or plated) and the type of media in which the cells are grown.

Conjugating Agents

Conjugating agents bind to the oligonucleotide in a covalent manner. In one embodiment, oligonucleotides can be derivatized or chemically modified by binding to a

conjugating agent to facilitate cellular uptake. For example, covalent linkage of a cholesterol moiety to an oligonucleotide can improve cellular uptake by 5- to 10-fold which in turn improves DNA binding by about 10-fold (Boutorin *et al.*, 1989, *FEBS Letters* 254:129-132). Conjugation of octyl, dodecyl, and octadecyl residues enhances cellular uptake by 3-, 4-, and 10-fold as compared to unmodified oligonucleotides (Vlassov *et al.*, 1994, Biochimica et Biophysica Acta 1197:95-108). Similarly, derivatization of oligonucleotides with poly-L-lysine can aid oligonucleotide uptake by cells (Schell, 1974, *Biochem. Biophys. Acta* 340:323, and Lemaitre *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* 84:648).

Certain protein carriers can also facilitate cellular uptake of oligonucleotides, including, for example, serum albumin, nuclear proteins possessing signals for transport to the nucleus, and viral or bacterial proteins capable of cell membrane penetration. Therefore, protein carriers are useful when associated with or linked to the oligonucleotides. Accordingly, the present invention provides for derivatization of oligonucleotides with groups capable of facilitating cellular uptake, including hydrocarbons and non-polar groups, cholesterol, long chain alcohols (*i.e.*, hexanol), poly-L-lysine and proteins, as well as other aryl or steroid groups and polycations having analogous beneficial effects, such as phenyl or naphthyl groups, quinoline, anthracene or phenanthracene groups, fatty acids, fatty alcohols and sesquiterpenes, diterpenes, and steroids. A major advantage of using conjugating agents is to increase the initial membrane interaction that leads to a greater cellular accumulation of oligonucleotides.

Other conjugating agents include various vitamins, such as fat soluble vitamins, which may be used as a conjugate to deliver RNAi constructs specifically into adipose tissue - the primary location where these vitamins are stored. These vitamin-based conjugating agents may be especially useful for targeting certain metabolic disease targets, such as diabetes / obesity. Of the fat soluble vitamins, such as vitamins A, D, E, K, *etc.*, vitamin K may be preferred in some embodiments, as there is no known upper intake level (although large doses could lead to breakdown of red blood cells and possibly to liver disease). In comparison, vitamins A and D have more defined toxicity and established upper intake levels.

In certain embodiments, gamma carboxyglutamic acid residues may be conjugated to the subject RNAi constructs to increased their membrane stickiness, and/or to slow clearance and improve general uptake (*infra*).

Certain conjugating agents that may be used with the instant constructs include those described in WO04048545A2 and US20040204377A1 (all incorporated herein by their entireties), such as a Tat peptide, a sequence substantially similar to the sequence of SEQ ID

NO: 12 of WO04048545A2 and US20040204377A1, a homeobox (hox) peptide, a MTS, VP22, MPG, at least one dendrimer (such as PAMAM), *etc.*

Other conjugating agents that may be used with the instant constructs include those described in WO07089607A2 (incorporated herein), which describes various nanotransporters and delivery complexes for use in delivery of nucleic acid molecules (such as the subject dsRNA constructs) and/or other pharmaceutical agents *in vivo* and *in vitro*. Using such delivery complexes, the subject dsRNA can be delivered while conjugated or associated with a nanotransporter comprising a core conjugated with at least one functional surface group. The core may be a nanoparticle, such as a dendrimer (*e.g.*, a polylysine dendrimer). The core may also be a nanotube, such as a single walled nanotube or a multi-walled nanotube. The functional surface group is at least one of a lipid, a cell type specific targeting moiety, a fluorescent molecule, and a charge controlling molecule. For example, the targeting moiety may be a tissue-selective peptide. The lipid may be an oleoyl lipid or derivative thereof. Exemplary nanotransporter include NOP-7 or HBOLD.

Encapsulating Agents

Encapsulating agents entrap oligonucleotides within vesicles. In another embodiment of the invention, an oligonucleotide may be associated with a carrier or vehicle, *e.g.*, liposomes or micelles, although other carriers could be used, as would be appreciated by one skilled in the art. Liposomes are vesicles made of a lipid bilayer having a structure similar to biological membranes. Such carriers are used to facilitate the cellular uptake or targeting of the oligonucleotide, or improve the oligonucleotide's pharmacokinetic or toxicologic properties.

For example, the oligonucleotides of the present invention may also be administered encapsulated in liposomes, pharmaceutical compositions wherein the active ingredient is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The oligonucleotides, depending upon solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phopholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, or other materials of a hydrophobic nature. The diameters of the liposomes generally range from about 15 nm to about 5 microns.

The use of liposomes as drug delivery vehicles offers several advantages. Liposomes

increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acids remain biologically active. For example, a lipid delivery vehicle originally designed as a research tool, such as Lipofectin or LIPOFECTAMINE™ 2000, can deliver intact nucleic acid molecules to cells.

Specific advantages of using liposomes include the following: they are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost-effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Complexing Agents

Complexing agents bind to the oligonucleotides of the invention by a strong but non-covalent attraction (*e.g.*, an electrostatic, van der Waals, pi-stacking, *etc.* interaction). In one embodiment, oligonucleotides of the invention can be complexed with a complexing agent to increase cellular uptake of oligonucleotides. An example of a complexing agent includes cationic lipids. Cationic lipids can be used to deliver oligonucleotides to cells.

The term "cationic lipid" includes lipids and synthetic lipids having both polar and non-polar domains and which are capable of being positively charged at or around physiological pH and which bind to polyanions, such as nucleic acids, and facilitate the delivery of nucleic acids into cells. In general cationic lipids include saturated and unsaturated alkyl and alicyclic ethers and esters of amines, amides, or derivatives thereof. Straight-chain and branched alkyl and alkenyl groups of cationic lipids can contain, *e.g.*, from 1 to about 25 carbon atoms. Preferred straight chain or branched alkyl or alkene groups have six or more carbon atoms. Alicyclic groups include cholesterol and other steroid groups. Cationic lipids can be prepared with a variety of counterions (anions) including, *e.g.*, Cl^-, Br^-, I^-, F^-, acetate, trifluoroacetate, sulfate, nitrite, and nitrate.

Examples of cationic lipids include polyethylenimine, polyamidoamine (PAMAM) starburst dendrimers, Lipofectin (a combination of DOTMA and DOPE), Lipofectase, LIPOFECTAMINE™ (*e.g.*, LIPOFECTAMINE™ 2000), DOPE, Cytofectin (Gilead Sciences,

Foster City, Calif.), and Eufectins (JBL, San Luis Obispo, Calif.). Exemplary cationic liposomes can be made from N-[1-(2,3-dioleoloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA), N-[1 -(2,3-dioleoloxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP), 3β-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol), 2,3,-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; and dimethyldioctadecylammonium bromide (DDAB). The cationic lipid N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), for example, was found to increase 1000-fold the antisense effect of a phosphothioate oligonucleotide. (Vlassov *et al.*, 1994, Biochimica et Biophysica Acta 1197:95-108). Oligonucleotides can also be complexed with, *e.g.*, poly (L-lysine) or avidin and lipids may, or may not, be included in this mixture, *e.g.*, steryl-poly (L-lysine).

Cationic lipids have been used in the art to deliver oligonucleotides to cells (see, *e.g.*, U.S. Pat. Nos. 5,855,910; 5,851,548; 5,830,430; 5,780,053; 5,767,099; Lewis *et al.* 1996. *Proc. Natl. Acad. Sci. USA* 93:3176; Hope *et al.* 1998. *Molecular Membrane Biology* 15:1). Other lipid compositions which can be used to facilitate uptake of the instant oligonucleotides can be used in connection with the claimed methods. In addition to those listed supra, other lipid compositions are also known in the art and include, *e.g.*, those taught in U.S. Pat. No. 4,235,871; U.S. Pat. Nos. 4,501,728; 4,837,028; 4,737,323.

In one embodiment lipid compositions can further comprise agents, *e.g.*, viral proteins to enhance lipid-mediated transfections of oligonucleotides (Kamata, *et al.*, 1994. *Nucl. Acids. Res.* 22:536). In another embodiment, oligonucleotides are contacted with cells as part of a composition comprising an oligonucleotide, a peptide, and a lipid as taught, *e.g.*, in U.S. patent 5,736,392. Improved lipids have also been described which are serum resistant (Lewis, *et al.*, 1996. *Proc. Natl. Acad. Sci.* 93:3176). Cationic lipids and other complexing agents act to increase the number of oligonucleotides carried into the cell through endocytosis.

In another embodiment N-substituted glycine oligonucleotides (peptoids) can be used to optimize uptake of oligonucleotides. Peptoids have been used to create cationic lipid-like compounds for transfection (Murphy, *et al.*, 1998. *Proc. Natl. Acad. Sci.* 95:1517). Peptoids can be synthesized using standard methods (*e.g.*, Zuckermann, R. N., *et al.* 1992. *J. Am. Chem. Soc.* 114:10646; Zuckermann, R. N., *et al.* 1992. *Int. J. Peptide Protein Res.* 40:497). Combinations of cationic lipids and peptoids, liptoids, can also be used to optimize uptake of the subject oligonucleotides (Hunag, *et al.*, 1998. *Chemistry and Biology.* 5:345). Liptoids can be

synthesized by elaborating peptoid oligonucleotides and coupling the amino terminal submonomer to a lipid via its amino group (Hunag, *et al.*, 1998. *Chemistry and Biology.* 5:345).

It is known in the art that positively charged amino acids can be used for creating highly active cationic lipids (Lewis *et al.* 1996. *Proc. Natl. Acad. Sci. US.A.* 93:3176). In one embodiment, a composition for delivering oligonucleotides of the invention comprises a number of arginine, lysine, histidine or ornithine residues linked to a lipophilic moiety (see *e.g.*, U.S. Pat. No. 5,777,153).

In another embodiment, a composition for delivering oligonucleotides of the invention comprises a peptide having from between about one to about four basic residues. These basic residues can be located, *e.g.*, on the amino terminal, C-terminal, or internal region of the peptide. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine (can also be considered non-polar), asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Apart from the basic amino acids, a majority or all of the other residues of the peptide can be selected from the non-basic amino acids, *e.g.*, amino acids other than lysine, arginine, or histidine. Preferably a preponderance of neutral amino acids with long neutral side chains are used.

In one embodiment, a composition for delivering oligonucleotides of the invention comprises a natural or synthetic polypeptide having one or more gamma carboxyglutamic acid residues, or γ-Gla residues. These gamma carboxyglutamic acid residues may enable the polypeptide to bind to each other and to membrane surfaces. In other words, a polypeptide having a series of γ-Gla may be used as a general delivery modality that helps an RNAi construct to stick to whatever membrane to which it comes in contact. This may at least slow RNAi constructs from being cleared from the blood stream and enhance their chance of homing to the target.

The gamma carboxyglutamic acid residues may exist in natural proteins (for example, prothrombin has 10 γ-Gla residues). Alternatively, they can be introduced into the purified, recombinantly produced, or chemically synthesized polypeptides by carboxylation using, for example, a vitamin K-dependent carboxylase. The gamma carboxyglutamic acid residues may be consecutive or non-consecutive, and the total number and location of such gamma

carboxyglutamic acid residues in the polypeptide can be regulated / fine tuned to achieve different levels of "stickiness"of the polypeptide.

In one embodiment, the cells to be contacted with an oligonucleotide composition of the invention are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 12 hours to about 24 hours. In another embodiment, the cells to be contacted with an oligonucleotide composition are contacted with a mixture comprising the oligonucleotide and a mixture comprising a lipid, *e.g.*, one of the lipids or lipid compositions described supra for between about 1 and about five days. In one embodiment, the cells are contacted with a mixture comprising a lipid and the oligonucleotide for between about three days to as long as about 30 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about five to about 20 days. In another embodiment, a mixture comprising a lipid is left in contact with the cells for at least about seven to about 15 days.

For example, in one embodiment, an oligonucleotide composition can be contacted with cells in the presence of a lipid such as cytofectin CS or GSV (available from Glen Research; Sterling, Va.), GS3815, GS2888 for prolonged incubation periods as described herein.

In one embodiment, the incubation of the cells with the mixture comprising a lipid and an oligonucleotide composition does not reduce the viability of the cells. Preferably, after the transfection period the cells are substantially viable. In one embodiment, after transfection, the cells are between at least about 70% and at least about 100% viable. In another embodiment, the cells are between at least about 80% and at least about 95% viable. In yet another embodiment, the cells are between at least about 85% and at least about 90% viable.

In one embodiment, oligonucleotides are modified by attaching a peptide sequence that transports the oligonucleotide into a cell, referred to herein as a "transporting peptide." In one embodiment, the composition includes an oligonucleotide which is complementary to a target nucleic acid molecule encoding the protein, and a covalently attached transporting peptide.

The language "transporting peptide" includes an amino acid sequence that facilitates the transport of an oligonucleotide into a cell. Exemplary peptides which facilitate the transport of the moieties to which they are linked into cells are known in the art, and include, *e.g.*, HIV TAT transcription factor, lactoferrin, Herpes VP22 protein, and fibroblast growth factor 2 (Pooga *et al.* 1998. *Nature Biotechnology.* 16:857; and Derossi *et al.* 1998. *Trends in Cell Biology.* 8:84; Elliott and O'Hare. 1997. Cell 88:223).

Oligonucleotides can be attached to the transporting peptide using known techniques,

e.g., (Prochiantz, A. 1996. *Curr. Opin. Neurobiol.* 6:629; Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Troy *et al.* 1996. *J. Neurosci.* 16:253), Vives *et al.* 1997. *J. Biol. Chem.* 272:16010). For example, in one embodiment, oligonucleotides bearing an activated thiol group are linked via that thiol group to a cysteine present in a transport peptide (*e.g.*, to the cysteine present in the β turn between the second and the third helix of the antennapedia homeodomain as taught, *e.g.*, in Derossi *et al.* 1998. *Trends Cell Biol.* 8:84; Prochiantz. 1996. *Current Opinion in Neurobiol.* 6:629; Allinquant *et al.* 1995. J Cell Biol. 128:919). In another embodiment, a Boc-Cys-(Npys)OH group can be coupled to the transport peptide as the last (N-terminal) amino acid and an oligonucleotide bearing an SH group can be coupled to the peptide (Troy *et al.* 1996. *J. Neurosci.* 16:253).

In one embodiment, a linking group can be attached to a nucleomonomer and the transporting peptide can be covalently attached to the linker. In one embodiment, a linker can function as both an attachment site for a transporting peptide and can provide stability against nucleases. Examples of suitable linkers include substituted or unsubstituted C_1-C_{20} alkyl chains, C_2-C_{20} alkenyl chains, C_2-C_{20} alkynyl chains, peptides, and heteroatoms (*e.g.*, S, O, NH, *etc.*). Other exemplary linkers include bifinctional crosslinking agents such as sulfosuccinimidyl-4-(maleimidophenyl)-butyrate (SMPB) (see, *e.g.*, Smith *et al.* Biochem J 1991.276: 417-2).

In one embodiment, oligonucleotides of the invention are synthesized as molecular conjugates which utilize receptor-mediated endocytotic mechanisms for delivering genes into cells (see, *e.g.*, Bunnell *et al.* 1992. *Somatic Cell and Molecular Genetics.* 18:559, and the references cited therein).

Targeting Agents

The delivery of oligonucleotides can also be improved by targeting the oligonucleotides to a cellular receptor. The targeting moieties can be conjugated to the oligonucleotides or attached to a carrier group (*i.e.*, poly(L-lysine) or liposomes) linked to the oligonucleotides. This method is well suited to cells that display specific receptor-mediated endocytosis.

For instance, oligonucleotide conjugates to 6-phosphomannosylated proteins are internalized 20-fold more efficiently by cells expressing mannose 6-phosphate specific receptors than free oligonucleotides. The oligonucleotides may also be coupled to a ligand for a cellular receptor using a biodegradable linker. In another example, the delivery construct is mannosylated streptavidin which forms a tight complex with biotinylated oligonucleotides. Mannosylated streptavidin was found to increase 20-fold the internalization of biotinylated

oligonucleotides. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

In addition specific ligands can be conjugated to the polylysine component of polylysine-based delivery systems. For example, transferrin-polylysine, adenovirus-polylysine, and influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides-polylysine conjugates greatly enhance receptor-mediated DNA delivery in eucaryotic cells. Mannosylated glycoprotein conjugated to poly(L-lysine) in aveolar macrophages has been employed to enhance the cellular uptake of oligonucleotides. Liang *et al.* 1999. *Pharmazie* 54:559-566.

Because malignant cells have an increased need for essential nutrients such as folic acid and transferrin, these nutrients can be used to target oligonucleotides to cancerous cells. For example, when folic acid is linked to poly(L-lysine) enhanced oligonucleotide uptake is seen in promyelocytic leukaemia (HL-60) cells and human melanoma (M-14) cells. Ginobbi *et al.* 1997. *Anticancer Res.* 17:29. In another example, liposomes coated with maleylated bovine serum albumin, folic acid, or ferric protoporphyrin IX, show enhanced cellular uptake of oligonucleotides in murine macrophages, KB cells, and 2.2.15 human hepatoma cells. Liang *et al.* 1999. *Pharmazie* 54:559-566.

Liposomes naturally accumulate in the liver, spleen, and reticuloendothelial system (so-called, passive targeting). By coupling liposomes to various ligands such as antibodies are protein A, they can be actively targeted to specific cell populations. For example, protein A-bearing liposomes may be pretreated with H-2K specific antibodies which are targeted to the mouse major histocompatibility complex-encoded H-2K protein expressed on L cells. (Vlassov *et al.* 1994. *Biochimica et Biophysica Acta* 1197:95-108).

V. *Administration*

The optimal course of administration or delivery of the oligonucleotides may vary depending upon the desired result and/or on the subject to be treated. As used herein "administration" refers to contacting cells with oligonucleotides and can be performed *in vitro* or *in vivo*. The dosage of oligonucleotides may be adjusted to optimally reduce expression of a protein translated from a target nucleic acid molecule, *e.g.*, as measured by a readout of RNA stability or by a therapeutic response, without undue experimentation.

For example, expression of the protein encoded by the nucleic acid target can be measured to determine whether or not the dosage regimen needs to be adjusted accordingly. In addition, an increase or decrease in RNA or protein levels in a cell or produced by a cell can be measured using any art recognized technique. By determining whether transcription has been

decreased, the effectiveness of the oligonucleotide in inducing the cleavage of a target RNA can be determined.

Any of the above-described oligonucleotide compositions can be used alone or in conjunction with a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" includes appropriate solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, it can be used in the therapeutic compositions. Supplementary active ingredients can also be incorporated into the compositions.

Oligonucleotides may be incorporated into liposomes or liposomes modified with polyethylene glycol or admixed with cationic lipids for parenteral administration. Incorporation of additional substances into the liposome, for example, antibodies reactive against membrane proteins found on specific target cells, can help target the oligonucleotides to specific cell types.

Moreover, the present invention provides for administering the subject oligonucleotides with an osmotic pump providing continuous infusion of such oligonucleotides, for example, as described in Rataiczak *et al.* (1992 *Proc. Natl. Acad. Sci. USA* 89:11823-11827). Such osmotic pumps are commercially available, *e.g.*, from Alzet Inc. (Palo Alto, Calif.). Topical administration and parenteral administration in a cationic lipid carrier are preferred.

With respect to *in vivo* applications, the formulations of the present invention can be administered to a patient in a variety of forms adapted to the chosen route of administration, *e.g.*, parenterally, orally, or intraperitoneally. Parenteral administration, which is preferred, includes administration by the following routes: intravenous; intramuscular; interstitially; intraarterially; subcutaneous; intra ocular; intrasynovial; trans epithelial, including transdermal; pulmonary via inhalation; ophthalmic; sublingual and buccal; topically, including ophthalmic; dermal; ocular; rectal; and nasal inhalation via insufflation.

Pharmaceutical preparations for parenteral administration include aqueous solutions of the active compounds in water-soluble or water-dispersible form. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, or dextran, optionally, the suspension may also contain

stabilizers. The oligonucleotides of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligonucleotides may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

Pharmaceutical preparations for topical administration include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids and powders. In addition, conventional pharmaceutical carriers, aqueous, powder or oily bases, or thickeners may be used in pharmaceutical preparations for topical administration.

Pharmaceutical preparations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. In addition, thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, or binders may be used in pharmaceutical preparations for oral administration.

For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligonucleotides are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligonucleotides of the invention are formulated into ointments, salves, gels, or creams as known in the art.

Drug delivery vehicles can be chosen *e.g.*, for *in vitro*, for systemic, or for topical administration. These vehicles can be designed to serve as a slow release reservoir or to deliver their contents directly to the target cell. An advantage of using some direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs that would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

The described oligonucleotides may be administered systemically to a subject. Systemic absorption refers to the entry of drugs into the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, and intranasal. Each of these administration routes delivers the oligonucleotide to accessible diseased cells. Following subcutaneous administration, the therapeutic agent drains into local lymph nodes and proceeds through the lymphatic network

into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the oligonucleotide at the lymph node. The oligonucleotide can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified oligonucleotide into the cell.

The chosen method of delivery will result in entry into cells. Preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, and other pharmaceutically applicable vehicles, and microinjection or electroporation (for ex vivo treatments).

The pharmaceutical preparations of the present invention may be prepared and formulated as emulsions. Emulsions are usually heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. The emulsions of the present invention may contain excipients such as emulsifiers, stabilizers, dyes, fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives, and anti-oxidants may also be present in emulsions as needed. These excipients may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase.

Examples of naturally occurring emulsifiers that may be used in emulsion formulations of the present invention include lanolin, beeswax, phosphatides, lecithin and acacia. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. Examples of finely divided solids that may be used as emulsifiers include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montrnorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

Examples of preservatives that may be included in the emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Examples of antioxidants that may be included in the emulsion formulations include free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

In one embodiment, the compositions of oligonucleotides are formulated as

microemulsions. A microemulsion is a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution. Typically microemulsions are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a 4th component, generally an intermediate chain-length alcohol to form a transparent system.

Surfactants that may be used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (S0750), decaglycerol decaoleate (DA0750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules.

Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C_8-C_{12}) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C_8-C_{10} glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both oil/water and water/oil) have been proposed to enhance the oral bioavailability of drugs.

Microemulsions offer improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, Pharmaceutical Research, 1994, 11:1385; Ho *et al.*, J. Pharm. Sci., 1996, 85:138-143). Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic

absorption of oligonucleotides from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

In an embodiment, the present invention employs various penetration enhancers to affect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to increasing the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also act to enhance the permeability of lipophilic drugs.

Five categories of penetration enhancers that may be used in the present invention include: surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Other agents may be utilized to enhance the penetration of the administered oligonucleotides include: glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-15 pyrrol, azones, and terpenes such as limonene, and menthone.

The oligonucleotides, especially in lipid formulations, can also be administered by coating a medical device, for example, a catheter, such as an angioplasty balloon catheter, with a cationic lipid formulation. Coating may be achieved, for example, by dipping the medical device into a lipid formulation or a mixture of a lipid formulation and a suitable solvent, for example, an aqueous-based buffer, an aqueous solvent, ethanol, methylene chloride, chloroform and the like. An amount of the formulation will naturally adhere to the surface of the device which is subsequently administered to a patient, as appropriate. Alternatively, a lyophilized mixture of a lipid formulation may be specifically bound to the surface of the device. Such binding techniques are described, for example, in K. Ishihara *et al.*, Journal of Biomedical Materials Research, Vol. 27, pp. 1309-1314 (1993), the disclosures of which are incorporated herein by reference in their entirety.

The useful dosage to be administered and the particular mode of administration will vary depending upon such factors as the cell type, or for *in vivo* use, the age, weight and the particular animal and region thereof to be treated, the particular oligonucleotide and delivery method used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or liposome, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased until the desired effect is achieved. When lipids are used to deliver the oligonucleotides, the amount of lipid compound that is administered can vary and generally depends upon the amount of oligonucleotide agent being administered. For example, the weight ratio of lipid compound to oligonucleotide agent is

preferably from about 1:1 to about 15:1, with a weight ratio of about 5:1 to about 10:1 being more preferred. Generally, the amount of cationic lipid compound which is administered will vary from between about 0.1 milligram (mg) to about 1 gram (g). By way of general guidance, typically between about 0.1 mg and about 10 mg of the particular oligonucleotide agent, and about 1 mg to about 100 mg of the lipid compositions, each per kilogram of patient body weight, is administered, although higher and lower amounts can be used.

The agents of the invention are administered to subjects or contacted with cells in a biologically compatible form suitable for pharmaceutical administration. By "biologically compatible form suitable for administration" is meant that the oligonucleotide is administered in a form in which any toxic effects are outweighed by the therapeutic effects of the oligonucleotide. In one embodiment, oligonucleotides can be administered to subjects. Examples of subjects include mammals, *e.g.*, humans and other primates; cows, pigs, horses, and farming (agricultural) animals; dogs, cats, and other domesticated pets; mice, rats, and transgenic non-human animals.

Administration of an active amount of an oligonucleotide of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, an active amount of an oligonucleotide may vary according to factors such as the type of cell, the oligonucleotide used, and for *in vivo* uses the disease state, age, sex, and weight of the individual, and the ability of the oligonucleotide to elicit a desired response in the individual. Establishment of therapeutic levels of oligonucleotides within the cell is dependent upon the rates of uptake and efflux or degradation. Decreasing the degree of degradation prolongs the intracellular half-life of the oligonucleotide. Thus, chemically-modified oligonucleotides, *e.g.*, with modification of the phosphate backbone, may require different dosing.

The exact dosage of an oligonucleotide and number of doses administered will depend upon the data generated experimentally and in clinical trials. Several factors such as the desired effect, the delivery vehicle, disease indication, and the route of administration, will affect the dosage. Dosages can be readily determined by one of ordinary skill in the art and formulated into the subject pharmaceutical compositions. Preferably, the duration of treatment will extend at least through the course of the disease symptoms.

Dosage regima may be adjusted to provide the optimum therapeutic response. For example, the oligonucleotide may be repeatedly administered, *e.g.*, several doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of

the therapeutic situation. One of ordinary skill in the art will readily be able to determine appropriate doses and schedules of administration of the subject oligonucleotides, whether the oligonucleotides are to be administered to cells or to subjects.

VI. *Assays of Oligonucleotide Stability*

Preferably, the double-stranded oligonucleotides of the invention are stabilized, *i.e.*, substantially resistant to endonuclease and exonuclease degradation. An oligonucleotide is defined as being substantially resistant to nucleases when it is at least about 3-fold more resistant to attack by an endogenous cellular nuclease, and is highly nuclease resistant when it is at least about 6-fold more resistant than a corresponding, single-stranded oligonucleotide. This can be demonstrated by showing that the oligonucleotides of the invention are substantially resistant to nucleases using techniques which are known in the art.

One way in which substantial stability can be demonstrated is by showing that the oligonucleotides of the invention function when delivered to a cell, *e.g.*, that they reduce transcription or translation of target nucleic acid molecules, *e.g.*, by measuring protein levels or by measuring cleavage of mRNA. Assays which measure the stability of target RNA can be performed at about 24 hours post-transfection (*e.g.*, using Northern blot techniques, RNase Protection Assays, or QC-PCR assays as known in the art). Alternatively, levels of the target protein can be measured. Preferably, in addition to testing the RNA or protein levels of interest, the RNA or protein levels of a control, non-targeted gene will be measured (*e.g.*, actin, or preferably a control with sequence similarity to the target) as a specificity control. RNA or protein measurements can be made using any art-recognized technique. Preferably, measurements will be made beginning at about 16-24 hours post transfection. (M. Y. Chiang, *et al.* 1991. J Biol Chem. 266:18162-71; T. Fisher, *et al.* 1993. Nucleic Acids Research. 21 3857).

The ability of an oligonucleotide composition of the invention to inhibit protein synthesis can be measured using techniques which are known in the art, for example, by detecting an inhibition in gene transcription or protein synthesis. For example, Nuclease S1 mapping can be performed. In another example, Northern blot analysis can be used to measure the presence of RNA encoding a particular protein. For example, total RNA can be prepared over a cesium chloride cushion (see, *e.g.*, Ausebel *et al.*, 1987. Current Protocols in Molecular Biology (Greene & Wiley, New York)). Northern blots can then be made using the RNA and probed (see, *e.g.*, Id.). In another example, the level of the specific mRNA produced by the target protein can be measured, *e.g.*, using PCR. In yet another example, Western blots can be used to

measure the amount of target protein present. In still another embodiment, a phenotype influenced by the amount of the protein can be detected. Techniques for performing Western blots are well known in the art, see, *e.g.*, Chen *et al.* J. Biol. Chem. 271:28259.

In another example, the promoter sequence of a target gene can be linked to a reporter gene and reporter gene transcription (*e.g.*, as described in more detail below) can be monitored. Alternatively, oligonucleotide compositions that do not target a promoter can be identified by fusing a portion of the target nucleic acid molecule with a reporter gene so that the reporter gene is transcribed. By monitoring a change in the expression of the reporter gene in the presence of the oligonucleotide composition, it is possible to determine the effectiveness of the oligonucleotide composition in inhibiting the expression of the reporter gene. For example, in one embodiment, an effective oligonucleotide composition will reduce the expression of the reporter gene.

A "reporter gene" is a nucleic acid that expresses a detectable gene product, which may be RNA or protein. Detection of mRNA expression may be accomplished by Northern blotting and detection of protein may be accomplished by staining with antibodies specific to the protein. Preferred reporter genes produce a readily detectable product. A reporter gene may be operably linked with a regulatory DNA sequence such that detection of the reporter gene product provides a measure of the transcriptional activity of the regulatory sequence. In preferred embodiments, the gene product of the reporter gene is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detectable signal based on color, fluorescence, or luminescence. Examples of reporter genes include, but are not limited to, those coding for chloramphenicol acetyl transferase (CAT), luciferase, beta-galactosidase, and alkaline phosphatase.

One skilled in the art would readily recognize numerous reporter genes suitable for use in the present invention. These include, but are not limited to, chloramphenicol acetyltransferase (CAT), luciferase, human growth hormone (hGH), and beta-galactosidase. Examples of such reporter genes can be found in F. A. Ausubel *et al.*, Eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, (1989). Any gene that encodes a detectable product, *e.g.*, any product having detectable enzymatic activity or against which a specific antibody can be raised, can be used as a reporter gene in the present methods.

One reporter gene system is the firefly luciferase reporter system. (Gould, S. J., and Subramani, S. 1988. Anal. Biochem., 7:404-408 incorporated herein by reference). The luciferase assay is fast and sensitive. In this assay, a lysate of the test cell is prepared and

combined with ATP and the substrate luciferin. The encoded enzyme luciferase catalyzes a rapid, ATP dependent oxidation of the substrate to generate a light-emitting product. The total light output is measured and is proportional to the amount of luciferase present over a wide range of enzyme concentrations.

CAT is another frequently used reporter gene system; a major advantage of this system is that it has been an extensively validated and is widely accepted as a measure of promoter activity. (Gorman C. M., Moffat, L. F., and Howard, B. H. 1982. Mol. Cell. Biol., 2:1044-1051). In this system, test cells are transfected with CAT expression vectors and incubated with the candidate substance within 2-3 days of the initial transfection. Thereafter, cell extracts are prepared. The extracts are incubated with acetyl CoA and radioactive chloramphenicol. Following the incubation, acetylated chloramphenicol is separated from nonacetylated form by thin layer chromatography. In this assay, the degree of acetylation reflects the CAT gene activity with the particular promoter.

Another suitable reporter gene system is based on immunologic detection of hGH. This system is also quick and easy to use. (Selden, R., Burke-Howie, K. Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986), Mol. Cell, Biol., 6:3173-3179 incorporated herein by reference). The hGH system is advantageous in that the expressed hGH polypeptide is assayed in the media, rather than in a cell extract. Thus, this system does not require the destruction of the test cells. It will be appreciated that the principle of this reporter gene system is not limited to hGH but rather adapted for use with any polypeptide for which an antibody of acceptable specificity is available or can be prepared.

In one embodiment, nuclease stability of a double-stranded oligonucleotide of the invention is measured and compared to a control, *e.g.*, an RNAi molecule typically used in the art (*e.g.*, a duplex oligonucleotide of less than 25 nucleotides in length and comprising 2 nucleotide base overhangs) or an unmodified RNA duplex with blunt ends.

VII. *Therapeutic use*

By inhibiting the expression of a gene, the oligonucleotide compositions of the present invention can be used to treat any disease involving the expression of a protein. Examples of diseases that can be treated by oligonucleotide compositions, just to illustrate, include: cancer, retinopathies, autoimmune diseases, inflammatory diseases (*i.e.*, ICAM-1 related disorders, Psoriasis, Ulcerative Colitus, Crohn's disease), viral diseases (*i.e.*, HIV, Hepatitis C), and cardiovascular diseases.

In one embodiment, *in vitro* treatment of cells with oligonucleotides can be used for ex vivo therapy of cells removed from a subject (*e.g.*, for treatment of leukemia or viral infection) or for treatment of cells which did not originate in the subject, but are to be administered to the subject (*e.g.*, to eliminate transplantation antigen expression on cells to be transplanted into a subject). In addition, *in vitro* treatment of cells can be used in non-therapeutic settings, *e.g.*, to evaluate gene function, to study gene regulation and protein synthesis or to evaluate improvements made to oligonucleotides designed to modulate gene expression or protein synthesis. *In vivo* treatment of cells can be useful in certain clinical settings where it is desirable to inhibit the expression of a protein. There are numerous medical conditions for which antisense therapy is reported to be suitable (see, *e.g.*, U.S. Pat. No. 5,830,653) as well as respiratory syncytial virus infection (WO 95/22,553) influenza virus (WO 94/23,028), and malignancies (WO 94/08,003). Other examples of clinical uses of antisense sequences are reviewed, *e.g.*, in Glaser. 1996. *Genetic Engineering News* 16:1. Exemplary targets for cleavage by oligonucleotides include, *e.g.*, protein kinase Ca, ICAM-1, c-raf kinase, p53, c-myb, and the bcr/abl fusion gene found in chronic myelogenous leukemia.

Mututions in SOD1 cause certain forms of familial ALS disease. Inhibition of SOD1 expression by antisense oligonucleotides or RNAi has been shown to slow the progression of ALS-like symtoms in animal model. The highly active, nuclease stable and specific compounds that are the subject of this invention are well suited for therapeutic application to ALS.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual,* 2nd Ed., ed. by Sambrook, J. *et al.* (Cold Spring Harbor Laboratory Press (1989)); *Short Protocols in Molecular Biology,* 3rd Ed., ed. by Ausubel, F. *et al.* (Wiley, N.Y. (1995)); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. (1984)); Mullis *et al.* U.S. Pat. No. 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. (1984)); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London (1987)); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds. (1986)); and Miller, J. *Experiments in Molecular Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1972)).

Examples

The invention now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Although most of the examples below use the SOD1 and/or PPIB genes as the target genes, the methods and reagents of the invention are generally applicable to any other genes, and are not so limited. For simplicity, the subject RNAi constructs are sometimes referred-to as "Alternate RNAi Compound" in the examples below.

Example 1 Alternate RNAi Compound is as Effective or Superior Compared to Standard siRNAs *in vitro*

In order to demonstrate that the subject modified RNAi constructs ("Alternate RNAi Compounds") are at least as effective, if not superior, when compared to standard siRNAs or siRNA with different chemical modification, the following side-by-side comparison experiment was conducted.

Alternate RNAi Compounds (5 nM) targeting SOD1 were reverse transfected into HEK293 cells in suspension, using LIPOFECTAMINE™ RNAiMAX (Invitrogen) as a transfection agent. Cells were lysed at 24 hrs, and SOD1 mRNA levels were quantified using a bDNA assay (Panomics QUANTIGENE®).

The bDNA assay is a sandwich nucleic acid hybridization method that uses bDNA molecules to amplify signal from captured target RNA. According to the manufacturer, bDNA technology forms the basis of the FDA-approved clinical diagnostic VERSANT 3.0 assays for HIV, HCV and HBV viral load, that are offered commercially by Siemens and have been in use for over a decade. Another advantage of bDNA assays is that RNA is measured directly from the sample source, without RNA purification or enzymatic manipulation, thereby avoiding inefficiencies and variability introduced by or errors inherent to these processes.

In this assay, SOD1 mRNA was normalized to Cyclophilin B (PPIB) mRNA. As shown in Figure 2, "R1 original chemistry" refers to double stranded 21-mer RNAi construct hybridizing to the SOD1 mRNA starting at nucleotide 436 of NCBI RefSeq ID NM_000454. The 5'-end of both the sense and antisense strands are phosphorylated, and the terminal

nucleotides for both strands are modified by 2'-fluoro and/or phosphothioate linkage. See the sequence indicated in Figure 1 as ID No. 10105.

The modified 2'-OMe version of R1 is indicated in Figure 1 as ID number 10104, in which 2'-F nucleotides of 10105 have been replaced with 2'-O-Me (ID No. 10104).

ID No. 10023 is a 25-mer blunt-ended Alternate RNAi Compound hybridizing to the SOD1 mRNA starting at nucleotide 434 (*i.e.*, adding two nucleotides to each end of the 21-mer R1 sequence). The 4 nucleotides at the most 5'-end and the 4 nucleotides at the most 3'-end are modified by 2'-O-methyl groups (and the antisense strand is not modified).

"MM ctrls" with related chemistries were used for the siRNAs or Alternate RNAi Compounds and shown as an average. As shown in Figure 2, applying 2'OMe chemistry and/or extending the length of the R1 sequence trends toward an improvement of activity. This can be beneficial, not only because the cost of making 2'OMe modification is generally less than making 2'-F modification, but also because siRNAs with 2'F modifications have resulted in toxicity in animals whereas an Alternate RNAi Compound with 2'OMe does not exhibit a toxicity even at a higher dose.

Thus, Alternate RNAi Compound is at least as effective as or superior compared to prior art 2'-F modified siRNAs *in vitro*.

Example 2 Activity of Exemplary SOD1 Alternate RNAi Compounds *in vitro*

This example demonstrates that some target regions on a target gene (in this case, the SOD1 gene) may be better target sites than other.

Different 25-mer Alternate RNAi Compounds (5 nM) targeting SOD1 were reverse transfected into HEK293 cells in suspension using LIPOFECTAMINE™ RNAiMAX (Invitrogen) as a transfection agent. Cells were lysed at 24 hrs and SOD1 mRNA levels were quantified using a bDNA assay (Panomics QUANTIGENE®). SOD1 mRNA was normalized to Cyclophilin B (PPIB) mRNA. MM-10025 indicates chemistry-matched mismatch control.

Although all the Alternate RNAi Compounds test here have the same modification chemistry (*e.g.*, sense strand modified at both ends with 2'-O-methyl groups), seven Alternate RNAi Compounds in the primary screen reduced normalized SOD1 levels by $\geq$ 90% compared to treatment with a chemistry-matched control, while the majority of the other Alternate RNAi Compounds reduced normalized SOD1 levels by $\geq$ 60%. See Figure 3.

Example 3 Alternate RNAi Compounds Against SOD1

This example demonstrates that certain subject Alternate RNAi Compounds (25-mer with blunt ends, with four 2'OMe on each end of the sense strand) are effective for SOD1 expression knock down.

Eight Alternate RNAi Compounds (5 nM) targeting human SOD1 were reverse transfected into HEK293 cells in suspension using LIPOFECTAMINE™ RNAiMAX (Invitrogen) as a transfection agent. Cells were lysed at 24 hrs and SOD1 mRNA levels were quantified using a bDNA assay (QUANTIGENE®). SOD1 mRNA levels were normalized to Cyclophilin B (PPIB) mRNA and made relative to a chemistry-matched control set at 100. MM-1025 is the chemistry-matched control in Alternate RNAi Compound chemistry. In addition, activity of R1 (modified 21-mer siRNA sequence and chemistry configuration, see Example 1) is given for comparison. MM-10032 is a chemistry-matched control for R1.

As shown in Figure 4, the subject Alternate RNAi Compounds had excellent activity against the SOD1 target gene. Activity was improved over the 21-mer modified duplex R1. These sequences also maintained activity against the mouse SOD1 gene.

Example 4 Dose Response Analysis for SOD1 Alternate RNAi Compounds.

HEK293 cells were transfected with Alternate RNAi Compounds targeting SOD1 using concentrations from 0.01 nM to 10 nM. In these studies, total duplex concentration at each concentration was maintained at 25 nM by the addition of non-targeting, filler RNA duplex species to the transfection reaction. This method enabled testing of very low concentrations of targeting duplex in a way that maintained transfection of the cells based on recommendations from the manufacturers. Human SOD1 mRNA was measured at 48 hrs post-transfection by QuantiGene bDNA Assay. Alternate RNAi Compound 10014 has been tested multiple times in previous studies, and was included here as a control for comparison. The other duplexes tested in this study were identified as hits in single concentration experiments and were here followed up for EC_{50} determination. Duplexes 10011, 10014 and 10097 are mouse/human homologous and have confirmed activity in mouse and human cell culture. The activity of duplexes 10089 and 10097 has not yet been confirmed in mouse cells, but these duplexes have human/mouse sequence homology.

Dose titration studies using optimized transfection conditions allow direct comparison of duplex potencies. Particularly active duplexes were identified in these studies with EC_{50} values improved over the original hits and with values <50 pM (see Figure 5A).

Figure 5B shows an exemplary dose titration curve comparison between an unmodified,

25-bp, blunt-ended duplex (filled circle) or a subject 25-bp, blunt-ended duplex with "4/4" 2'-O-Me passenger strand modifications (open square). The human HEK293 cells were separately transfected with either construct. Both RNA duplexes target the SOD1 gene and were designed to a region of the gene that is homologous in both human and murine cells. Cells were lysed 48 h after transfection and mRNA levels were measured using a bDNA hybridization assay as described herein. The EC_{50} values for the unmodified 25-bp duplex and "4/4" 2'-O-Me chemistry 25-bp duplex are 0.072 nM ± 20 nM and 0.062 nM ± 0.031 nM, respectively.

Figure 5C shows essentially the same experiment conducted in murine NIH3T3 cells. The EC_{50} values for the unmodified 25-bp duplex and "4/4" 2'-O-Me chemistry 25-bp duplex are 0.079 nM ± 0.042 nM and 0.037 nM ± 0.013 nM, respectively.

These experiments show that silencing activity of the subject 25-bp modified RNA duplexes is consistently in the picomolar concentration range, and is equivalent to the silencing activity of an unmodified 25-bp duplex of the same sequence.

Example 5 Alternate RNAi Compounds with High Potency against PPIB

The result in Example 4 is neither site-specific nor gene specific, since potent gene silencing activity is also observed when comparing modified and unmodified duplexes targeting other sites in the SOD1 gene or the cyclophillin B (PPIB) gene, a ubiquitous gene expressed in most (if not all) tissues.

HEK293 cells were transfected with siRNA or Alternate RNAi Compound and incubated for 24 hours. Gene expression was measured using a bDNA assay (Panomics) with probes specific to human PPIB or SOD1. Analysis was performed by normalizing PPIB expression to SOD1 expression (control gene). The percent PPIB knockdown was adjusted using the negative control of Luciferase siRNA or Alternate RNAi Compound.

Figure 6A shows that the 25-mer Alternate RNAi Compound ID NO. 10457 (blunt-ended 25-mer with 4 2'OMe at each end of sense strand - "4/4") targeting PPIB maintains or improves upon the high potency demonstrated for an unmodified 21-mer siRNA. Construct 10167 is an unmodified 21-mer siRNA from literature that targets PPIB. Construct 10169 has the same sequence as 10167, but also has 2'-O-Me modification on the first 4 and last 4 nucleotides on its sense strand.

Figure 6B shows an exemplary dose titration curve comparison among a 19-bp + 2 nt siRNA (Figure 6B, filled circle); 19-bp + 2 nt siRNA with 2'-O-Me on the passenger strand (Figure 6B, open circle); 25-bp, blunt-ended duplex with 2'-O-Me passenger strand modification

(Figure 6B, open square); or 27-bp, blunt-ended duplex with 2'-O-Me passenger strand modification (Figure 6B, open triangle). The RNA duplexes target the PPIB gene in a region that is homologous in both human and mouse. Additionally, the duplexes were designed to preserve the same mRNA cleavage site regardless of length. The EC_{50} values are: unmodified 19-bp + 2 nt siRNA = 0.043 nM ± 0.019 nM, 19-bp + 2 nt siRNA with 2'-O-Me = 0.276 nM ± 0.144 nM, 25-bp duplex 2'-O-Me = 0.025 nM ± 0.009 nM, 27-bp duplex 2'-O-Me = 0.072 nM ± 0.035 nM.

The same experiment was also repeated in murine NIH3T3 cells (Figure 6C). The EC_{50} values are: unmodified 19-bp + 2 nt siRNA = 0.482 nM ± 0.076 nM, 19-bp + 2 nt siRNA with 2'-O-Me = 1.235 nM ± 0.194 nM, 25-bp duplex 2'-O-Me = 0.219 nM ± 0.044 nM, 27-bp duplex 2'-O-Me = 0.518 nM ± 0.099 nM.

Example 6 Chemical Modifications Prevent Dicer Processing of Alternate RNAi Compound

One of the key discoveries made by the Applicants is that certain modified double stranded RNAs are not cleaved by Dicer, despite the prior art teaching that dsRNA longer than 21-mer is cleaved by Dicer to 21-mer products. In addition, Applicants have shown that the antisense strand in the Dicer-resistant dsRNA can be incorporated into the RISC complex and serve as the guide sequence for RNA interference. The 5'-end nucleotide (rather than the 3'-end or other nucleotides) of this Dicer-resistant dsRNA lines up with the 5'-end of the Dicer-cleaved 21-mer in the RISC complex.

In the first experiment, several RNAi duplexes, including several 25-mer Alternate RNAi Compounds with different modification chemistries (see below), were incubated with or without recombinant human Dicer enzyme (0.5 units) (Genlantis, San Diego, CA) for 8 hours at 37°C in 250 mM NaCl, 30 mM HEPES (pH 8.0), 0.05 mM EDTA, 2.5 mM $MgCl_2$ reaction buffer. After incubation, the reaction was stopped by adding loading buffer and snap freezing with liquid nitrogen. An aliquot of each sample (15 pmoles) was run on a native 20% polyacrylamide gel in TBE buffer. Samples were loaded alternating without and with Dicer enzyme as indicated at the top of the gel. As shown in Figure 7, various 2'-O-Me modifications to blunt-ended 25-mer duplexes resist cleavage by Dicer, in contrast to a 25-mer blunt-ended duplex that has not been modified.

This indicates that Alternate RNAi Compound duplexes would be expected to function in cells as a uniform species and not be cleaved to multiple different 21-mer duplexes or other

shorter species to have activity. These findings have important implications for clinical development because the active drug is be a singular species. This is in contrast to current models which suggest that duplexes longer than 21-mer are processed to be that length. Furthermore, data from 27-mer duplexes showing modification on one side of the duplexed RNA shows processing. For example, Kubo *et al.* (*Oligonucleotides* 17(4): 445-464, 2007) showed that dicer will process long duplexes with modifications on the same side.

Here, Applicants have demonstrated that certain sense and/or antisense strand modification of longer dsRNA, such as blunt, 25-mer duplexes with 4 x 2' O-methyl modifications on either end of the sequence, are not cleaved by the Dicer enzyme. It is noted that other modification strategies as described herein can also eliminate ability to be cleaved by Dicer. Modifications are generally included on both the 5' and 3' ends of the sense strand.

Example 7 Northern Blot of Immunoprecipitated Ago2 Loaded with Alternate RNAi Compound.

This example demonstrates that the Dicer-resistant dsRNAs of the invention are loaded onto the RISC complex, and has gene silencing activity.

293S cells stably expressing myc-Ago2 (courtesy of the Hannon Lab, CSHL) were transfected with either 25-mer (10174 in Table 4) or 26-mer (10175 in Table 4) Alternate RNAi Compound duplexes. After 24 hours, cell lysates were collected and immunoprecipitated using agarose beads conjugated to anti-c-myc antibodies (Sigma) to precipitate myc-Ago2 protein. RNA that was loaded into Ago2 was precipitated and loaded onto a 15% polyacrylamide gel. ^{32}P end-labeled single stranded RNA markers from 21-26 nt were included on the gel to determine size. RNA from the polyacrylamide gel was transferred to a nylon membrane and UV cross-linked to the membrane. The membrane was incubated overnight with ^{32}P-labeled LNA probes specific to the Alternate RNAi Compound that was transfected. After washing, the blots were visualized using a phosphor imagining system (Fuji BAS-2500) (Figure 8A).

This result further supports the conclusion that 25-mer or 26-mer Alternate RNAi Compounds are not cleaved by Dicer as part of the RNAi process. In addition, the 25-mer and 26-mer duplexes were found to be associated with the RNAi silencing Ago2 in the RISC complex. Sequences are shown in Table 4.

In a similar experiment, 293 cells expressing c-myc Ago2 were transfected with various duplexes against SOD1 (*e.g.*, 21-mer with no modification; 25-mer with no modification; and 25-mer with 4/4 2'-O-Me modification). The cells were harvested, lysed, and c-myc Ago2 was

immunoprecipitated as described before. After immunoprecipitation, the RNA from the IP fractions was extracted and precipitated. RNA was loaded onto a denaturing polyacrylamide gel, transferred to a nylon membrane, and detected using LNA probes specific to the guide strand of the transfected RNA duplexes as described herein.

Figure 8B shows a Northern blot of the guide strand of SOD1 targeting RNA duplexes. Single stranded RNAs were run alongside captured guide strand RNA. The size markers are underlined and correspond to sizes from 21 to 25 nt long. By comparison to single-stranded RNA markers of 21-25 nt, the Ago2:RISC-associated guide strand of the two tested 25-bp duplexes was determined to be 25 nt in length. These data confirm that the modified guide strand is bound by Ago2:RISC in its full length form. Note that in this study, the unmodified 25-bp duplex was not efficiently cleaved to the expected smaller product that would have resulted from efficient Dicer processing in the cell. Since these engineered 293 cell lines dramatically over-express c-myc tagged Ago2 protein, it is possible that the abundance of the Ago2:RISC complex causes the RNA duplex to be loaded before being able to be processed by Dicer.

Potent activity was confirmed in these cells by measuring target mRNA reduction in an aliquot of the cell lysate prior to immunoprecipitation. Figure 8C shows expression of SOD1 after transfection of 293 cells expressing c-myc Ago2. Before immunoprecipitation of c-myc Ago2, a fraction of the cell lysate was taken for total cell RNA purification. The RNA was purified and gene expression was measured using a bDNA assay as described in the methods. The levels of SOD1 expression are normalized to PPIB and adjusted to a luciferase targeting control duplex. The final concentration of transfected SOD1 targeting RNA duplex was 25 nM.

Example 8 mRNA Cleavage Assay of a Synthetic Substrate Using Purified RISC

This example demonstrates that the 5'-end of the Dicer-resistant antisense strand (guide sequence) in the Alternate RNAi Compound aligns with the 5'-end of the Dicer cleavage product (21-mer), and that the cleavage site (on the target mRNA) is between the 10th and the 11th nucleotides from the 5'-end of the guide sequence.

In one experiment, 293S cells stably expressing myc-Ago2 (courtesy of the Hannon Lab, CSHL) were transfected with several Alternate RNAi Compound duplexes (10023 and 10174), along with the 21-mer modified dsRNA 10036 (R1). After 24 hours, cell lysates were collected and immunoprecipitated using agarose beads conjugated to anti-c-myc antibodies (Sigma). Immunoprecipitation (IP) samples were incubated for 2 hours at 37° C with a synthetic,

radiolabeled 50 nt substrate (Table 5). Samples were then run on a 15% polyacrylamide gel and visualized using a phosphor imager (Fuji BAS-2500).

Note that the 25-mer Alternate RNAi Compound 10023 has 4 extra 5'-end nucleotides compared to R1/10036, and thus its predicted cleavage product would be 4 nucleotides longer than that of the 10036. The 25-mer Alternate RNAi Compound 10174 has 4 extra 3'-end nucleotides compared to R1/10036, but has the same 5'-end, and thus its predicted cleavage product would be the same as that of the 10036.

Cleavage of the target synthetic substrate occurs at a uniform point 10 nt from the 5'-end of the antisense strand for non-Dicer processed duplexes longer than 21 nt (data not shown). There is a single cleavage position which results not in a family of bands, but one product band. This result has implications for the design of active duplexes and enables one to position chemical and sequence modifications on any length duplexes with knowledge of the cleavage position. Key residues are defined by this result.

The result was further confirmed with three other duplexes designed to direct cleavage of a different site on the SOD1 gene (see Figures 9A and 9B). Specifically, a synthetic substrate was chemically synthesized to correspond to a 50 nt region of the SOD1 gene containing the target sequence for the RNA duplexes tested. Figure 9A is a schematic of the synthetic substrate and predicted cleavage position and products. In Figure 9B, RNA duplexes targeting SOD1 were transfected into 293 cells expressing c-myc Ago2 as described in methods. The cells were harvested, lysed, c-myc Ago2 was immunoprecipitated, and reconstituted in buffer. The immunoprecipitates were incubated with a 50 nt ^{32}-P-labeled synthetic substrate for 2 h at 30 °C as described in methods. After 2 hr incubations, samples were loaded onto a denaturing, polyacrylamide gel along with size markers (shown underlined). Sample letters correspond to the following duplexes shown in the schematic in panel a. A = unmodified 19-bp + 2 nt siRNA, B = unmodified 25-bp duplex, C = 25-bp duplex with 4/4 2'OMe, D = 25-bp duplex with 4/4 2'OMe, E = Luciferase Ctrl duplex.

Example 9 Chemical Modifications Prevent Dicer Processing

The above studies show that the 25-bp RNA duplexes are able to achieve potent gene silencing, load fully into Ago2:RISC, and cleave intended targets across from nucleotide 10 of the guide strand. Previous studies have shown that a methylation modification of nucleic acids can prevent endonuclease activity and it could potentially inhibit Dicer's ability to process duplexes >21-bp. To explore Dicer processing with these chemically modified RNA duplexes, a

panel of modification configurations was tested for susceptibility to Dicer cleavage in vitro (Figure 17A). Since activity and target specificity are defined by the 5' position of the guide strand sequence that is loaded into RISC, an understanding of the cleavage products, if any, would help elucidate the role of Dicer processing in RNAi activity.

A series of duplexes targeting the SOD1 gene were modified on both ends (5' and 3') of the passenger strand with varying numbers of 2'OMe nucleotides. These duplexes were incubated with recombinant human Dicer enzyme and analyzed for processing (Figure 17B). The unmodified 25-bp duplex and the duplex with one 2'OMe modification on both ends of the passenger strand (1/1) are completely processed to the 21-bp siRNA product. The duplex with two 2'OMe on both ends of the passenger strand (2/2) showed partial processing under these conditions (16 h incubation) with about 30% of the duplex remaining unprocessed in this study (Figure 17C). However, the duplex with either three or four 2'OMe modified nucleotides on both ends of the passenger strand (3/3 or 4/4) is not processed by the Dicer enzyme.

It has been reported that the presence of a blocking group on the 3' ends of both strands of a Dicer substrate can block processing. Dicer processing of this same SOD1 targeting duplex sequence bearing additional modification configurations was explored. Configurations tested included four 2'OMe modifications on both 5' ends, both 3' ends, or both ends (5' and 3') of each individual strand of the duplex (passenger or guide strand). Dicer processing is blocked by all of these modification patterns (Figure 17D). Similar results are obtained with 2'-O-Me modified 25-bp duplexes targeting a different sequence in the SOD1 gene (Figure 17E). Specifically, a 25-bp duplex that targets the human SOD1 sequence (see Table 4) was incubated with Dicer enzyme for 16 hr, and loaded onto a TBE-polyacrylamide gel as described herein. The gel was visualized by SYBR green staining and UV transluminator. The sequence shown has a high C and G base content on both ends of the duplex which helps it to be more nuclease resistant. The "M" designates a commercially available siRNA marker described herein.

Example 9 Activity Comparison of 2'OMe-containing Alternate RNAi Compounds

This example describes the many possible designs for the subject Alternate RNAi Compound constructs, and provides exemplary activity comparison data for the various 2'OMe-containing Alternate RNAi Compounds.

A variety of Alternate RNAi Compounds based on either 10015 or 10023 sequence (see Figure 10A) were compared with chemistry modifications as indicated in Figure 10B. For example, "0811" refers to 008 chemistry for the sense strand, and 011 chemistry for the

antisense strand as indicated in Figure 10B. For comparison, 0111 *i.e.*, 001-011, is the original Alternate RNAi Compound screening chemistry used in many earlier examples. The results show that some modifications lead to higher activity at reducing SOD1 activity levels (Figure 11).

Activity Comparison of Varying amounts of 2'OMe Modifications on Sense strand of Alternate RNAi Compounds. To understand the positional effect that 2'OMe on the sense strand has on the degree of activity of a given duplex, a variety of Alternate RNAi Compounds based on either 10015 or 10023 sequence were compared with varying amounts of 2'OMe modifications to the sense strand (Figures 12A, 12B, 12C). All duplexes had unmodified antisense strands (*i.e.*, chemistry 011, see Figure 10B). Figure 12A shows relative activity normalized to PPIB (housekeeping gene) of silencing SOD1. Figures 12B and 12C are visual maps showing what position (in respect to the antisense strand numbering) a 2'OMe was placed and compared to the original 001 chemistry (see Figure 10B). The graphs illustrate the activity of each duplex as a relative percentage above or below to the 011 chemistry. IDs for the 10015 sequence (Figure 12B) corresponding to 025, 024, 009, 036, 021, 010, 039, 033, and 008 showed better activity, and some were selected for further activity analysis. Analogous analysis for the 10023 sequence is also shown in Figure 12C.

This study was performed by custom annealing different strands together and transfecting HEK293 cells with 5nM duplex. The activity of the duplex against SOD1 was measured 24 hours post-transfection using bDNA assay and normalizing to PPIB. Mechanism of action studies described above allowed for predictive positioning of other modifications to improve activity and functionality.

Results from two constructs (10015 and 10023) with non-overlapping sequences are remarkably consistent with respect to the specific modification chemistries tested (*e.g.*, 10015 modified by the 019 chemistry has virtually the same result as 10023 modified by the 019 chemistry, *etc.*). This suggests that the modification chemistry (rather than the targeting sequence) is the main reason for the observed differences in RNAi mediated gene silencing.

Having identified several preferred sense-strand modification schemes, Applicants tested below a few follow-up sense-antisense modification combinations.

Activity comparison of different 2'OMe modifications patterns. A variety of Alternate RNAi Compounds based on either 10015 or 10023 sequence were tested as a follow-up to the extensive sense-only 2'OMe analysis. Figures 13A (24 hours) and 13B (48 hours) show relative activity normalized to PPIB (housekeeping gene) of silencing SOD1. Figures 14C

and 14D are visual maps showing the relative position of a 2'OMe (in respect to the antisense strand numbering) and compared to the original chemistry (001). The graphs are colored to represent the type of antisense chemistry present on the duplex. The activity presented in these figures is in relation to 001-011 (original Alternate RNAi Compound). IDs corresponding to the duplexes containing antisense strand with 042 chemistry (2'OMe at position 2) showed better activity in this experiment.

This experiment was performed by custom annealing different strands together and transfecting HEK293 cells with 5 nM duplex. The activity of the duplex against SOD1 was measured 24 or 48 hours post-transfection using bDNA assay and normalizing to PPIB. Mechanism of action studies described above enabled us to position other modifications to improve activity and functionality.

These results confirm that better or equivalent activity is achieved with having increased 2'OMe modification on the sense strand. Further, there are additional designs with beneficial features that improve activity. We have found, surprisingly, that modification of the 25-mer sense strand with 12 2'OMe on the 5' end and 10 2'OMe on the 3' end (*i.e.*, sense chemistry 039) resulted in increased activity. This almost completely modified version has improved activity in more than one duplex and more than one study. The following chemistries found to have increased activities in the studies above are highlighted:

- 001-011
- 001-042
- 001-013
- 039-011
- 039-042
- 039-013
- MM001-011

Furthermore, as shown in Figure 14D, modification on nucleotide 2 of the antisense strand showed increased activity. Modification on nucleotide 2 of the antisense strand has been reported to increase specificity of target cleavage, restricting mRNA reduction more exclusively to the target gene.

In addition, mismatches in the sense strand (chemistry MM001) opposite nucleotides 2-8 of the 5' antisense sequence allow more efficient loading into the RISC complex. We have confirmed that a mismatch at nucleotide 2 on the 3'end of the sense strand leads to more potent mRNA reducing activity. The mechanism of action studies demonstrated above allow for correct positioning of modifications on a duplex longer than 21 nt that is not processed by Dicer.

Example 10 ***In vitro*** **Stability Assays**

This example shows that, in addition to the benefits described herein above, the subject Alternate RNAi Compound constructs are more stable in serum, and are thus expected to have better *in vivo* efficacy.

Duplexes based on sequences 10023 (Figure 14A) or 10015 (Figure 14B) with various modifications as indicated were incubated in 10% human serum at 37°C for up to two hours (given in minutes at bottom of panel). Samples were quenched with chloroform and the aqueous phase was run on a 20% TBE gel. Gels were stained with SYBR green. "M" denotes siRNA marker (25mer, 21mer, 17mer duplexes), while "C" indicates duplex without incubation in serum. For the 10015 sequence, all modifications resulted in a more stable duplex as compared to the unmodified 811 duplex (008 sense chemistry, 011 antisense chemistry) under the same conditions. The Alternate RNAi Compound modification 111 *i.e.*, 001 sense, 011 antisense, is one of the most stable tested. Modification with 039 chemistry on the sense strand (3911) also stabilized the duplex and showed increased activity. Modification of the 10023 sequence, however, did not change the stability under the same conditions as compared to the unmodified sequence (811). The 039 modification (3911) displays mild inhibition of degradation (comparing the intensity of the major degradation product to the full length) as compared to other modifications.

Example 11 RNAi with 21-27 bp Duplexes in the Absence of Dicer

This example demonstrates that Dicer is not required to effect RNAi with 21-27 bp duplexes. Here, silencing of target genes in a mouse embryonic stem cell line deficient for the Dicer enzyme (Murchison et al., 2005) was studied to enable a more direct test of the role of Dicer in RNAi with synthetic, chemically modified duplexes. Dicer homozygous null (-/-) or heterozygous (+/-) mutant cells were transfected with RNA duplexes and reduction of the target mRNA was quantified.

Mouse derived embryonic stem cells that are homozygous null or heterozygous for the enzyme Dicer were generated by Murchison and co-workers and cultured as previously described (Murchison et al., 2005). Dose response transfections were carried out by adapting previously described conditions (Schaniel et al., 2006) to a 96-well dose response method described herein. Active RNA duplexes that target the human SOD1 gene that had 100% homology to mouse SOD1 were transfected in concentrations ranging from 0.05 nM to 10 nM in

a final RNA concentration of 25nM. Transfection efficiency was monitored using a DY547 labeled version of the non-targeting control duplex. Cells were incubated for 48 h after transfection and gene silencing activity was measured using the QuantiGene bDNA hybridization assay (Panomics) as described above.

The bars in Figure 15A represent the mRNA levels of SOD1 48 hours post transfection as measured by bDNA hybridization assay. Expression of SOD1 levels are normalized to the PPIB gene and adjusted as a percent of the luciferase control duplex. The three duplexes tested were 19-bp + 2 nt siRNA (solid white bar), 25-bp blunt-ended duplex unmodified (black solid bar), and 25-bp blunt-ended duplex with "4/4" 2'-O-Me modification (striped bar). The exact sequences used in this study are shown in Figure 15B.

Consistent with previously published data, the 19-bp + 2 nt siRNA duplexes efficiently silence the target gene in the absence of Dicer (Murchison et al., 2005). Interestingly, 25-bp duplexes (modified and unmodified) silence the target gene in the Dicer null cells with at least the same efficiency (Figure 15A). In fact, gene silencing activity appeared slightly higher in the Dicer null cells as compared to cells that contained the Dicer protein. This observation might be explained by the fact that Dicer null cells have no endogenous miRNAs to compete for occupancy of Ago2:RISC and thus the transfected duplexes could appear to be more potent. These results confirm that Dicer is not required for efficient silencing with RNA duplexes.

This observation was extended to duplexes up to 27-bp in length by testing a sequence to the PPIB gene (Figure 16A). Here, the solid white bars are a 19-bp + 2 nt siRNA, the dotted bars are an unmodified 25-bp duplex, the solid black bars are a 25-bp duplex with 2'-O-Me chemistry, and the striped bars are a 27-bp duplex with 2'-O-Me chemistry. The silencing activity achieved similar results as seen with the SOD1 targeting duplexes in the Dicer null cells. Sequences are shown in Figure 16B.

Methods

Certain methods lised in the examples herein are provided below for illustration purpose only.

Chemically synthesized RNAs. Single stranded RNA was synthesized by Integrated DNA technologies or ThermoFisher Dharmacon products. RNA duplexes were formed by mixing equal molar ratios of single stranded RNA and incubating at 90 °C for 1 min and then incubating at 37 °C for 1 h. All single stranded RNA and RNA duplexes were stored at -20 °C. See **Table 3** and **Table 4** online for RNA sequence information.

Dose response transfections. HEK293 cells (ATCC) were cultured in Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin. NIH3T3 Cells (ATCC) was cultured in DMEM with 10% Bovine Calf Serum and 1% Penicillin-Streptomycin. All cells were incubated at 37 °C with 10% CO_2 as recommended by ATCC. RNA duplexes were reverse-transfected in a dose dependent manner (0.005 nM to 5 nM) using Lipofectamine RNAiMAX (Invitrogen) reagent as described by the manufacturer and optimized conditions. The RNA duplexes were complexed along with a non-targeting control duplex to make a final concentration of 25 nM RNA duplex transfected. The non-targeting, chemistry, and length matched control duplexes target the luciferase gene. Transfections were performed in 96-well plates with media containing no antibiotics and were incubated for 48 h under normal growth conditions. Gene silencing was measured using the QuantiGene bDNA hybridization assay (Panomics). Cells were lysed and mRNA levels were measured under assay conditions described by the manufacturer. Gene expression was measured for the SOD1 gene and the PPIB gene using species specific probe sets (Panomics). The gene expression values were normalized to the PPIB gene which was used as a housekeeping control since its expression is not affected by SOD1 silencing. The percent silencing and EC_{50} values are based on the normalized SOD1 expression of the non-targeting control duplexes. The values are calculated and graphically displayed using KaleidaGraph (Synergy Software).

Argonaute-2 immunoprecipitation and extraction of complexed RNA from RISC. RNA duplexes were annealed as described and transfected into 293T cells stably expressing c-myc Ago2 (Hannon lab, Cold Spring Harbor Labs). 293 cells were cultured as described above in the presence of 0.5 ug/ml G418 to selectively express c-myc Ago2. Transfection of RNA duplexes was carried out using Lipofectamine RNAiMAX (Invitrogen) in a 10 cm plate as described by the manufacturer. The cells were transfected in media containing no antibiotics and having a final concentration of RNA duplex of 25 nM. Cells were incubated for 48 hours before harvesting and immunoprecipitation (IP) of c-myc Ago2. Cell harvest and IP was performed as previously described. Cells were collected from plates and washed once with 1X PBS and once in 2 ml of Hypotonic Lysis Buffer (HLB) (10 mM Tris pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 5 mM DTT, and protease inhibitor). Cells were then reconstituted in 0.5 ml of HLB and allowed to swell on ice for 15 min. A fraction of the cell lysate (50 ul) was taken and added to 200 ul Trizol (Invitrogen) for subsequent gene silencing assays using the QuantiGene bDNA hybridization assay (Panomics). The cell lysate was added to a 1 ml dounce homogenizer with tight pestle and cells were homogenized for 30 strokes on ice. Cell lysates were clarified by

centrifugation (14,000 rpm, 30 min at 4 °C) and supernatant was transferred to a new tube. The supernatant or cytosolic fraction had 1 ml of buffer (LB650) (0.5% NP40, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Tris pH 7.5, 5 mM DTT, 650 mM KCl, and protease inhibitors) added. Anti-c-myc antibody conjugated to agarose beads (Sigma) were added to each tube and tubes were incubated overnight at 4 °C while rotating. After overnight incubation, IP reactions were spun down for 2 min at 3,000 rpm and then beads were washed three times in LB650 buffer. After washing the beads, 200 ul of Trizol (Invitrogen) was added to disassociate the RNA from the antibody captured Ago2. RNA was precipitated as described by manufacturer instructions forgoing an ethanol wash. RNA was reconstituted in 20 ul of TE buffer.

Northern blot of RNA captured from Ago2 immunoprecipitation. RNA was loaded on to a 15% polyacrylamide, TBE-Urea denaturing gel. Previously labeled ^{32}P-end size makers ranging from 21 to 25 nt were run alongside IP reactions to determine the size of the captured RNA. The gel was transferred to a nylon membrane and RNA was UV cross-linked. Membrane was pre-hybridized using UltraHyb-Oligo Buffer (Ambion) for 30 min at 42 °C and then previously prepared ^{32}P-labeled locked nucleic acid probes complimentary to the guide strand were added to the hybridization buffer. After overnight incubation at 42 °C, membrane was washed twice for 30 min in wash buffer (1X SSC buffer, 0.1% SDS). The blot was visualized by exposing to BioMAX autoradiograph film (Kodak). Film was developed using automatic film processing unit.

mRNA cleavage position assay. 293 cells expressing c-myc Ago2 were transfected with RNA duplexes as described above. Ago2 was immunoprecipitation was also carried out as described above. After the final wash, the agarose beads containing Ago2 complexes loaded with transfected RNAs were re-constituted in 10ul buffer (100 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris pH 7.5). A chemically synthesized, synthetic substrate that matched a 50 nt region of the human SOD1 gene to which the transfected 25-bp duplexes or 19-bp + 2 nt siRNAs target was ^{32}P-5'end labeled and gel purified. The synthetic substrate was designed specifically to have base 21 correspond to position 10 of the guide strand of the 19-bp + 2 nt siRNA or 25-bp duplex. The labeled RNA was gel purified and precipitated in isopropanol. The cleavage reaction was setup with a final volume of 20 ul. The IP reaction (10 ul) was added to 4 ul of labeled synthetic substrate, 1 ul RNAsin, and 5 ul buffer (100 mM KCl, 2 mM $MgCl_2$, and 10 mM Tris pH 7.5). The reaction was incubated for 2 h at 30 °C. After incubation the reactions were run on a 15% polyacrylamide, TBE-urea gel. Gel was exposed to autoradiograph film and developed using methods described above. Size of cleavage product was determined using ^{32}P-

labeled size marker RNAs that were run alongside cleavage assay reactions.

Dicer processing assay. RNA duplexes were incubated with recombinant human Dicer enzyme (Genlantis) according to manufacturer recommendations and previously described conditions. Samples were incubated overnight (~16 h) at 37 °C with and without Dicer enzyme. Reactions were stopped by the addition of TBE gel loading buffer and snap freezing in liquid nitrogen. A fraction (16 pmoles) of the stopped reaction was run on a native, TBE buffered, 20% polyacrylamide gel. A siRNA marker (New England Biolabs) was run alongside samples on the gel. The gel was stained using SYBR green II (Invitrogen) for 20 min and then visualized using a UV transluminator and CCD camera. UV images and relative quantification analysis were carried out using the UVP BioChemi imaging station with LabWorks software (UVP).

Dicer null cell transfections. Dicer null cells were generated and cultured as previously described. Dose response transfections were carried out by adapting previously described conditions to a 96-well dose response method described above. RNA duplexes that targeted the human SOD1 gene that had 100% homology to mouse SOD1 were transfected in concentrations ranging from 0.05 nM to 10 nM. Transfection efficiency was monitored using a DY547 labeled control duplex. Cells were incubated for 48 h after transfection and then gene silencing activity was measured using the QuantiGene bDNA hybridization assay (Panomics). Cells were lysed and assay was carried out as described above and according to manufacturer's recommendations.

Table 3

Sequence Name	Polarity	Sequence (5'->3')
Figure 9		
A (11892)	PS	G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.U.U
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U
B (11893)	PS	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
C (11897)	PS	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA

	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
D (10357)	PS	mA.mG.mG.mU.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.mA.mA.mA.mG
	GS	C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U
E (10461)	PS	mG.mC.mA.mC.U.C.U.G.A.U.U.G.A.C.A.A.A.U.A.C.G.mA.mU.mU.mU
	GS	A.A.A.U.C.G.U.A.U.U.U.G.U.C.A.A.U.C.A.G.A.G.U.G.C
Figure 15B		
19-bp + 2 nt duplex (11892)	PS	G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.U.U
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U
0 / 0 (11893)	PS	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
4 / 4 (11897)	PS	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
Figure 17B		
0 / 0 (11893)	PS	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
1 / 1 (11894)	PS	mG.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
2 / 2 (11895)	PS	mG.mG.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.mU.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
3 / 3 (11896)	PS	mG.mG.mC.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.mG.mU.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
4 / 4 (11897)	PS	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA

	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
Figure 17D		
4 / 4 PS (11897)	PS	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
4 / 4 AS (11904)	PS	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	GS	mU.mA.mC.mU.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.mU.mG.mC.mC
4 / 0 – 4 / 0 (11906)	PS	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	GS	mU.mA.mC.mU.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
0 / 4 – 0 / 4 (11907)	PS	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA
	GS	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.mU.mG.mC.mC

Table 4

Sequence Name	Polarity	Sequence (5'->3')
Fig. 6C		
19-bp + 2 nt unmodified (10167)	PS	G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.A.A.A.A.U.U
	GS	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.U.U
19-bp + 2 nt with 2'OMe (10459)	PS	mG.mG.mA.mA.A.G.A.C.U.G.U.U.C.C.A.A.A.mA.mA.mU.mU
	GS	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.U.U
25-mer with 2'OMe (10460)	PS	mC.mU.mC.mU.U.C.G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.mA.mA.mA.mA
	GS	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.G.A.A.G.A.G
27-mer with	PS	mG.mU.mC.mU.C.U.U.C.G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.mA.mA.mA.mA

2'OMe (10462)		
	GS	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.G.A.A.G.A.G.A.C
Example 7		
10036	PS	C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
	GS	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
10023	PS	mG.mC.mC.mG.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.mU.mC.mU.mG
	GS	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
10174	PS	mU.mG.mU.mG.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
	GS	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A
10175	PS	mG.mU.mG.mU.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
	GS	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C
Figure 17E		
19-bp + 2 nt siRNA (10036)	PS	C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
	GS	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
25-bp 4 / 4 Sense Only (10023)	PS	mG.mC.mC.mG.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.mU.mC.mU.mG
	GS	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
25-bp 0 / 0 (10777)	PS	G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G
	GS	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C

Table 5

Sequence Name	Sequence (5'->3')
Size Markers	
21 nt	AGAGCAGUGGCUGGUUGAGAU
22 nt	AGAGCAGUGGCUGGUUGAGAUU
23 nt	AGAGCAGUGGCUGGUUGAGAUUU
24 nt	AGAGCAGUGGCUGGUUGAGAUUUA

25 nt	AGAGCAGUGGCUGGUUGAGAUUUAA
26 nt	AGAGCAGUGGCUGGUUGAGAUUUAAU
Synthetic Substrates	
Synthetic Substrate Fig. 9A	ACUUGGGCAAAGGUGGAAAUGAAGAAAGUACAAAGACAGGAAACGCUGGA
Synthetic Substrate Example 8	GAUGGUGUGGCCGAUGUGUCUAUUGAAGAUUCUGUGAUCUCACUCUCAGG

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. The entire contents of all patents, published patent applications and other references cited herein are hereby

expressly incorporated by reference in their entireties.

TABLE 1: Alternative RNAi Compound Sequences to SOD1 and PPIB			
Full Sequence Name	**ID Number**	**Polarity**	**Sequence (5'->3')**
SOD1-125-25-10001	10001	Sense	P.G.A.C.C.U.C.G.G.C.G.U.G.G.C.C.U.A.G.C.G.A.G.U.U.A
		Antisense	U.A.A.C.U.C.G.C.U.A.G.G.C.C.A.C.G.C.C.G.A.G.G.U.C
SOD1-376-25-10002	10002	Sense	P.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C
		Antisense	G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C
SOD1-371-25-10003	10003	Sense	P.C.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G
		Antisense	C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G.G
SOD1-533-25-10004	10004	Sense	P.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A
		Antisense	U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U
SOD1-062-25-10005	10005	Sense	P.G.U.G.C.U.G.G.U.U.U.G.C.G.U.C.G.U.A.G.U.C.U.C.C.U
		Antisense	A.G.G.A.G.A.C.U.A.C.G.A.C.G.C.A.A.A.C.C.A.G.C.A.C
SOD1-074-25-10006	10006	Sense	P.G.U.C.G.U.A.G.U.C.U.C.C.U.G.C.A.G.C.G.U.C.U.G.G.G
		Antisense	C.C.C.A.G.A.C.G.C.U.G.C.A.G.G.A.G.A.C.U.A.C.G.A.C
SOD1-202-25-10007	10007	Sense	P.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U
		Antisense	A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G
SOD1-201-25-10008	10008	Sense	P.U.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G
		Antisense	C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G.A
SOD1-320-25-10009	10009	Sense	P.U.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A
		Antisense	U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A.C.A
SOD1-325-25-10010	10010	Sense	P.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U
		Antisense	A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G
SOD1-396-25-10011	10011	Sense	P.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A
		Antisense	U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C
SOD1-431-25-10012	10012	Sense	P.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U
		Antisense	A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C
SOD1-440-25-10013	10013	Sense	P.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U
		Antisense	A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C
SOD1-457-25-10014	10014	Sense	P.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U
		Antisense	A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G.A.G.A.U.C.A.C.A
SOD1-530-25-10015	10015	Sense	P.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
		Antisense	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
SOD1-520-25-10016	10016	Sense	P.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U
		Antisense	A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C.A.U.C.U
SOD1-568-25-10017	10017	Sense	P.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U
		Antisense	A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G
SOD1-609-25-10018	10018	Sense	P.A.A.U.A.A.A.C.A.U.U.C.C.C.U.U.G.G.A.U.G.U.A.G.U.C
		Antisense	G.A.C.U.A.C.A.U.C.C.A.A.G.G.G.A.A.U.G.U.U.U.A.U.U
SOD1-662-25-10019	10019	Sense	P.G.C.U.A.G.C.U.G.U.A.G.A.A.A.U.G.U.A.U.C.C.U.G.A.U

		Antisense	A.U.C.A.G.G.A.U.A.C.A.U.U.U.C.U.A.C.A.G.C.U.A.G.C
SOD1-751-25-10020	10020	Sense	P.C.C.U.G.U.A.G.U.G.A.G.A.A.A.C.U.G.A.U.U.U.A.U.G.A
		Antisense	U.C.A.U.A.A.A.U.C.A.G.U.U.U.C.U.C.A.C.U.A.C.A.G.G
SOD1-844-25-10021	10021	Sense	P.C.C.A.G.A.C.U.U.A.A.A.U.C.A.C.A.G.A.U.G.G.G.U.A.U
		Antisense	A.U.A.C.C.C.A.U.C.U.G.U.G.A.U.U.U.A.A.G.U.C.U.G.G
SOD1-858-25-10022	10022	Sense	P.C.A.G.A.U.G.G.G.U.A.U.U.A.A.A.C.U.U.G.U.C.A.G.A.A
		Antisense	U.U.C.U.G.A.C.A.A.G.U.U.U.A.A.U.A.C.C.C.A.U.C.U.G
SOD1-434-25-10023	10023	Sense	P.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G
		Antisense	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
SOD1-261-25-10024	10024	Sense	P.G.A.C.U.G.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U
		Antisense	A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G.U.C.A.G.U.C
SOD1-434-25-10026	10026	Sense	P.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G
		Antisense	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
SOD1-125-25-10027	10027	Sense	P.G.A.C.C.U.C.G.G.C.G.U.G.G.C.C.U.A.G.C.G.A.G.U.U.A
		Antisense	U.A.A.C.U.C.G.C.U.A.G.G.C.C.A.C.G.C.C.G.A.G.G.U.C
SOD1-751-25-10028	10028	Sense	P.C.C.U.G.U.A.G.U.G.A.G.A.A.A.C.U.G.A.U.U.U.A.U.G.A
		Antisense	U.C.A.U.A.A.A.U.C.A.G.U.U.U.C.U.C.A.C.U.A.C.A.G.G
SOD1-376-25-10029	10029	Sense	P.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C
		Antisense	G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C
SOD1-533-25-10030	10030	Sense	P.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A
		Antisense	U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U
SOD1-436-21-10033 (R1 var)	10033	Sense	P.C*G*A*fU.G.fU.G.U.C.U.A.U.U.G.A.A.G*A.fU*fU*C
		Antisense	P.A.fU.fC.fU.U.C.A.A.U.A.G.A.C.A.fC.A*fU*fC*G*G*C
SOD1-395-21-10034 (R2)	10034	Sense	P.G*G*A*G.A.fC.fU.U.G.G.G.C.A.A.fU.G.fU*G*A*fU*U
		Antisense	P.U.fC.A.fC.A.fU.fU.G.C.C.C.A.A.G.fU.fC.fU*fC*fC*U*U
SOD1-436-21-10036 (R1 unmod)	10036	Sense	P.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
		Antisense	P.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
SOD1-395-21-10037 (R2 unmod)	10037	Sense	P.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.U.U
		Antisense	P.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.U.U
SOD1-318-25-10078	10078	Sense	P.G.C.U.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U
		Antisense	A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A.C.A.G.C
SOD1-319-25-10079	10079	Sense	P.C.U.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U
		Antisense	A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A.C.A.G
SOD1-321-25-10080	10080	Sense	P.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A
		Antisense	U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A.C
SOD1-322-25-10081	10081	Sense	P.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U
		Antisense	A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A
SOD1-432-25-10082	10082	Sense	P.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
		Antisense	G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A
SOD1-433-25-10083	10083	Sense	P.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U
		Antisense	A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C
SOD1-435-25-10084	10084	Sense	P.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U

		Antisense	A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G
SOD1-436-25-10085	10085	Sense	P.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G
		Antisense	C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
SOD1-528-25-10086	10086	Sense	P.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G
		Antisense	C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A
SOD1-529-25-10087	10087	Sense	P.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U
		Antisense	A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C
SOD1-531-25-10088	10088	Sense	P.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C
		Antisense	G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C
SOD1-532-25-10089	10089	Sense	P.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A
		Antisense	U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G
SOD1-566-25-10090	10090	Sense	P.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G
		Antisense	C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U
SOD1-567-25-10091	10091	Sense	P.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G
		Antisense	C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U
SOD1-569-25-10092	10092	Sense	P.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G
		Antisense	C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C
SOD1-570-25-10093	10093	Sense	P.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U
		Antisense	A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G
SOD1-369-25-10094	10094	Sense	P.G.G.C.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U
		Antisense	A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G.G.C.C
SOD1-370-25-10095	10095	Sense	P.G.C.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U
		Antisense	A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G.G.C
SOD1-372-25-10096	10096	Sense	P.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G
		Antisense	C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G
SOD1-373-25-10097	10097	Sense	P.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A
		Antisense	U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U
SOD1-436-21-10104 (R1 with 2'Ome)	10104	Sense	P.C*G*A*mU.G.mU.G.U.C.U.A.U.U.G.A.A.G*A.mU*mU*C
		Anti-sense	P.A.mU.mC.mU.U.C.A.A.U.A.G.A.C.A.mC.A*mU*mC*G*G*C
SOD1-436-21-10105 (R1)	10105	Sense	P.C*G*A*fU.G.fU.G.U.C.U.A.U.U.G.A.A.G*A*fU*fU*C
		Anti-sense	P.A.fU.fC.fU.U.C.A.A.U.A.G.A.C.A.fC.A*fU*fC*G*G*C
SOD1-430-25-10174	10174	Sense	5'-P.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A
SOD1-429-26-10175	10175	Sense	5'-P.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C
PPIB-10167	10167	Antisense	5'-U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C
PPIB-10169	10169	Antisense	5'-mU.mU.mU.mU.U.G.G.A.A.C.A.G.U.C.U.mU.mU.mC.mC

TABLE 2: Additional Alternative RNAi Compound Sequences to SOD1		
Additional Sequences Designed		
10255	Sense	P.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A

	Antisense	U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U
10256	Sense	P.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A
	Antisense	U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U
10257	Sense	P.C.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U
	Antisense	A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A.G
10258	Sense	P.A.U.G.A.A.A.A.A.G.C.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A
	Antisense	U.U.G.C.C.C.C.A.A.G.U.C.A.U.C.U.G.C.U.U.U.U.U.C.A.U
10259	Sense	P.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A
	Antisense	U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A.G.C.A.G
10260	Sense	P.G.G.C.C.G.C.A.C.A.C.U.G.G.U.G.G.U.C.C.A.U.G.A.A.A
	Antisense	U.U.U.C.A.U.G.G.A.C.C.A.C.C.A.G.U.G.U.G.C.G.G.C.C
10261	Sense	P.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A.U.U
	Antisense	A.A.U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U
10262	Sense	P.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U
	Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A
10263	Sense	P.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A
	Antisense	U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U
10264	Sense	P.G.U.G.G.G.C.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A
	Antisense	U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G.G.C.C.C.A.C
10265	Sense	P.C.U.U.G.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A
	Antisense	U.U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C.C.C.A.A.G
10266	Sense	P.U.C.U.A.U.C.C.A.G.A.A.A.A.C.A.C.G.G.U.G.G.G.C.C.A
	Antisense	U.G.G.C.C.C.A.C.C.G.U.G.U.U.U.U.C.U.G.G.A.U.A.G.A
10267	Sense	P.A.A.A.A.A.G.C.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G
	Antisense	C.C.U.U.U.G.C.C.C.A.A.G.U.C.A.U.C.U.G.C.U.U.U.U.U
10268	Sense	P.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G.U.G.U.G.G.G.G.A.A
	Antisense	U.U.C.C.C.C.C.A.C.A.C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U
10269	Sense	P.U.G.U.U.A.U.C.C.U.G.C.U.A.G.C.U.G.U.A.G.A.A.A.U.G
	Antisense	C.A.U.U.U.C.U.A.C.A.G.C.U.A.G.C.A.G.G.A.U.A.A.C.A
10270	Sense	P.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A
	Antisense	U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G
10271	Sense	P.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A
	Antisense	U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U
10272	Sense	P.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A
	Antisense	U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G
10273	Sense	P.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U
	Antisense	A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A
10274	Sense	P.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G
	Antisense	C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U
10275	Sense	P.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G
	Antisense	C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A
10276	Sense	P.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A
	Antisense	U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U
10277	Sense	P.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A
	Antisense	U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U
10278	Sense	P.U.U.G.G.C.C.G.C.A.C.A.C.U.G.G.U.G.G.U.C.C.A.U.G.A
	Antisense	U.C.A.U.G.G.A.C.C.A.C.C.A.G.U.G.U.G.C.G.G.C.C.A.A
10279	Sense	P.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A
	Antisense	U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A
10280	Sense	P.G.A.A.A.A.A.G.C.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G
	Antisense	C.U.U.U.G.C.C.C.A.A.G.U.C.A.U.C.U.G.C.U.U.U.U.U.C
10281	Sense	P.C.C.C.A.G.U.G.C.A.G.G.G.C.A.U.C.A.U.C.A.A.U.U.U.C
	Antisense	G.A.A.A.U.U.G.A.U.G.A.U.G.C.C.C.U.G.C.A.C.U.G.G.G
10282	Sense	P.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C
	Antisense	G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U
10283	Sense	P.G.G.G.G.A.A.G.C.A.U.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A
	Antisense	U.C.A.G.U.C.A.G.U.C.C.U.U.U.A.A.U.G.C.U.U.C.C.C.C

10284	Sense	P.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G
	Antisense	C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C
10285	Sense	P.G.U.G.U.G.G.G.G.A.A.G.C.A.U.U.A.A.A.G.G.A.C.U.G.A
	Antisense	U.C.A.G.U.C.C.U.U.U.A.A.U.G.C.U.U.C.C.C.C.A.C.A.C
10286	Sense	P.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A.U.U.G
	Antisense	C.A.A.U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G
10287	Sense	P.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A
	Antisense	U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G
10288	Sense	P.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A
	Antisense	U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C.A
10289	Sense	P.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G.U
	Antisense	A.C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U
10290	Sense	P.G.G.C.C.C.A.G.U.G.C.A.G.G.G.C.A.U.C.A.U.C.A.A.U.U
	Antisense	A.A.U.U.G.A.U.G.A.U.G.C.C.C.U.G.C.A.C.U.G.G.G.C.C
10291	Sense	P.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A
	Antisense	U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A
10292	Sense	P.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G
	Antisense	C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U
10293	Sense	P.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C
	Antisense	G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A
10294	Sense	P.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C
	Antisense	G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U
10295	Sense	P.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A
	Antisense	U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G.A
10296	Sense	P.A.G.G.G.C.A.U.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A
	Antisense	U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G.A.U.G.C.C.C.U
10297	Sense	P.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A.U
	Antisense	A.U.U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G
10298	Sense	P.A.A.A.C.A.U.U.C.C.C.U.U.G.G.A.U.G.U.A.G.U.C.U.G.A
	Antisense	U.C.A.G.A.C.U.A.C.A.U.C.C.A.A.G.G.G.A.A.U.G.U.U.U
10299	Sense	P.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A
	Antisense	U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A
10300	Sense	P.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U.A
	Antisense	U.A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A
10301	Sense	P.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A
	Antisense	U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A
10302	Sense	P.G.C.C.C.A.G.U.G.C.A.G.G.G.C.A.U.C.A.U.C.A.A.U.U.U
	Antisense	A.A.A.U.U.G.A.U.G.A.U.G.C.C.C.U.G.C.A.C.U.G.G.G.C
10303	Sense	P.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A
	Antisense	U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U
10304	Sense	P.G.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U
	Antisense	A.U.C.U.U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C.C.C
10305	Sense	P.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G.A.A.A
	Antisense	U.U.U.C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G
10306	Sense	P.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A
	Antisense	U.U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A
10307	Sense	P.G.G.G.A.A.G.C.A.U.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A.A
	Antisense	U.U.C.A.G.U.C.A.G.U.C.C.U.U.U.A.A.U.G.C.U.U.C.C.C
10308	Sense	P.C.C.A.G.U.G.C.A.G.G.G.C.A.U.C.A.U.C.A.A.U.U.U.C.G
	Antisense	C.G.A.A.A.U.U.G.A.U.G.A.U.G.C.C.C.U.G.C.A.C.U.G.G
10309	Sense	P.G.C.A.U.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A
	Antisense	U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G.A.U.G.C
10310	Sense	P.C.A.U.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A
	Antisense	U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G.A.U.G
10311	Sense	P.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U
	Antisense	A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G
10312	Sense	P.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A

	Antisense	U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U
10313	Sense	P.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G
	Antisense	C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.C.U.U.C
10314	Sense	P.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G.U.G.U
	Antisense	A.C.A.C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C
10315	Sense	P.C.A.U.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A.A.G.G.C.C.U.G
	Antisense	C.A.G.G.C.C.U.U.C.A.G.U.C.A.G.U.C.C.U.U.U.A.A.U.G
10316	Sense	P.G.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U
	Antisense	A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G.U.C
10317	Sense	P.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G
	Antisense	C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G.U
10318	Sense	P.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U
	Antisense	A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A
10319	Sense	P.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C
	Antisense	G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C
10320	Sense	P.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U
	Antisense	A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U
10321	Sense	P.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A
	Antisense	U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C
10322	Sense	P.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G
	Antisense	C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G
10323	Sense	P.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U
	Antisense	A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A
10324	Sense	P.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G
	Antisense	C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G
10325	Sense	P.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A
	Antisense	U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C
10326	Sense	P.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A.U.A.C.A.G.C.A
	Antisense	U.G.C.U.G.U.A.U.U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A
10327	Sense	P.G.G.A.G.A.U.A.A.U.A.C.A.G.C.A.G.G.C.U.G.U.A.C.C.A
	Antisense	U.G.G.U.A.C.A.G.C.C.U.G.C.U.G.U.A.U.U.A.U.C.U.C.C
10328	Sense	P.G.A.U.A.A.U.A.C.A.G.C.A.G.G.C.U.G.U.A.C.C.A.G.U.G
	Antisense	C.A.C.U.G.G.U.A.C.A.G.C.C.U.G.C.U.G.U.A.U.U.A.U.C
10329	Sense	P.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A
	Antisense	U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A
10330	Sense	P.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A
	Antisense	U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C
10331	Sense	P.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G.A
	Antisense	U.C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A
10332	Sense	P.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G.A.A
	Antisense	U.U.C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G
10333	Sense	P.U.G.G.G.C.C.A.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U
	Antisense	A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U.U.G.G.C.C.C.A
10334	Sense	P.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U
	Antisense	A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C
10335	Sense	P.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U
	Antisense	A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C
10336	Sense	P.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G
	Antisense	C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C
10337	Sense	P.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U
	Antisense	A.G.C.A.G.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A
10338	Sense	P.G.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G
	Antisense	C.A.U.C.U.U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C.C
10339	Sense	P.G.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G.G
	Antisense	C.C.A.U.C.U.U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G.C
10340	Sense	P.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G
	Antisense	C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A

10341	Sense	P.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U
	Antisense	A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C
10342	Sense	P.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U
	Antisense	A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C
10343	Sense	P.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U
	Antisense	A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G
10344	Sense	P.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U
	Antisense	A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U
10345	Sense	P.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U
	Antisense	A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U
10346	Sense	P.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G
	Antisense	C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C
10347	Sense	P.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A
	Antisense	U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A
10348	Sense	P.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A
	Antisense	U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C
10349	Sense	P.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A
	Antisense	U.G.A.G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A
10350	Sense	P.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A.G
	Antisense	C.U.G.A.G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C
10351	Sense	P.U.C.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U
	Antisense	A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G.A.G.A
10352	Sense	P.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A.U
	Antisense	A.U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G
10353	Sense	P.A.C.C.A.U.U.G.C.A.U.C.A.U.U.G.G.C.C.G.C.A.C.A.C.U
	Antisense	A.G.U.G.U.G.C.G.G.C.C.A.A.U.G.A.U.G.C.A.A.U.G.G.U
10354	Sense	P.G.C.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A
	Antisense	U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C.A.U.C.U.G.C
10355	Sense	P.C.A.G.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A
	Antisense	U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C.A.U.C.U.G
10356	Sense	P.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G
	Antisense	C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C
10357	Sense	P.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G
	Antisense	C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U
10358	Sense	P.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A
	Antisense	U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C
10359	Sense	P.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G
	Antisense	C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C
10360	Sense	P.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U
	Antisense	A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U
10361	Sense	P.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A
	Antisense	U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U
10362	Sense	P.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U
	Antisense	A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U
10363	Sense	P.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U
	Antisense	A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U
10364	Sense	P.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U
	Antisense	A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U
10365	Sense	P.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U
	Antisense	A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C
10366	Sense	P.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G
	Antisense	C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C
10367	Sense	P.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U.A.A
	Antisense	U.U.A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C
10368	Sense	P.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U.A.A.U
	Antisense	A.U.U.A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C
10369	Sense	P.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U.A.A.U.U

	Antisense	A.A.U.U.A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U
10370	Sense	P.U.G.U.G.G.U.G.U.A.A.U.U.G.G.G.A.U.C.G.C.C.C.A.A.U
	Antisense	A.U.U.G.G.G.C.G.A.U.C.C.C.A.A.U.U.A.C.A.C.C.A.C.A
10371	Sense	P.U.G.G.U.G.U.A.A.U.U.G.G.G.A.U.C.G.C.C.C.A.A.U.A.A
	Antisense	U.U.A.U.U.G.G.G.C.G.A.U.C.C.C.A.A.U.U.A.C.A.C.C.A
10372	Sense	P.C.U.G.G.C.C.U.A.U.A.A.A.G.U.A.G.U.C.G.C.G.G.A.G.A
	Antisense	U.C.U.C.C.G.C.G.A.C.U.A.C.U.U.U.A.U.A.G.G.C.C.A.G
10373	Sense	P.G.G.C.C.A.G.A.G.U.G.G.G.C.G.A.G.G.C.G.C.G.G.A.G.G
	Antisense	C.C.U.C.C.G.C.G.C.C.U.C.G.C.C.C.A.C.U.C.U.G.G.C.C
10374	Sense	P.C.C.A.G.A.G.U.G.G.G.C.G.A.G.G.C.G.C.G.G.A.G.G.U.C
	Antisense	G.A.C.C.U.C.C.G.C.G.C.C.U.C.G.C.C.C.A.C.U.C.U.G.G
10375	Sense	P.A.G.U.G.G.G.C.G.A.G.G.C.G.C.G.G.A.G.G.U.C.U.G.G.C
	Antisense	G.C.C.A.G.A.C.C.U.C.C.G.C.G.C.C.U.C.G.C.C.C.A.C.U
10376	Sense	P.G.G.C.G.A.G.G.C.G.C.G.G.A.G.G.U.C.U.G.G.C.C.U.A.U
	Antisense	A.U.A.G.G.C.C.A.G.A.C.C.U.C.C.G.C.G.C.C.U.C.G.C.C
10377	Sense	P.A.G.G.C.G.C.G.G.A.G.G.U.C.U.G.G.C.C.U.A.U.A.A.A.G
	Antisense	C.U.U.U.A.U.A.G.G.C.C.A.G.A.C.C.U.C.C.G.C.G.C.C.U
10378	Sense	P.G.C.G.G.A.G.G.U.C.U.G.G.C.C.U.A.U.A.A.A.G.U.A.G.U
	Antisense	A.C.U.A.C.U.U.U.A.U.A.G.G.C.C.A.G.A.C.C.U.C.C.G.C
10379	Sense	P.A.G.G.U.C.U.G.G.C.C.U.A.U.A.A.A.G.U.A.G.U.C.G.C.G
	Antisense	C.G.C.G.A.C.U.A.C.U.U.U.A.U.A.G.G.C.C.A.G.A.C.C.U
10380	Sense	P.G.C.C.U.A.U.A.A.A.G.U.A.G.U.C.G.C.G.G.A.G.A.C.G.G
	Antisense	C.C.G.U.C.U.C.C.G.C.G.A.C.U.A.C.U.U.U.A.U.A.G.G.C
10381	Sense	P.G.G.U.G.C.U.G.G.U.U.U.G.C.G.U.C.G.U.A.G.U.C.U.C.C
	Antisense	G.G.A.G.A.C.U.A.C.G.A.C.G.C.A.A.A.C.C.A.G.C.A.C.C
10382	Sense	P.C.U.G.G.U.U.U.G.C.G.U.C.G.U.A.G.U.C.U.C.C.U.G.C.A
	Antisense	U.G.C.A.G.G.A.G.A.C.U.A.C.G.A.C.G.C.A.A.A.C.C.A.G
10383	Sense	P.U.U.U.G.C.G.U.C.G.U.A.G.U.C.U.C.C.U.G.C.A.G.C.G.U
	Antisense	A.C.G.C.U.G.C.A.G.G.A.G.A.C.U.A.C.G.A.C.G.C.A.A.A
10384	Sense	P.C.G.U.C.G.U.A.G.U.C.U.C.C.U.G.C.A.G.C.G.U.C.U.G.G
	Antisense	C.C.A.G.A.C.G.C.U.G.C.A.G.G.A.G.A.C.U.A.C.G.A.C.G
10385	Sense	P.G.G.U.U.U.C.C.G.U.U.G.C.A.G.U.C.C.U.C.G.G.A.A.C.C
	Antisense	G.G.U.U.C.C.G.A.G.G.A.C.U.G.C.A.A.C.G.G.A.A.A.C.C
10386	Sense	P.U.C.C.G.U.U.G.C.A.G.U.C.C.U.C.G.G.A.A.C.C.A.G.G.A
	Antisense	U.C.C.U.G.G.U.U.C.C.G.A.G.G.A.C.U.G.C.A.A.C.G.G.A
10387	Sense	P.U.U.G.C.A.G.U.C.C.U.C.G.G.A.A.C.C.A.G.G.A.C.C.U.C
	Antisense	G.A.G.G.U.C.C.U.G.G.U.U.C.C.G.A.G.G.A.C.U.G.C.A.A
10388	Sense	P.A.G.U.C.C.U.C.G.G.A.A.C.C.A.G.G.A.C.C.U.C.G.G.C.G
	Antisense	C.G.C.C.G.A.G.G.U.C.C.U.G.G.U.U.C.C.G.A.G.G.A.C.U
10389	Sense	P.C.U.C.G.G.A.A.C.C.A.G.G.A.C.C.U.C.G.G.C.G.U.G.G.C
	Antisense	G.C.C.A.C.G.C.C.G.A.G.G.U.C.C.U.G.G.U.U.C.C.G.A.G
10390	Sense	P.G.A.A.C.C.A.G.G.A.C.C.U.C.G.G.C.G.U.G.G.C.C.U.A.G
	Antisense	C.U.A.G.G.C.C.A.C.G.C.C.G.A.G.G.U.C.C.U.G.G.U.U.C
10391	Sense	P.C.A.G.G.A.C.C.U.C.G.G.C.G.U.G.G.C.C.U.A.G.C.G.A.G
	Antisense	C.U.C.G.C.U.A.G.G.C.C.A.C.G.C.C.G.A.G.G.U.C.C.U.G
10392	Sense	P.A.C.C.U.C.G.G.C.G.U.G.G.C.C.U.A.G.C.G.A.G.U.U.A.U
	Antisense	A.U.A.A.C.U.C.G.C.U.A.G.G.C.C.A.C.G.C.C.G.A.G.G.U
10393	Sense	P.C.G.G.C.G.U.G.G.C.C.U.A.G.C.G.A.G.U.U.A.U.G.G.C.G
	Antisense	C.G.C.C.A.U.A.A.C.U.C.G.C.U.A.G.G.C.C.A.C.G.C.C.G
10394	Sense	P.G.U.G.G.C.C.U.A.G.C.G.A.G.U.U.A.U.G.G.C.G.A.C.G.A
	Antisense	U.C.G.U.C.G.C.C.A.U.A.A.C.U.C.G.C.U.A.G.G.C.C.A.C
10395	Sense	P.C.C.U.A.G.C.G.A.G.U.U.A.U.G.G.C.G.A.C.G.A.A.G.G.C
	Antisense	G.C.C.U.U.C.G.U.C.G.C.C.A.U.A.A.C.U.C.G.C.U.A.G.G
10396	Sense	P.G.C.G.A.G.U.U.A.U.G.G.C.G.A.C.G.A.A.G.G.C.C.G.U.G
	Antisense	C.A.C.G.G.C.C.U.U.C.G.U.C.G.C.C.A.U.A.A.C.U.C.G.C
10397	Sense	P.G.U.U.A.U.G.G.C.G.A.C.G.A.A.G.G.C.C.G.U.G.U.G.C.G
	Antisense	C.G.C.A.C.A.C.G.G.C.C.U.U.C.G.U.C.G.C.C.A.U.A.A.C

10398	Sense	P.U.G.G.C.G.A.C.G.A.A.G.G.C.C.G.U.G.U.G.C.G.U.G.C.U
	Antisense	A.G.C.A.C.G.C.A.C.A.C.G.G.C.C.U.U.C.G.U.C.G.C.C.A
10399	Sense	P.G.A.C.G.A.A.G.G.C.C.G.U.G.U.G.C.G.U.G.C.U.G.A.A.G
	Antisense	C.U.U.C.A.G.C.A.C.G.C.A.C.A.C.G.G.C.C.U.U.C.G.U.C
10400	Sense	P.A.C.G.A.A.G.G.C.C.G.U.G.U.G.C.G.U.G.C.U.G.A.A.G.G
	Antisense	C.C.U.U.C.A.G.C.A.C.G.C.A.C.A.C.G.G.C.C.U.U.C.G.U
10401	Sense	P.G.G.C.A.U.C.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G
	Antisense	C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U.G.A.U.G.C.C
10402	Sense	P.A.U.C.A.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A
	Antisense	U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U.U.G.A.U
10403	Sense	P.A.U.U.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G
	Antisense	C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A.A.A.U
10404	Sense	P.U.C.G.A.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C
	Antisense	G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C.U.C.G.A
10405	Sense	P.G.C.A.G.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G
	Antisense	C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U.C.U.G.C
10406	Sense	P.A.A.G.G.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G
	Antisense	C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U.C.C.U.U
10407	Sense	P.A.A.A.G.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G.U.G.U.G
	Antisense	C.A.C.A.C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A.C.U.U.U
10408	Sense	P.U.A.A.U.G.G.A.C.C.A.G.U.G.A.A.G.G.U.G.U.G.G.G.G.A
	Antisense	U.C.C.C.C.A.C.A.C.C.U.U.C.A.C.U.G.G.U.C.C.A.U.U.A
10409	Sense	P.G.G.A.A.G.C.A.U.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A.A.G
	Antisense	C.U.U.C.A.G.U.C.A.G.U.C.C.U.U.U.A.A.U.G.C.U.U.C.C
10410	Sense	P.G.C.A.U.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A.A.G.G.C.C.U
	Antisense	A.G.G.C.C.U.U.C.A.G.U.C.A.G.U.C.C.U.U.U.A.A.U.G.C
10411	Sense	P.U.A.A.A.G.G.A.C.U.G.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U
	Antisense	A.U.G.C.A.G.G.C.C.U.U.C.A.G.U.C.A.G.U.C.C.U.U.U.A
10412	Sense	P.G.G.A.C.U.G.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U
	Antisense	A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G.U.C.A.G.U.C.C
10413	Sense	P.U.G.A.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A
	Antisense	U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G.U.C.A
10414	Sense	P.C.U.G.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U
	Antisense	A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U.C.A.G
10415	Sense	P.A.A.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A
	Antisense	U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C.U.U
10416	Sense	P.G.G.C.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G
	Antisense	C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G.G.C.C
10417	Sense	P.C.U.G.C.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U
	Antisense	A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U.G.C.A.G
10418	Sense	P.A.U.G.G.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G
	Antisense	C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U.C.C.A.U
10419	Sense	P.A.U.U.C.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U
	Antisense	A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G.G.A.A.U
10420	Sense	P.C.A.U.G.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A.U.A
	Antisense	U.A.U.U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A.C.A.U.G
10421	Sense	P.U.U.C.A.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A.U.A.C.A.G.C
	Antisense	G.C.U.G.U.A.U.U.A.U.C.U.C.C.A.A.A.C.U.C.A.U.G.A.A
10422	Sense	P.U.G.A.G.U.U.U.G.G.A.G.A.U.A.A.U.A.C.A.G.C.A.G.G.C
	Antisense	G.C.C.U.G.C.U.G.U.A.U.U.A.U.C.U.C.C.A.A.A.C.U.C.A
10423	Sense	P.U.U.U.G.G.A.G.A.U.A.A.U.A.C.A.G.C.A.G.G.C.U.G.U.A
	Antisense	U.A.C.A.G.C.C.U.G.C.U.G.U.A.U.U.A.U.C.U.C.C.A.A.A
10424	Sense	P.G.A.G.A.U.A.A.U.A.C.A.G.C.A.G.G.C.U.G.U.A.C.C.A.G
	Antisense	C.U.G.G.U.A.C.A.G.C.C.U.G.C.U.G.U.A.U.U.A.U.C.U.C
10425	Sense	P.U.A.A.U.A.C.A.G.C.A.G.G.C.U.G.U.A.C.C.A.G.U.G.C.A
	Antisense	U.G.C.A.C.U.G.G.U.A.C.A.G.C.C.U.G.C.U.G.U.A.U.U.A
10426	Sense	P.A.C.A.G.C.A.G.G.C.U.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C

	Antisense	G.A.C.C.U.G.C.A.C.U.G.G.U.A.C.A.G.C.C.U.G.C.U.G.U
10427	Sense	P.C.A.G.G.C.U.G.U.A.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A
	Antisense	U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G.U.A.C.A.G.C.C.U.G
10428	Sense	P.C.C.A.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C
	Antisense	G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C.U.G.G
10429	Sense	P.G.U.G.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U
	Antisense	A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G.C.A.C
10430	Sense	P.C.A.G.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C
	Antisense	G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C.C.U.G
10431	Sense	P.G.U.C.C.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G
	Antisense	C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A.G.G.A.C
10432	Sense	P.U.C.A.C.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G.A.A.A.A
	Antisense	U.U.U.U.C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A.G.U.G.A
10433	Sense	P.U.U.U.A.A.U.C.C.U.C.U.A.U.C.C.A.G.A.A.A.A.C.A.C.G
	Antisense	C.G.U.G.U.U.U.U.C.U.G.G.A.U.A.G.A.G.G.A.U.U.A.A.A
10434	Sense	P.A.U.C.C.U.C.U.A.U.C.C.A.G.A.A.A.A.C.A.C.G.G.U.G.G
	Antisense	C.C.A.C.C.G.U.G.U.U.U.U.C.U.G.G.A.U.A.G.A.G.G.A.U
10435	Sense	P.A.A.G.G.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G
	Antisense	C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U.C.C.U.U
10436	Sense	P.A.U.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U
	Antisense	A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C.A.U
10437	Sense	P.G.A.A.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G
	Antisense	C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C.U.U.C
10438	Sense	P.G.A.G.A.G.G.C.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A
	Antisense	U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U.G.C.C.U.C.U.C
10439	Sense	P.A.U.G.U.U.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.C
	Antisense	G.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.A.A.C.A.U
10440	Sense	P.C.A.A.U.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G.G.U
	Antisense	A.C.C.A.U.C.U.U.U.G.U.C.A.G.C.A.G.U.C.A.C.A.U.U.G
10441	Sense	P.G.U.G.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G
	Antisense	C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A.G.C.A.G.U.C.A.C
10442	Sense	P.A.C.U.G.C.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G
	Antisense	C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A.G.C.A.G.U
10443	Sense	P.G.C.U.G.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G
	Antisense	C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U.C.A.G.C
10444	Sense	P.A.C.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C
	Antisense	G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U.G.U
10445	Sense	P.A.A.A.G.A.U.G.G.U.G.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A
	Antisense	U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C.A.U.C.U.U.U
10446	Sense	P.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U
	Antisense	A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U
10447	Sense	P.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C
	Antisense	G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A
10448	Sense	P.U.A.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C
	Antisense	G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A.U.A
10449	Sense	P.U.U.G.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C
	Antisense	G.A.G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U.C.A.A
10450	Sense	P.A.A.G.A.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A.G.G
	Antisense	C.C.U.G.A.G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A.U.C.U.U
10451	Sense	P.U.U.C.U.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C
	Antisense	G.U.C.U.C.C.U.G.A.G.A.G.U.G.A.G.A.U.C.A.C.A.G.A.A
10452	Sense	P.G.U.G.A.U.C.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U
	Antisense	A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G.A.G.A.U.C.A.C
10453	Sense	P.A.U.C.U.C.A.C.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A
	Antisense	U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A.G.U.G.A.G.A.U
10454	Sense	P.U.C.U.C.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A.U.U.G.G
	Antisense	C.C.A.A.U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U.G.A.G.A

10455	Sense	P.A.G.G.A.G.A.C.C.A.U.U.G.C.A.U.C.A.U.U.G.G.C.C.G.C
	Antisense	G.C.G.G.C.C.A.A.U.G.A.U.G.C.A.A.U.G.G.U.C.U.C.C.U
10456	Sense	P.G.A.C.C.A.U.U.G.C.A.U.C.A.U.U.G.G.C.C.G.C.A.C.A.C
	Antisense	G.U.G.U.G.C.G.G.C.C.A.A.U.G.A.U.G.C.A.A.U.G.G.U.C
10464	Sense	P.A.A.A.G.A.A.U.C.C.A.A.A.U.U.C.A.A.A.C.U.A.A.A.A.A
	Antisense	U.U.U.U.U.A.G.U.U.U.G.A.A.U.U.U.G.G.A.U.U.C.U.U.U
10465	Sense	P.A.U.U.A.A.A.A.G.A.A.U.C.C.A.A.A.U.U.C.A.A.A.C.U.A
	Antisense	A.U.U.A.A.A.A.G.A.A.U.C.C.A.A.A.U.U.C.A.A.A.C.U.A
10466	Sense	P.G.G.C.U.A.U.U.A.A.A.A.G.A.A.U.C.C.A.A.A.U.U.C.A.A
	Antisense	U.U.G.A.A.U.U.U.G.G.A.U.U.C.U.U.U.U.A.A.U.A.G.C.C
10467	Sense	P.A.U.G.A.G.G.C.U.A.U.U.A.A.A.A.G.A.A.U.C.C.A.A.A.U
	Antisense	A.U.U.U.G.G.A.U.U.C.U.U.U.U.A.A.U.A.G.C.C.U.C.A.U
10468	Sense	P.U.A.U.U.A.U.G.A.G.G.C.U.A.U.U.A.A.A.A.G.A.A.U.C.C
	Antisense	G.G.A.U.U.C.U.U.U.U.A.A.U.A.G.C.C.U.C.A.U.A.A.U.A
10469	Sense	P.C.A.C.U.U.A.U.U.A.U.G.A.G.G.C.U.A.U.U.A.A.A.A.G.A
	Antisense	U.C.U.U.U.U.A.A.U.A.G.C.C.U.C.A.U.A.A.U.A.A.G.U.G
10470	Sense	P.A.U.G.G.C.A.C.U.U.A.U.U.A.U.G.A.G.G.C.U.A.U.U.A.A
	Antisense	U.U.A.A.U.A.G.C.C.U.C.A.U.A.A.U.A.A.G.U.G.C.C.A.U
10471	Sense	P.C.U.G.U.A.U.G.G.C.A.C.U.U.A.U.U.A.U.G.A.G.G.C.U.A
	Antisense	U.A.G.C.C.U.C.A.U.A.A.U.A.A.G.U.G.C.C.A.U.A.C.A.G
10472	Sense	P.U.C.A.U.U.C.A.A.G.C.C.U.G.U.G.A.A.U.A.A.A.A.A.C.C
	Antisense	G.G.U.U.U.U.U.A.U.U.C.A.C.A.G.G.C.U.U.G.A.A.U.G.A
10473	Sense	P.U.G.U.C.A.U.U.C.A.A.G.C.C.U.G.U.G.A.A.U.A.A.A.A.A
	Antisense	U.U.U.U.U.A.U.U.C.A.C.A.G.G.C.U.U.G.A.A.U.G.A.C.A
10474	Sense	P.U.U.U.G.U.C.A.U.U.C.A.A.G.C.C.U.G.U.G.A.A.U.A.A.A
	Antisense	U.U.U.A.U.U.C.A.C.A.G.G.C.U.U.G.A.A.U.G.A.C.A.A.A
10475	Sense	P.U.C.U.U.U.G.U.C.A.U.U.C.A.A.G.C.C.U.G.U.G.A.A.U.A
	Antisense	U.A.U.U.C.A.C.A.G.G.C.U.U.G.A.A.U.G.A.C.A.A.A.G.A
10476	Sense	P.U.U.U.C.U.U.U.G.U.C.A.U.U.C.A.A.G.C.C.U.G.U.G.A.A
	Antisense	U.U.C.A.C.A.G.G.C.U.U.G.A.A.U.G.A.C.A.A.A.G.A.A.A
10477	Sense	P.A.G.A.A.U.U.U.C.U.U.U.G.U.C.A.U.U.C.A.A.G.C.C.U.G
	Antisense	C.A.G.G.C.U.U.G.A.A.U.G.A.C.A.A.A.G.A.A.A.U.U.C.U
10478	Sense	P.U.G.U.C.A.G.A.A.U.U.U.C.U.U.U.G.U.C.A.U.U.C.A.A.G
	Antisense	C.U.U.G.A.A.U.G.A.C.A.A.A.G.A.A.A.U.U.C.U.G.A.C.A
10479	Sense	P.A.A.C.U.U.G.U.C.A.G.A.A.U.U.U.C.U.U.U.G.U.C.A.U.U
	Antisense	A.A.U.G.A.C.A.A.A.G.A.A.A.U.U.C.U.G.A.C.A.A.G.U.U
10480	Sense	P.A.U.U.A.A.A.C.U.U.G.U.C.A.G.A.A.U.U.U.C.U.U.U.G.U
	Antisense	A.C.A.A.A.G.A.A.A.U.U.C.U.G.A.C.A.A.G.U.U.U.A.A.U
10481	Sense	P.G.G.U.A.U.U.A.A.A.C.U.U.G.U.C.A.G.A.A.U.U.U.C.U.U
	Antisense	A.A.G.A.A.A.U.U.C.U.G.A.C.A.A.G.U.U.U.A.A.U.A.C.C
10482	Sense	P.U.U.U.G.C.C.A.G.A.C.U.U.A.A.A.U.C.A.C.A.G.A.U.G.G
	Antisense	C.C.A.U.C.U.G.U.G.A.U.U.U.A.A.G.U.C.U.G.G.C.A.A.A
10483	Sense	P.A.U.U.U.U.G.C.C.A.G.A.C.U.U.A.A.A.U.C.A.C.A.G.A.U
	Antisense	A.U.C.U.G.U.G.A.U.U.U.A.A.G.U.C.U.G.G.C.A.A.A.A.U
10484	Sense	P.G.U.A.U.U.U.U.G.C.C.A.G.A.C.U.U.A.A.A.U.C.A.C.A.G
	Antisense	C.U.G.U.G.A.U.U.U.A.A.G.U.C.U.G.G.C.A.A.A.A.U.A.C
10485	Sense	P.C.U.G.U.A.U.U.U.U.G.C.C.A.G.A.C.U.U.A.A.A.U.C.A.C
	Antisense	G.U.G.A.U.U.U.A.A.G.U.C.U.G.G.C.A.A.A.A.U.A.C.A.G
10486	Sense	P.A.C.C.U.G.U.A.U.U.U.U.G.C.C.A.G.A.C.U.U.A.A.A.U.C
	Antisense	G.A.U.U.U.A.A.G.U.C.U.G.G.C.A.A.A.A.U.A.C.A.G.G.U
10487	Sense	P.A.A.U.G.A.C.C.U.G.U.A.U.U.U.U.G.C.C.A.G.A.C.U.U.A
	Antisense	U.A.A.G.U.C.U.G.G.C.A.A.A.A.U.A.C.A.G.G.U.C.A.U.U
10488	Sense	P.U.U.U.C.A.A.U.G.A.C.C.U.G.U.A.U.U.U.U.G.C.C.A.G.A
	Antisense	U.C.U.G.G.C.A.A.A.A.U.A.C.A.G.G.U.C.A.U.U.G.A.A.A
10489	Sense	P.U.C.U.G.U.U.U.C.A.A.U.G.A.C.C.U.G.U.A.U.U.U.U.G.C
	Antisense	G.C.A.A.A.A.U.A.C.A.G.G.U.C.A.U.U.G.A.A.A.C.A.G.A
10490	Sense	P.A.A.U.G.U.C.U.G.U.U.U.C.A.A.U.G.A.C.C.U.G.U.A.U.U

	Antisense	A.A.U.A.C.A.G.G.U.C.A.U.U.G.A.A.A.C.A.G.A.C.A.U.U
10491	Sense	P.U.U.A.A.A.A.U.G.U.C.U.G.U.U.U.C.A.A.U.G.A.C.C.U.G
	Antisense	C.A.G.G.U.C.A.U.U.G.A.A.A.C.A.G.A.C.A.U.U.U.U.A.A
10492	Sense	P.U.C.A.G.U.U.A.A.A.A.U.G.U.C.U.G.U.U.U.C.A.A.U.G.A
	Antisense	U.C.A.U.U.G.A.A.A.C.A.G.A.C.A.U.U.U.U.A.A.C.U.G.A
10493	Sense	P.A.A.A.C.U.C.A.G.U.U.A.A.A.A.U.G.U.C.U.G.U.U.U.C.A
	Antisense	U.G.A.A.A.C.A.G.A.C.A.U.U.U.U.A.A.C.U.G.A.G.U.U.U
10494	Sense	P.U.A.U.A.A.A.A.C.U.C.A.G.U.U.A.A.A.A.A.U.G.U.C.U.G.U
	Antisense	A.C.A.G.A.C.A.U.U.U.U.A.A.C.U.G.A.G.U.U.U.U.A.U.A
10495	Sense	P.G.U.U.U.U.A.U.A.A.A.A.C.U.C.A.G.U.U.A.A.A.A.U.G.U
	Antisense	A.C.A.U.U.U.U.A.A.C.U.G.A.G.U.U.U.U.A.U.A.A.A.A.C
10496	Sense	P.U.A.U.A.G.U.U.U.U.A.U.A.A.A.A.C.U.C.A.G.U.U.A.A.A
	Antisense	U.U.U.A.A.C.U.G.A.G.U.U.U.U.A.U.A.A.A.A.C.U.A.U.A
10497	Sense	P.U.U.U.G.U.A.U.A.G.U.U.U.U.A.U.A.A.A.A.C.U.C.A.G.U
	Antisense	A.C.U.G.A.G.U.U.U.U.A.U.A.A.A.A.C.U.A.U.A.C.A.A.A
10498	Sense	P.A.A.G.A.U.U.U.G.U.A.U.A.G.U.U.U.U.A.U.A.A.A.A.C.U
	Antisense	A.G.U.U.U.U.A.U.A.A.A.A.C.U.A.U.A.C.A.A.A.U.C.U.U
10499	Sense	P.U.U.G.G.A.A.G.A.U.U.U.G.U.A.U.A.G.U.U.U.U.A.U.A.A
	Antisense	U.U.A.U.A.A.A.A.C.U.A.U.A.C.A.A.A.U.C.U.U.C.C.A.A
10500	Sense	P.U.C.A.C.U.U.G.G.A.A.G.A.U.U.U.G.U.A.U.A.G.U.U.U.U
	Antisense	A.A.A.A.C.U.A.U.A.C.A.A.A.U.C.U.U.C.C.A.A.G.U.G.A
10501	Sense	P.A.U.G.A.U.C.A.C.U.U.G.G.A.A.G.A.U.U.U.G.U.A.U.A.G
	Antisense	C.U.A.U.A.C.A.A.A.U.C.U.U.C.C.A.A.G.U.G.A.U.C.A.U
10502	Sense	P.A.U.U.U.A.U.G.A.U.C.A.C.U.U.G.G.A.A.G.A.U.U.U.G.U
	Antisense	A.C.A.A.A.U.C.U.U.C.C.A.A.G.U.G.A.U.C.A.U.A.A.A.U
10503	Sense	P.A.C.U.G.A.U.U.U.A.U.G.A.U.C.A.C.U.U.G.G.A.A.G.A.U
	Antisense	A.U.C.U.U.C.C.A.A.G.U.G.A.U.C.A.U.A.A.A.U.C.A.G.U
10504	Sense	P.A.G.A.A.A.C.U.G.A.U.U.U.A.U.G.A.U.C.A.C.U.U.G.G.A
	Antisense	U.C.C.A.A.G.U.G.A.U.C.A.U.A.A.A.U.C.A.G.U.U.U.C.U
10505	Sense	P.A.G.U.G.A.G.A.A.A.C.U.G.A.U.U.U.A.U.G.A.U.C.A.C.U
	Antisense	A.G.U.G.A.U.C.A.U.A.A.A.U.C.A.G.U.U.U.C.U.C.A.C.U
10506	Sense	P.C.U.G.U.A.G.U.G.A.G.A.A.A.C.U.G.A.U.U.U.A.U.G.A.U
	Antisense	A.U.C.A.U.A.A.A.U.C.A.G.U.U.U.C.U.C.A.C.U.A.C.A.G
10507	Sense	P.G.U.A.C.C.U.G.U.A.G.U.G.A.G.A.A.A.C.U.G.A.U.U.U.A
	Antisense	U.A.A.A.U.C.A.G.U.U.U.C.U.C.A.C.U.A.C.A.G.G.U.A.C
10508	Sense	P.U.A.A.A.G.U.A.C.C.U.G.U.A.G.U.G.A.G.A.A.A.C.U.G.A
	Antisense	U.C.A.G.U.U.U.C.U.C.A.C.U.A.C.A.G.G.U.A.C.U.U.U.A
10509	Sense	P.G.C.U.U.U.A.A.A.G.U.A.C.C.U.G.U.A.G.U.G.A.G.A.A.A
	Antisense	U.U.U.C.U.C.A.C.U.A.C.A.G.G.U.A.C.U.U.U.A.A.A.G.C
10510	Sense	P.A.G.U.U.G.C.U.U.U.A.A.A.G.U.A.C.C.U.G.U.A.G.U.G.A
	Antisense	U.C.A.C.U.A.C.A.G.G.U.A.C.U.U.U.A.A.A.G.C.A.A.C.U
10511	Sense	P.U.C.A.G.A.G.U.U.G.C.U.U.U.A.A.A.G.U.A.C.C.U.G.U.A
	Antisense	U.A.C.A.G.G.U.A.C.U.U.U.A.A.A.G.C.A.A.C.U.C.U.G.A
10512	Sense	P.U.U.U.U.U.C.A.G.A.G.U.U.G.C.U.U.U.A.A.A.G.U.A.C.C
	Antisense	G.G.U.A.C.U.U.U.A.A.A.G.C.A.A.C.U.C.U.G.A.A.A.A.A
10513	Sense	P.U.G.A.C.U.U.U.U.U.C.A.G.A.G.U.U.G.C.U.U.U.A.A.A.G
	Antisense	C.U.U.U.A.A.A.G.C.A.A.C.U.C.U.G.A.A.A.A.A.G.U.C.A
10514	Sense	P.U.G.U.G.U.G.A.C.U.U.U.U.U.C.A.G.A.G.U.U.G.C.U.U.U
	Antisense	A.A.A.G.C.A.A.C.U.C.U.G.A.A.A.A.A.G.U.C.A.C.A.C.A
10515	Sense	P.U.A.A.U.U.G.U.G.U.G.A.C.U.U.U.U.U.C.A.G.A.G.U.U.G
	Antisense	C.A.A.C.U.C.U.G.A.A.A.A.A.G.U.C.A.C.A.C.A.A.U.U.A
10516	Sense	P.A.G.U.G.U.A.A.U.U.G.U.G.U.G.A.C.U.U.U.U.U.C.A.G.A
	Antisense	U.C.U.G.A.A.A.A.A.G.U.C.A.C.A.C.A.A.U.U.A.C.A.C.U
10517	Sense	P.U.A.A.A.A.G.U.G.U.A.A.U.U.G.U.G.U.G.A.C.U.U.U.U.U
	Antisense	A.A.A.A.A.G.U.C.A.C.A.C.A.A.U.U.A.C.A.C.U.U.U.U.A
10518	Sense	P.A.U.C.U.U.A.A.A.A.G.U.G.U.A.A.U.U.G.U.G.U.G.A.C.U
	Antisense	A.G.U.C.A.C.A.C.A.A.U.U.A.C.A.C.U.U.U.U.A.A.G.A.U

10519	Sense	P.U.G.U.A.A.U.C.U.U.A.A.A.A.G.U.G.U.A.A.U.U.G.U.G.U
	Antisense	A.C.A.C.A.A.U.U.A.C.A.C.U.U.U.U.A.A.G.A.U.U.A.C.A
10520	Sense	P.A.C.A.C.U.G.U.A.A.U.C.U.U.A.A.A.A.G.U.G.U.A.A.U.U
	Antisense	A.A.U.U.A.C.A.C.U.U.U.U.A.A.G.A.U.U.A.C.A.G.U.G.U
10521	Sense	P.U.U.A.A.A.C.A.C.U.G.U.A.A.U.C.U.U.A.A.A.A.G.U.G.U
	Antisense	A.C.A.C.U.U.U.U.A.A.G.A.U.U.A.C.A.G.U.G.U.U.U.A.A
10522	Sense	P.A.A.C.A.U.U.A.A.A.C.A.C.U.G.U.A.A.U.C.U.U.A.A.A.A
	Antisense	U.U.U.U.A.A.G.A.U.U.A.C.A.G.U.G.U.U.U.A.A.U.G.U.U
10523	Sense	P.G.A.U.A.A.A.C.A.U.U.A.A.A.C.A.C.U.G.U.A.A.U.C.U.U
	Antisense	A.A.G.A.U.U.A.C.A.G.U.G.U.U.U.A.A.U.G.U.U.U.A.U.C
10524	Sense	P.U.C.C.U.G.A.U.A.A.A.C.A.U.U.A.A.A.C.A.C.U.G.U.A.A
	Antisense	U.U.A.C.A.G.U.G.U.U.U.A.A.U.G.U.U.U.A.U.C.A.G.G.A
10525	Sense	P.U.G.U.A.U.C.C.U.G.A.U.A.A.A.C.A.U.U.A.A.A.C.A.C.U
	Antisense	A.G.U.G.U.U.U.A.A.U.G.U.U.U.A.U.C.A.G.G.A.U.A.C.A
10526	Sense	P.G.A.A.A.U.G.U.A.U.C.C.U.G.A.U.A.A.A.C.A.U.U.A.A.A
	Antisense	U.U.U.A.A.U.G.U.U.U.A.U.C.A.G.G.A.U.A.C.A.U.U.U.C
10527	Sense	P.U.G.U.A.G.A.A.A.U.G.U.A.U.C.C.U.G.A.U.A.A.A.C.A.U
	Antisense	A.U.G.U.U.U.A.U.C.A.G.G.A.U.A.C.A.U.U.U.C.U.A.C.A
10528	Sense	P.U.A.G.C.U.G.U.A.G.A.A.A.U.G.U.A.U.C.C.U.G.A.U.A.A
	Antisense	U.U.A.U.C.A.G.G.A.U.A.C.A.U.U.U.C.U.A.C.A.G.C.U.A
10529	Sense	P.C.U.G.C.U.A.G.C.U.G.U.A.G.A.A.A.U.G.U.A.U.C.C.U.G
	Antisense	C.A.G.G.A.U.A.C.A.U.U.U.C.U.A.C.A.G.C.U.A.G.C.A.G
10530	Sense	P.U.A.U.C.C.U.G.C.U.A.G.C.U.G.U.A.G.A.A.A.U.G.U.A.U
	Antisense	A.U.A.C.A.U.U.U.C.U.A.C.A.G.C.U.A.G.C.A.G.G.A.U.A
10531	Sense	P.C.U.G.U.U.A.U.C.C.U.G.C.U.A.G.C.U.G.U.A.G.A.A.A.U
	Antisense	A.U.U.U.C.U.A.C.A.G.C.U.A.G.C.A.G.G.A.U.A.A.C.A.G
10532	Sense	P.U.C.A.U.C.U.G.U.U.A.U.C.C.U.G.C.U.A.G.C.U.G.U.A.G
	Antisense	C.U.A.C.A.G.C.U.A.G.C.A.G.G.A.U.A.A.C.A.G.A.U.G.A
10533	Sense	P.U.A.A.C.U.C.A.U.C.U.G.U.U.A.U.C.C.U.G.C.U.A.G.C.U
	Antisense	A.G.C.U.A.G.C.A.G.G.A.U.A.A.C.A.G.A.U.G.A.G.U.U.A
10534	Sense	P.C.C.U.U.A.A.C.U.C.A.U.C.U.G.U.U.A.U.C.C.U.G.C.U.A
	Antisense	U.A.G.C.A.G.G.A.U.A.A.C.A.G.A.U.G.A.G.U.U.A.A.G.G
10535	Sense	P.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U.A.A.U.U.G.G
	Antisense	C.C.A.A.U.U.A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C
10536	Sense	P.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U.G.G.U.G.U
	Antisense	A.C.A.C.C.A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G
10537	Sense	P.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C.U.U.G.U
	Antisense	A.C.A.A.G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U
10538	Sense	P.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G.G.C
	Antisense	G.C.C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G
10539	Sense	P.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U.U.U.G
	Antisense	C.A.A.A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C
10540	Sense	P.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C.G.U
	Antisense	A.C.G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U
10541	Sense	P.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G.U.C
	Antisense	G.A.C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U
10542	Sense	P.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A.A.G
	Antisense	C.U.U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C
10543	Sense	P.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G.A
	Antisense	U.C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U
10544	Sense	P.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G.G
	Antisense	C.C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U
10545	Sense	P.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C.G.C.U.G
	Antisense	C.A.G.C.G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C
10546	Sense	P.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G.A.A.A.C
	Antisense	G.U.U.U.C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A
10547	Sense	P.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A.G.A.C.A.G.G

	Antisense	C.C.U.G.U.C.U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C
10548	Sense	P.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.C.A.A.A
	Antisense	U.U.U.G.U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U
10549	Sense	P.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A
	Antisense	U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A
10550	Sense	P.A.U.G.A.C.U.U.G.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A
	Antisense	U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C.C.A.A.G.U.C.A.U
10551	Sense	P.G.U.C.C.A.U.G.A.A.A.A.A.G.C.A.G.A.U.G.A.C.U.U.G.G
	Antisense	C.C.A.A.G.U.C.A.U.C.U.G.C.U.U.U.U.U.C.A.U.G.G.A.C
10552	Sense	P.G.G.U.G.G.U.C.C.A.U.G.A.A.A.A.A.G.C.A.G.A.U.G.A.C
	Antisense	G.U.C.A.U.C.U.G.C.U.U.U.U.U.C.A.U.G.G.A.C.C.A.C.C
10553	Sense	P.C.A.C.U.G.G.U.G.G.U.C.C.A.U.G.A.A.A.A.A.G.C.A.G.A
	Antisense	U.C.U.G.C.U.U.U.U.U.C.A.U.G.G.A.C.C.A.C.C.A.G.U.G
10554	Sense	P.G.C.A.C.A.C.U.G.G.U.G.G.U.C.C.A.U.G.A.A.A.A.A.G.C
	Antisense	G.C.U.U.U.U.U.C.A.U.G.G.A.C.C.A.C.C.A.G.U.G.U.G.C
10555	Sense	P.U.G.G.C.C.G.C.A.C.A.C.U.G.G.U.G.G.U.C.C.A.U.G.A.A
	Antisense	U.U.C.A.U.G.G.A.C.C.A.C.C.A.G.U.G.U.G.C.G.G.C.C.A

We Claim:

1. A double-stranded RNA (dsRNA) construct of 12-49 nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising:

(1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and,

(2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand includes a 2'-modified ribose sugar at the 2nd nucleotide from the 5'-end of the antisense strand,

wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

2. A double-stranded RNA (dsRNA) construct of 12-49 nucleotides in length, for inhibiting expression of a target gene, said dsRNA comprising:

(1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and,

(2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand comprises, at the 3'-end of the antisense strand,

(i) at least four consecutive 2'-modified ribose sugars with non-hydrolyzable internucleotide linkages,

(ii) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 2'-modified ribose sugars, preferably 2'-O-methyl modified ribose sugars, or,

(iii) a protective group,

wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

3. A double-stranded RNA (dsRNA) construct of 12-49 nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising:

(1) a sense strand having a 5'-end and a 3'-end, wherein one or more nucleotides at each of said 5'- and 3'-ends of said sense strand have 2'-modified ribose sugars, and said sense strand comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand, and,

(2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene,

wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

4. A double-stranded RNA (dsRNA) construct of 12-49 nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising:

(1) a sense strand having a 5'-end and a 3'-end, wherein four consecutive 2'-O-methyl nucleotides are present at each of said 5'- and 3'-ends of said sense strand, and,

(2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand:

(a) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages; or,

(b) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides,

wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

5. A double-stranded RNA (dsRNA) construct of 12-49 nucleotides in length, for inhibiting expression of a target gene, the dsRNA comprising:

(1) a sense strand having a 5'-end and a 3'-end, wherein the sense strand comprises 12 and 10 consecutive 2'-O-methyl nucleotides at the 5'-end and the 3'-end, respectively, and,

(2) an antisense strand having a 5'-end and a 3'-end, which hybridizes to said sense strand and to mRNA of said target gene, wherein said antisense strand:

(a) is unmodified;

(b) comprises four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages; or,

(c) comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end and no other modified nucleotides,

wherein (a) said dsRNA is resistant to cleavage by Dicer, (b) the antisense strand associates with RISC, and (c) the dsRNA inhibits expression of the target gene in a sequence-dependent manner.

6. The dsDNA of any one of claims 1-5, wherein the antisense strand directs the uniform cleavage of the target gene mRNA at a single site between the 10^{th} and 11^{th} nucleotides from the 5'-end of the antisense strand.

7. The dsDNA of any one of claims 1-5, wherein the sense strand of the dsRNA is cleavable by RISC at a single site between the 10th and the 11th nucleotides from the 3'-end of the sense strand.

8. The dsDNA of any one of claims 1-5, wherein the dsRNA construct is blunt-ended.

9. The dsDNA of any one of claims 1-4, wherein the 5'-end 12 nucleotides and the 3'-end 10 nucleotides of the sense strand are 2'-modified ribose sugars.

10. The dsDNA of any one of claims 1-5, wherein each end of the sense strand comprises a continuous stretch of 2'-modified ribose sugars.

11. The dsDNA of any one of claims 1-5, wherein each end of the sense strand comprises a continuous stretch of four 2'-modified ribose sugars.

12. The dsDNA of any one of claims 1-3, wherein the antisense strand comprises discontinuous 2'-modified ribose sugars, wherein the 10th and 11th antisense nucleotides are not modified.

13. The dsRNA of claim 12, wherein the antisense strand comprises 2'-modified ribose sugars for each 2, 3, 4, 5, 6, 7, 8, or 9 nucleotides.

14. The dsRNA of claim 13, wherein the most 5'-end 2'-modified ribose sugar on the antisense strand is the 2nd nucleotide.

15. The dsRNA of any one of claims 1-5, wherein the dsRNA construct is: 12-35 nucleotides in length; 25-30 nucleotides in length; 25, 26, 27, 28, 29, or 30 nucleotides in length; >22 nucleotides in length; >25 nucleotides in length; or 31-49 nucleotides in length.

16. The dsRNA of any one of claims 1-5, wherein each end of the sense strand comprises, independently, 4-16 2'-modified ribose sugars and/or non-hydrolyzable internucleotide linkages.

17. The dsRNA of claim 16, wherein each end of the sense strand comprises a symetrical or an asymetrical number of 2'-modified ribose sugars.

18. The dsRNA of claim 16, wherein the 2'-modified ribose sugars are 2'-O-alkyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy nucleotides, 2'-H (deoxyribonucleotides), or combination thereof.

19. The dsRNA of claim 18, wherein the 2'-O-alkyl nucleotides are 2'-O-methyl nucleotides.

20. The dsRNA of claim 18, wherein the 2'-O-alkyl nucleotides are 2'-O-allyl nucleotides.

21. The dsRNA of any one of claims 1 and 3-5, wherein the antisense strand comprises a 2'-O-methyl modified nucleotide at the 2nd nucleotide on the 5'-end of the antisense strand and no other modified nucleotides.

22. The dsRNA of claim 21, wherein the dsRNA has enhanced target specificty or reduced off-target silencing compared to a similar construct without the 2'-modification at said position(s).

23. The dsRNA of any one of claims 1, and 3-5, wherein the antisense strand comprises at least four consecutive 2'-O-methyl modified 3'-end nucleotides with phosphothioate linkages.

24. The dsRNA of any one of claims 1, 4, and 5, wherein the sense strand of the dsRNA comprises a mismatch nucleotide at the 2nd nucleotide from the 3'-end of the sense strand.

25. The dsRNA of any of claims 1-5, wherein the dsRNA has improved stability in serum and/or cerebral spinal fluid compared to an unmodified dsRNA having the same sequence.

26. The dsRNA of any of claims 1-5, wherein the last 2nd-8th nucleotides at the 3'-end of the sense strand mis-match their corresponding antisense strand nucleotides.

27. The dsRNA of any of claims 1-5, wherein the dsRNA does not induce interferon response in primary cells.

28. The dsRNA of any of claims 1-5, wherein either end of the sense strand and/or the 3'-end of the antisense strand is blocked by a protective group.

29. The dsRNA of claim 28, wherein the protective group is an inverted nucleotide, an inverted abasic moiety, or an amino-end modified nucleotide.

30. The dsRNA of claim 29, wherein said inverted nucleotide comprises an inverted deoxynucleotide.

31. The dsRNA of claim 29, wherein said inverted abasic moiety comprises an inverted deoxyabasic moiety.

32. The dsRNA of claim 31, wherein said inverted deoxyabasic moiety is a 3',3'-linked or 5',5'-linked deoxyabasic moiety.

33. The dsRNA of claim 1 or 3, wherein alternating nucleotides on the ends of the sense and/or antisense strands comprise 2'-modified ribose sugars, and wherein each of the 2'-modified ribose sugars faces an unmodified nucleotide on the opposite strand.

34. The dsRNA of claim 33, wherein the first 2'-modified antisense nucleotide is the most 5'-end antisense nucleotide or the 2nd nucleotide from the 5'-end of the antisense strand.

35. The dsRNA of any of claims 1-5, wherein the target gene is SOD1, PPIB, RIP140, PCSK9, TNFα, AP2 (adipocyte lipid-binding protein), or MAP4K4.

36. The dsRNA of any of claims 1-5, wherein the sense strand nucleotides between the 2'-modified ribose nucleotides are 2'-F modified.

37. The dsRNA of any of claims 1-5, wherein the sense strand nucleotides between the 2'-modified ribose nucleotides are purine nucleotides, optionally having 2'-F modification and/or phosphorothioate linkage.

38. The dsRNA of any of claims 1-5, wherein the sense strand nucleotides between the 2'-modified ribose nucleotides form one or more bulges of 1-5 nucleotides each.

39. The dsRNA of claim 10, wherein each said continuous stretch of 2'-modified ribose nucleotides independently starts from the terminal nucleotide, the second nucleotide from the terminal nucleotide, or the third nucleotide from the terminal nucleotide.

40. The dsRNA of any of claims 1-5, wherein between 50-100% of the pyrimidine nucleotides of the antisense strand are, independently, 2'-F modified or 2'-O-methyl-modified.

41. The dsRNA of any of claims 1-5, wherein the 5'-end of the antisense strand is phosphorylated.

42. An RNA construct for inhibiting expression of a target gene, wherein said construct is identical to the dsRNA of any of claims 1-41, except for a single nick on the sense strand.

43. The RNA construct of claim 42, wherein the nick occupies the opposite position of the nucleotide about 10 bases from the 5' end of the antisense strand.

44. The RNA construct of claim 42, wherein the nick occupies the opposite position of a nucleotide about 5-15 bases from the 5' end of the antisense strand.

45. The RNA construct of any of claims 42-44, wherein the ΔG of each duplex region is less than about -13 kcal/mole.

46. A vector expressing at least one strand of the dsRNA of any of claims 1-5.

47. A cell comprising the vector of claim 46, or the dsRNA of any of claims 1-5.

48. The cell of claim 47, wherein the cell is a mammalian cell in culture.

49. The cell of claim 47, wherein the cell is a human cell.

50. A composition comprising the dsRNA of any of claims 1-5, and a pharmaceutically acceptable carrier or diluent.

51. A method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with the dsRNA construct of any of claims 1-5.

52. The method of claim 51, wherein said mammalian cell is in culture.

53. The method of claim 51, wherein said mammalian cell is a human cell.

54. The method of claim 51, wherein the mammalian cell is contacted in the presence of a delivery reagent.

55. The method of claim 54, wherein said delivery reagent is a lipid.

56. The method of claim 55, wherein said lipid is a cationic lipid.

57. The method of claim 54, wherein said delivery reagent is a liposome.

58. A method for inhibiting the expression of a target gene in a mammalian cell, comprising contacting the mammalian cell with a vector expressing at least one strand of the dsRNA construct of any of claims 1-5.

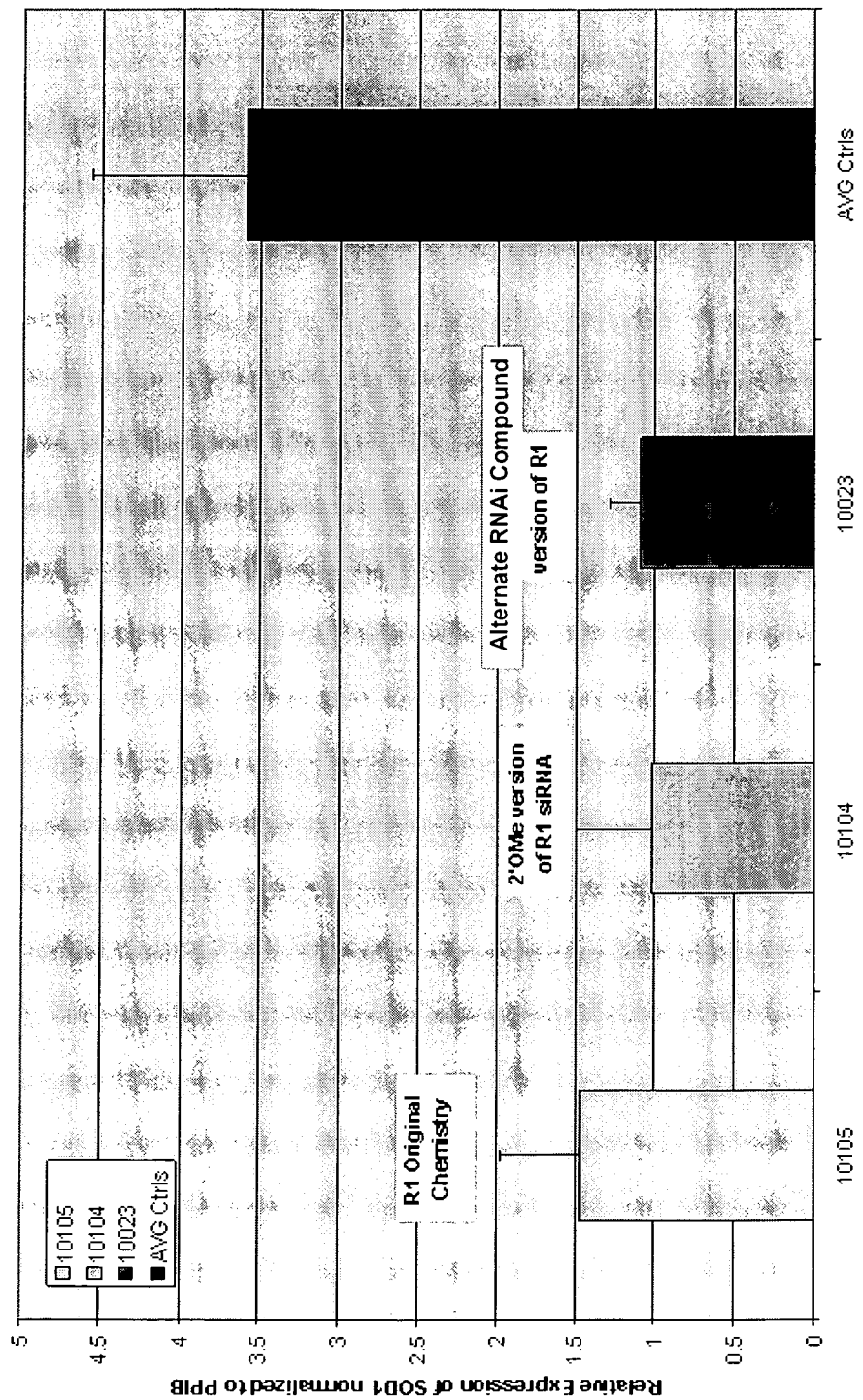
59. A method for improving the gene silencing effect of a small interference RNA (siRNA), comprising modifying the sense and/or antisense nucleotides of the siRNA to become the dsRNA construct of any of claims 1-5.

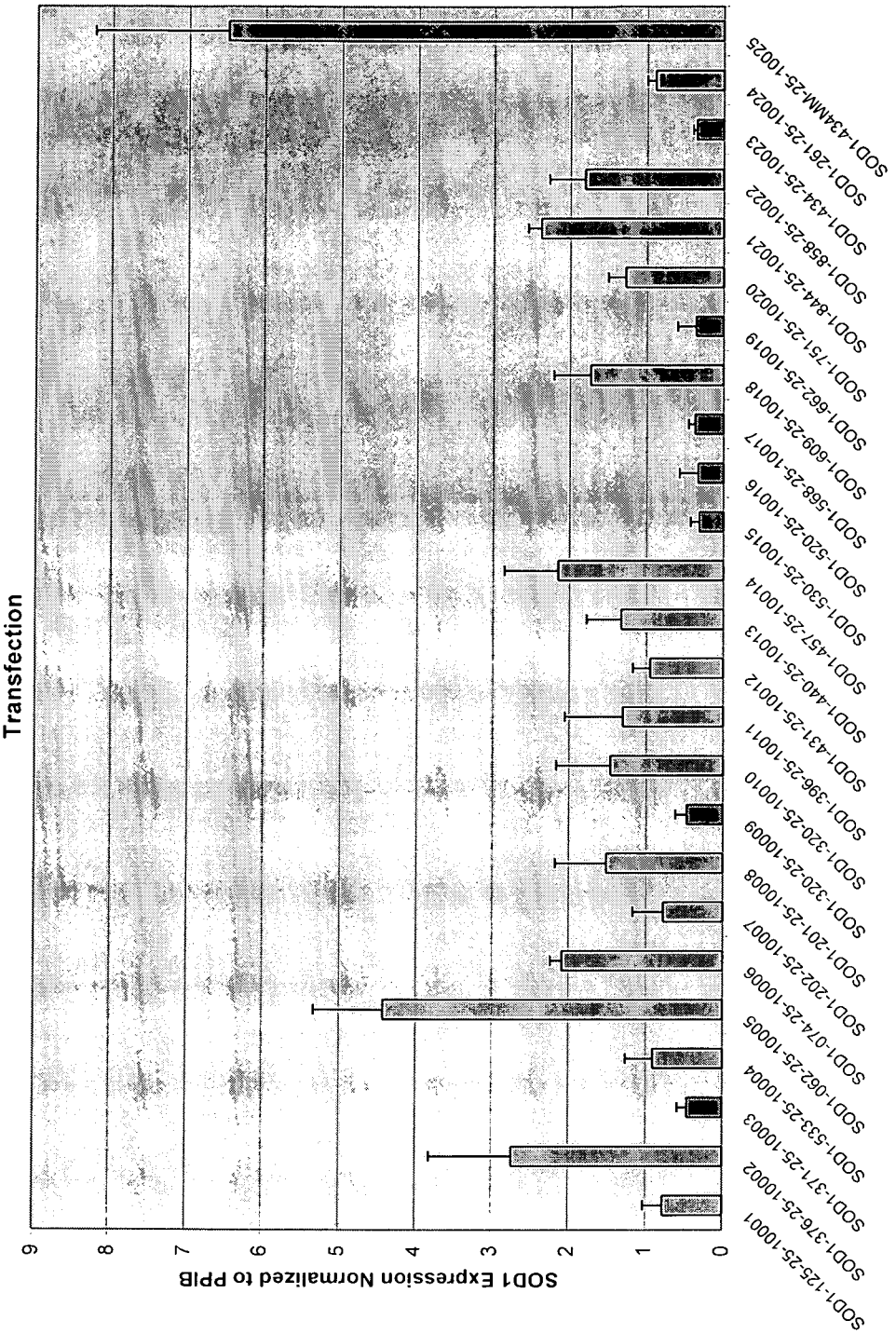
60. A method for evaluating *in vivo* delivery of an siRNA construct to a target site, comprising co-delivering the siRNA construct with a dsRNA construct of any of claims 1-5 targeting PPIB, and assaying the inhibition of PPIB function at the target site, wherein successful inhibition of the PPIB function at the target site is indicative of successful *in vivo* delivery of the siRNA construct to the target site.

Figure 1

Full Sequence Name	ID Numb	Polarity	Sequence (5'->3')
Sequence Designed and Tested			
SOD1-436-21-10033 (R1 var)	10033	Sense	P.C*G*A*fU.G.fU.G.U.C.U.A.U.U.G.A.A.G*A.fU*fU*C
		Antisense	P.A.fU.fC.fU.U.C.A.A.U.A.G.A.C.A.fC.A*fU*fC*G*G*C
SOD1-395-21-10034 (R2)	10034	Sense	P.G*G*A*G.A.fC.fU.U.G.G.G.C.A.A.fU.G.fU*G*A*fU*U
		Antisense	P.U.fC.A.fC.A.fU.fU.G.C.C.C.A.A.G.fU.fC.fU*fC*fC*U*U
SOD1-436-21-10036 (R1 unmod)	10036	Sense	P.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
		Antisense	P.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
SOD1-395-21-10037 (R2 unmod)	10037	Sense	P.G.G.A.G.A.C.U.U.G.G.G.C.A.A.U.G.U.G.A.U.U
		Antisense	P.U.C.A.C.A.U.U.G.C.C.C.A.A.G.U.C.U.C.C.U.U
SOD1-436-21-10104 (R1 var with 2'Ome)	10104	Sense	P.C*G*A*mU.G.mU.G.U.C.U.A.U.U.G.A.A.G*A.mU*mU*C
		Antisense	P.A.mU.mC.mU.U.C.A.A.U.A.G.A.C.A.mC.A*mU*mC*G*G*C
SOD1-436-21-10105 (R1)	10105	Sense	P.C*G*A*fU.G.fU.G.U.C.U.A.U.U.G.A.A.G*A*fU*fU*C
		Antisense	P.A.fU.fC.fU.U.C.A.A.U.A.G.A.C.A.fC.A*fU*fC*G*G*C
Additional Sequence Designed			
(R1 unmod no Overhangs)	10881	Sense	P.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U
		Antisense	P.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
(R1 unmod no Overhangs or 5'P)	10882	Sense	C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
(R1 unmod no 5'P)	10883	Sense	C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C
(R1 with 2'Ome replacing 2'F)	10884	Sense	P.C*G*A*mU.G.mU.G.U.C.U.A.U.U.G.A.A.G*A*mU*mU*C
		Antisense	P.A.mU.mC.mU.U.C.A.A.U.A.G.A.C.A.mC.A*mU*mC*G*G*C
(R1 with 2'Ome replacing 2'F, no Overhangs)	10885	Sense	C*G*A*mU.G.mU.G.U.C.U.A.U.U.G.A*A*G*A*mU
		Antisense	A.mU.mC.mU.U.C.A.A.U.A.G.A.C.A*mC*A*mU*mC*G
R1 w/ 001-011 Chem, blunt	10886	Sense	mC.mG.mA.mU.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
R1 w/ 001-042 Chem, blunt	10887	Sense	mC.mG.mA.mU.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.mU.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
R1 w/ MM001-011 Chem, blunt	10888	Sense	mC.mG.mA.mU.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mG.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
R1 w/ Extensive 2'Ome Sense, blunt	10889	Sense	mC.mG.mA.mU.mG.mU.mG.mU.C.U.A.U.mU.mG.mA.mA.mG.mG.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G
R1 original chemistry, blunt	10891	Sense	P.C*G*A*fU.G.fU.G.U.C.U.A.U.U.G.A.A.G*A*fU
		Antisense	P.A.fU.fC.fU.U.C.A.A.U.A.G.A.C.A.fC.A*fU*fC*G
R1 w/ 011-013 chem	10892	Sense	mC.mG.mA.mU.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C*mA*mU*mC*mG
Conversion of R1 into Alternate RNAi Compounds (Longer Length)			
SOD1-430-25-10174 (Tested)	10174	Sense	5'-P.mU.mG.mU.mG.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A
SOD1-429-26-10175 (Tested)	10175	Sense	5'-P.mG.mU.mG.mU.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C
SOD1-428-27-10890 (Designed)	10890	Sense	5'-P.mG.mG.mU.mG.U.G.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.mA.mG.mA.mU
		Antisense	A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C.C.A.C.A.C.C

Figure 2


SOD1 Expression 24hrs Post-Transfection with R1 siRNA or Alternate RNAi Compounds
Relative Expression of SOD1 normalized to PPIB
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
10105
10104
10023
AVG Ctrls
R1 Original Chemistry
2'OMe version of R1 siRNA
Alternate RNAi Compound version of R1


WS.002.1009 SOD1 Primary Screen in HEK 293 Cells mRNA reduction 24 hours post Transfection
SOD1 Expression Normalized to PPIB
0
1
2
3
4
5
6
7
8
9
SOD1-125-25-10001
SOD1-376-25-10002
SOD1-371-25-10003
SOD1-533-25-10004
SOD1-062-25-10005
SOD1-074-25-10006
SOD1-202-25-10007
SOD1-201-25-10008
SOD1-320-25-10009
SOD1-320-25-10010
SOD1-396-25-10011
SOD1-431-25-10012
SOD1-440-25-10013
SOD1-457-25-10014
SOD1-530-25-10015
SOD1-520-25-10016
SOD1-568-25-10017
SOD1-609-25-10018
SOD1-662-25-10019
SOD1-751-25-10020
SOD1-844-25-10021
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SOD1-434-25-10023
SOD1-261-25-10024
SOD1-434MM-25-10025

Figure 3

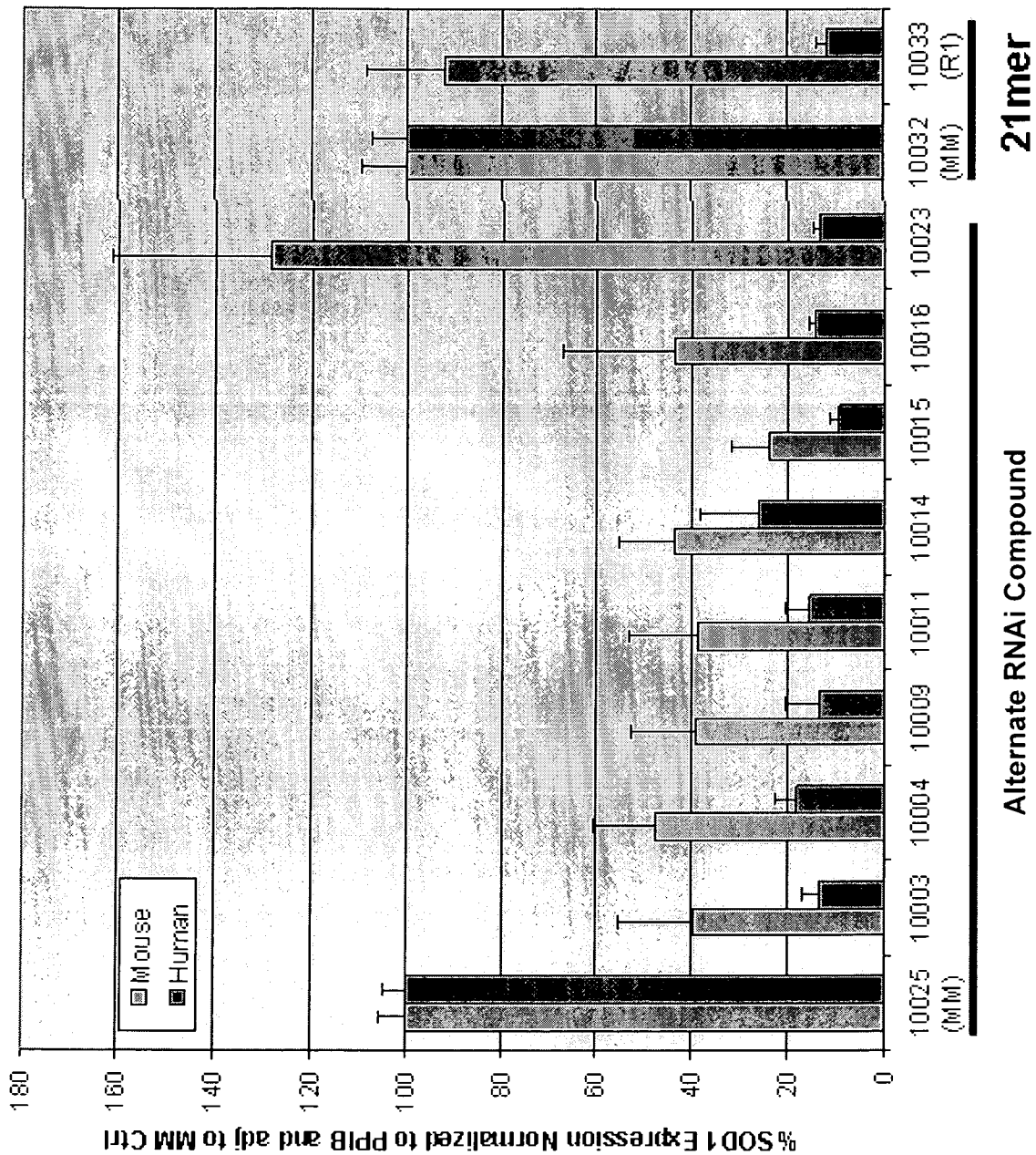

% SOD1 Expression Normalized to PPIB and adj to MM Ctrl
180
160
140
120
100
80
60
40
20
0
Mouse
Human
10025 (MM)
10003
10004
10009
10011
10014
10015
10016
10023
10032 (MM)
10033 (R1)
Alternate RNAi Compound
21mer

Figure 4

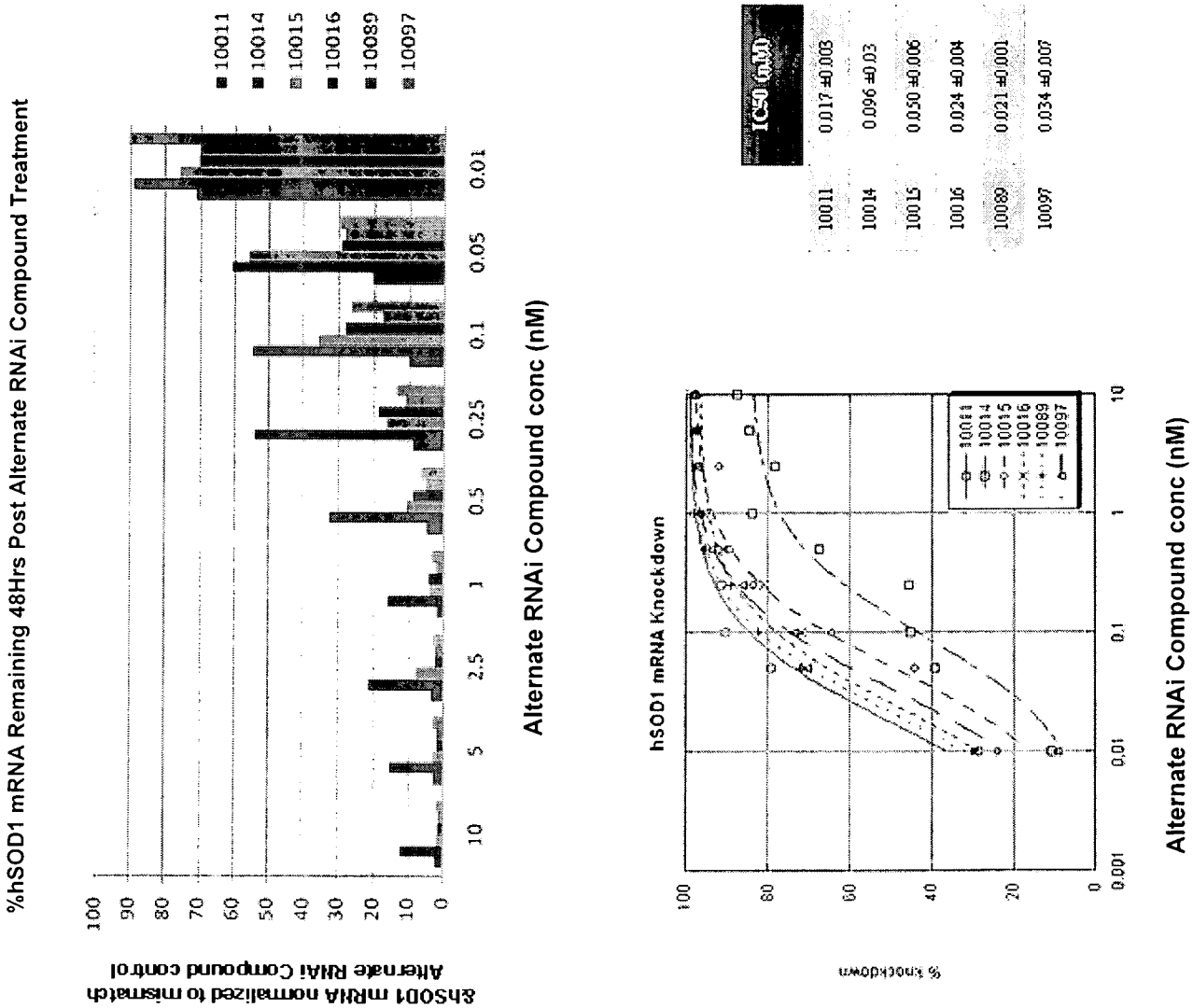

%hSOD1 mRNA Remaining 48Hrs Post Alternate RNAi Compound Treatment
%hSOD1 mRNA normalized to mismatch Alternate RNAi Compound control
Alternate RNAi Compound conc (nM)
10011
10014
10015
10016
10089
10097
100
90
80
70
60
50
40
30
20
10
0
10
5
2.5
1
0.5
0.25
0.1
0.05
0.01
hSOD1 mRNA Knockdown
% knockdown
IC50 (nM)
10011 0.017 ±0.003
10014 0.096 ±0.03
10015 0.050 ±0.006
10016 0.024 ±0.004
10089 0.021 ±0.001
10097 0.034 ±0.007
0.001
0.01
0.1
1
10
Alternate RNAi Compound conc (nM)

Figure 5A

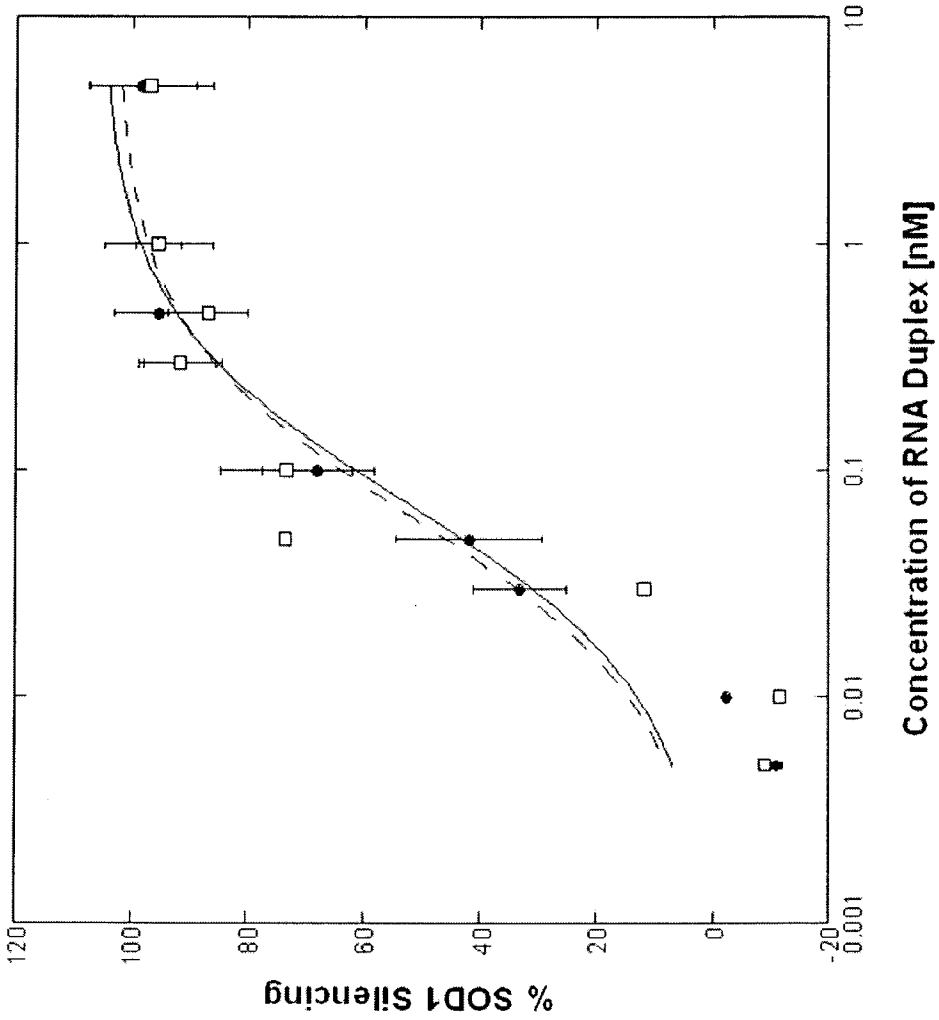

% SOD1 Silencing
120
100
80
60
40
20
0
-20
0.001
0.01
0.1
1
10
Concentration of RNA Duplex [nM]

Figure 5B

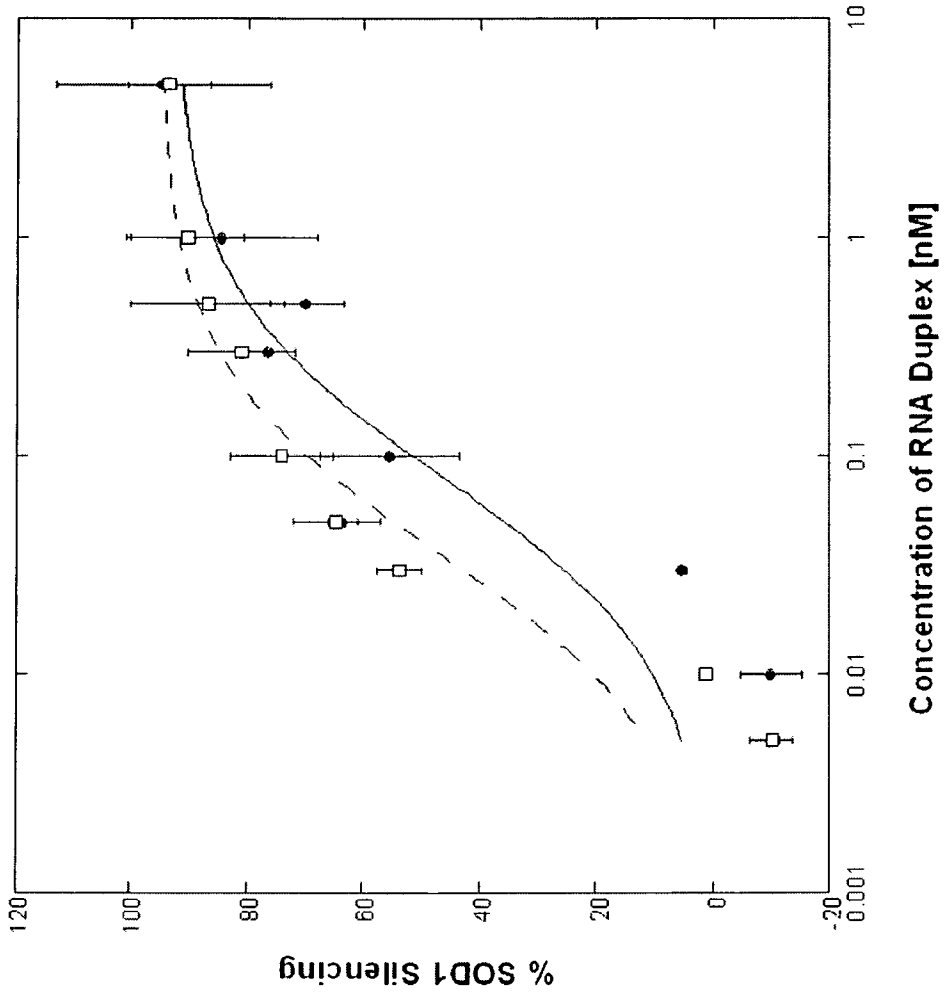
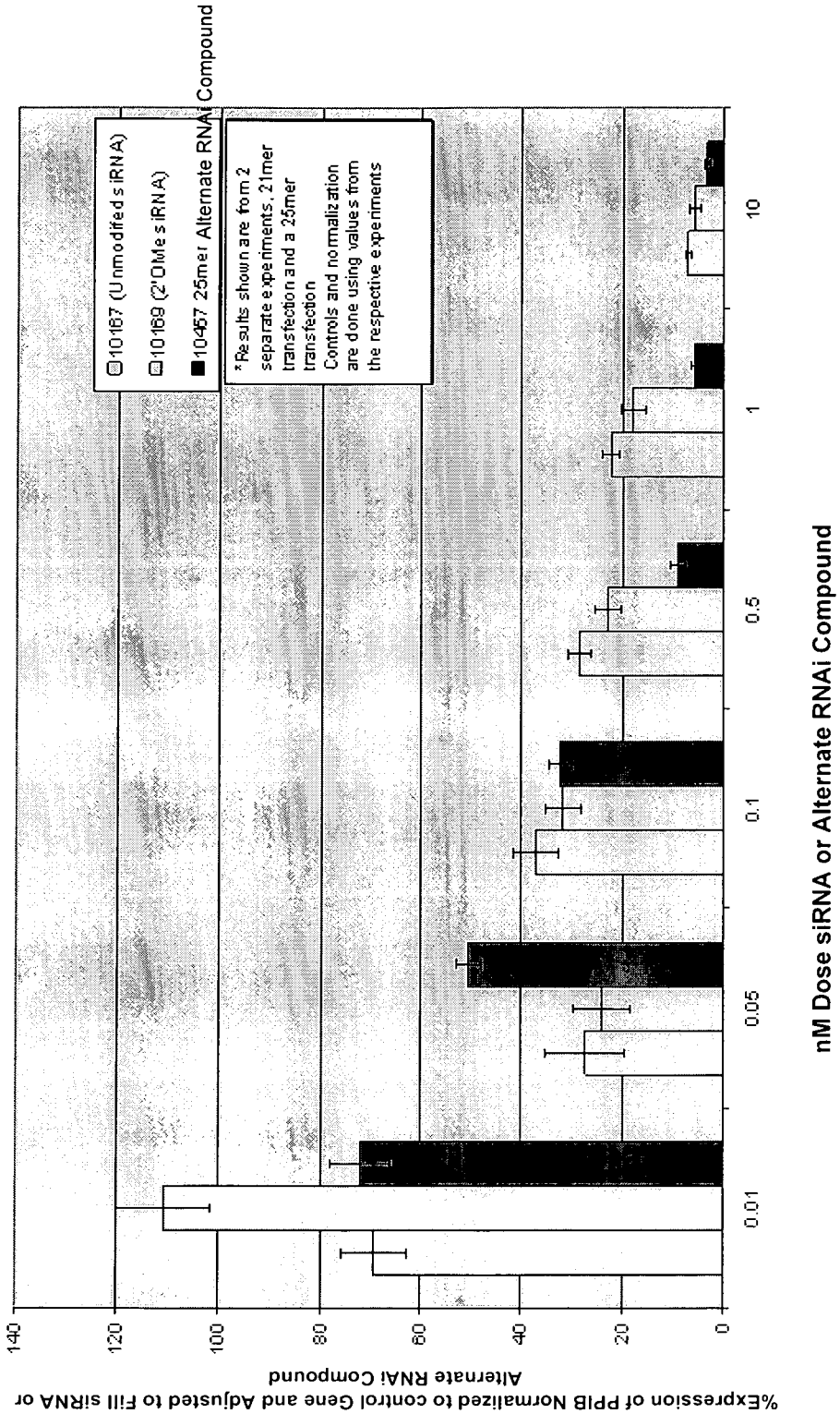

% SOD1 Silencing
120
100
80
60
40
20
0
-20
0.001
0.01
0.1
1
10
Concentration of RNA Duplex [nM]

Figure 5C

Figure 6A


Expression of PPIB 24hrs post-transfection
%Expression of PPIB Normalized to control Gene and Adjusted to Fill siRNA or Alternate RNAi Compound
140
120
100
80
60
40
20
0
0.01
0.05
0.1
0.5
1
10
nM Dose siRNA or Alternate RNAi Compound
10167 (Unmodified siRNA)
10169 (2'OMe siRNA)
10457 25mer Alternate RNAi Compound
*Results shown are from 2 separate experiments, 21mer transfection and a 25mer transfection
Controls and normalization are done using values from the respective experiments

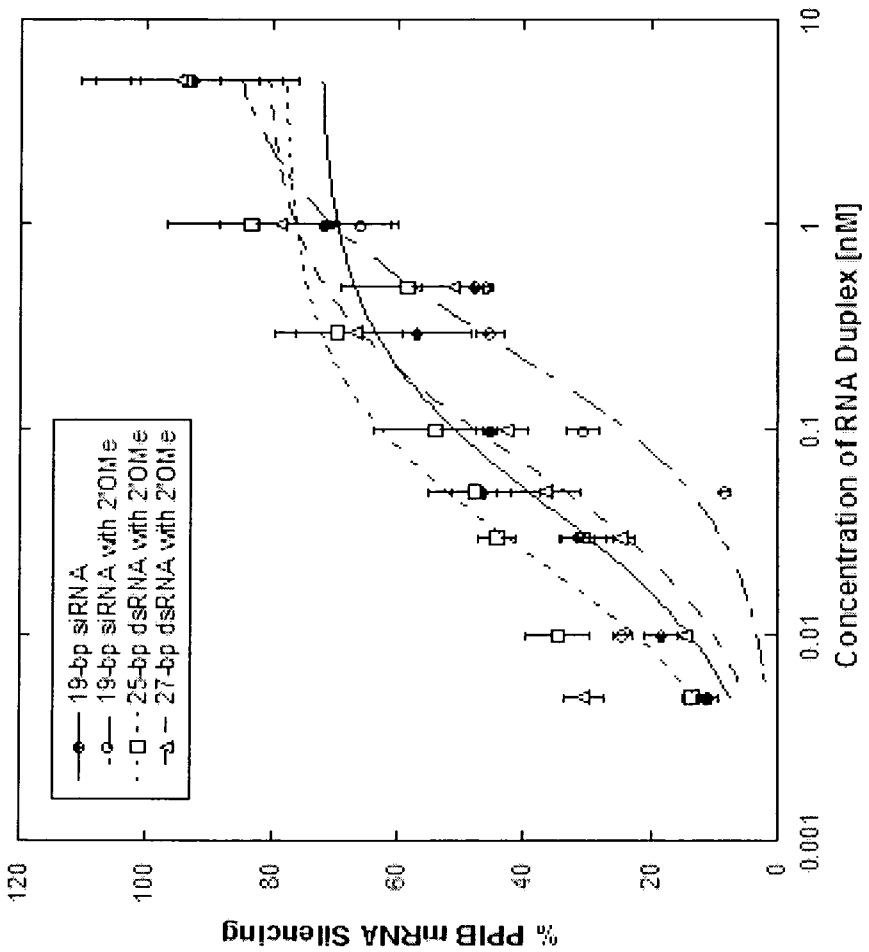

% PPIB mRNA Silencing
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
Concentration of RNA Duplex [nM]
19-bp siRNA
19-bp siRNA with 2'OMe
25-bp dsRNA with 2'OMe
27-bp dsRNA with 2'OMe

Figure 6B

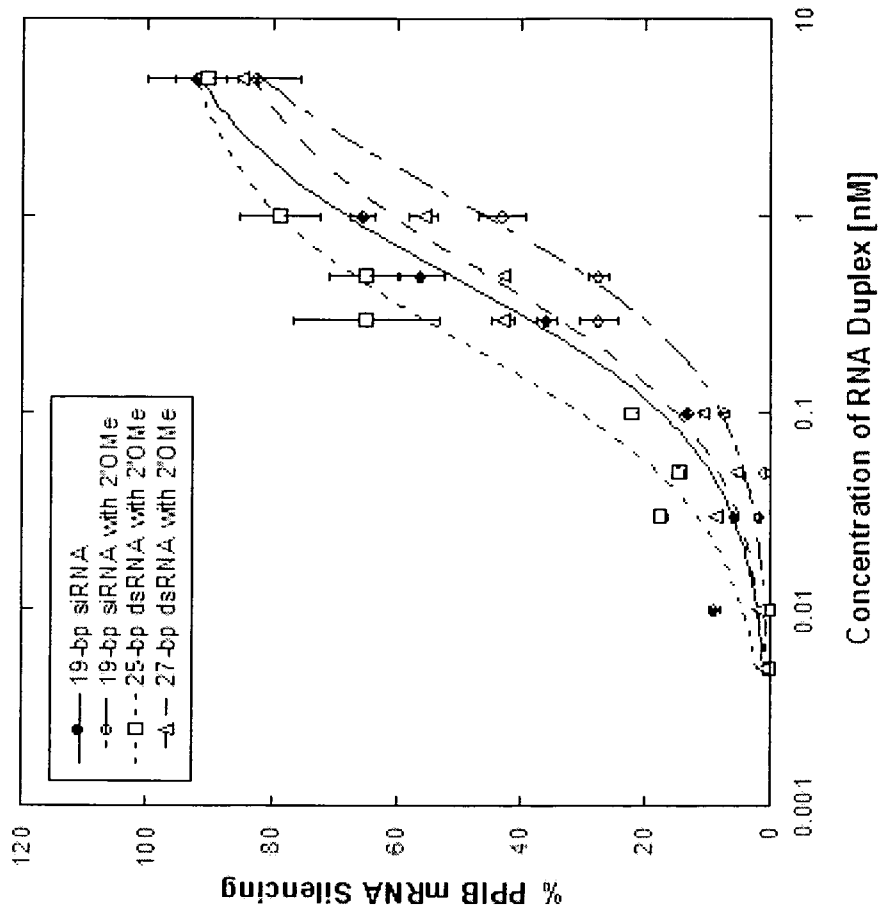

% PPIB mRNA Silencing
Concentration of RNA Duplex [nM]
19-bp siRNA
19-bp siRNA with 2'OMe
25-bp dsRNA with 2'OMe
27-bp dsRNA with 2'OMe
120
100
80
60
40
20
0
0.0001
0.01
0.1
1
10

Figure 6C

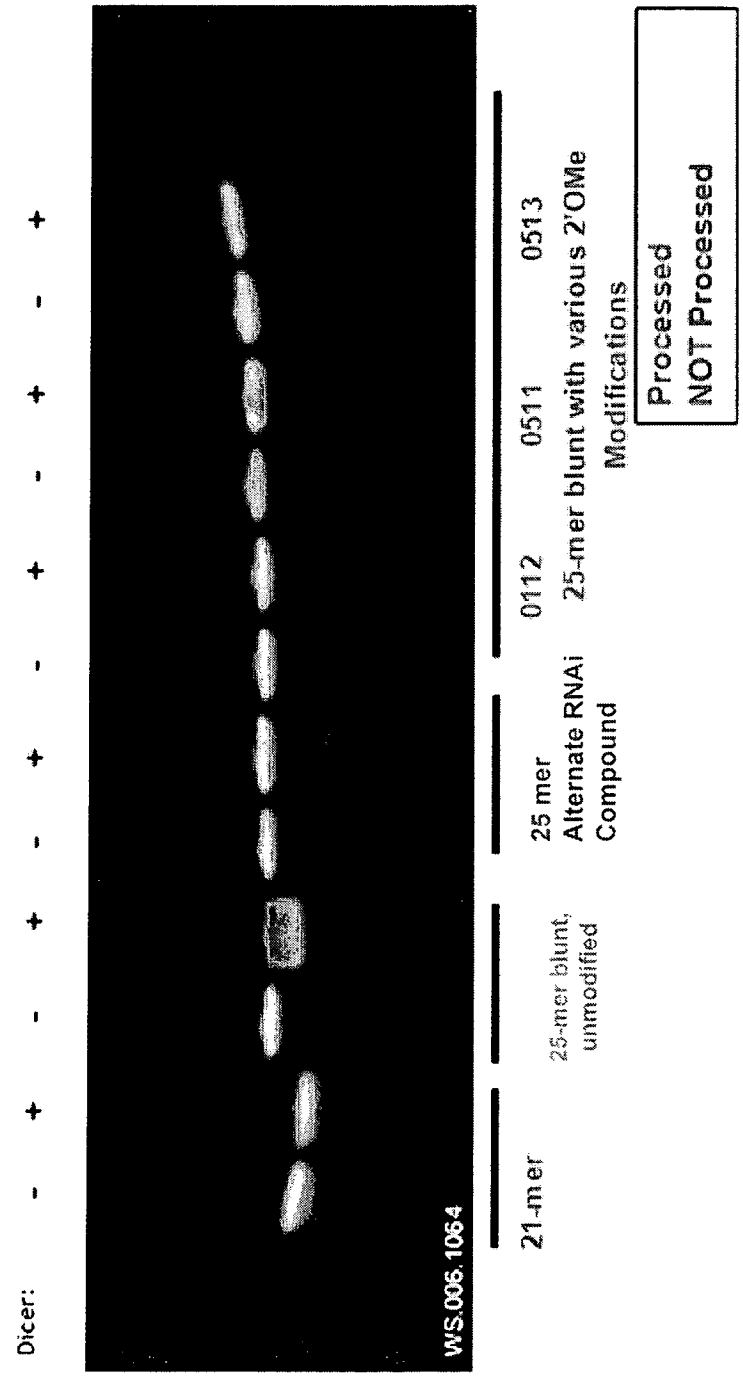

Dicer: - + - + - + - + - + - +
WS.006.1064
21-mer
25-mer blunt, unmodified
25 mer Alternate RNAi Compound
0112
0511
0513
25-mer blunt with various 2'OMe Modifications
Processed
NOT Processed

Figure 7

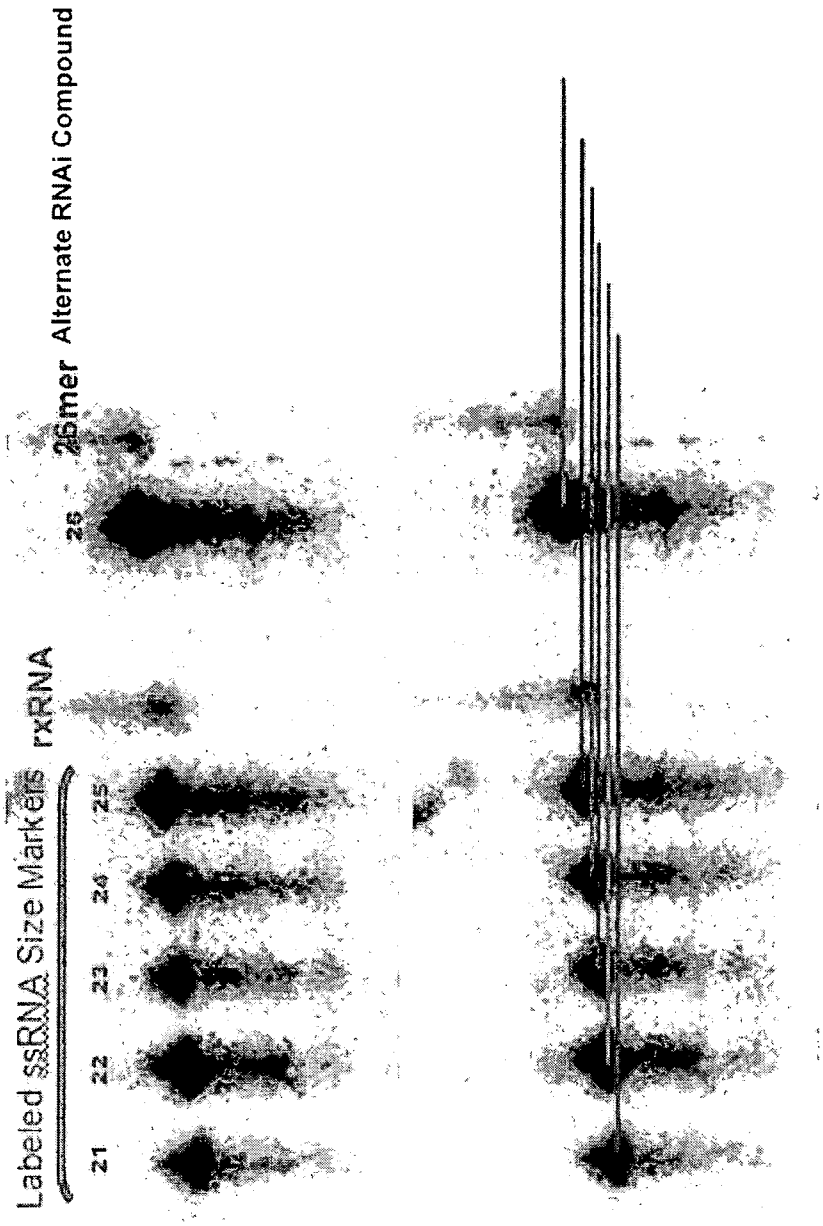

Labeled ssRNA Size Markers
21
22
23
24
25
rxRNA
25
26mer Alternate RNAi Compound

Figure 8A

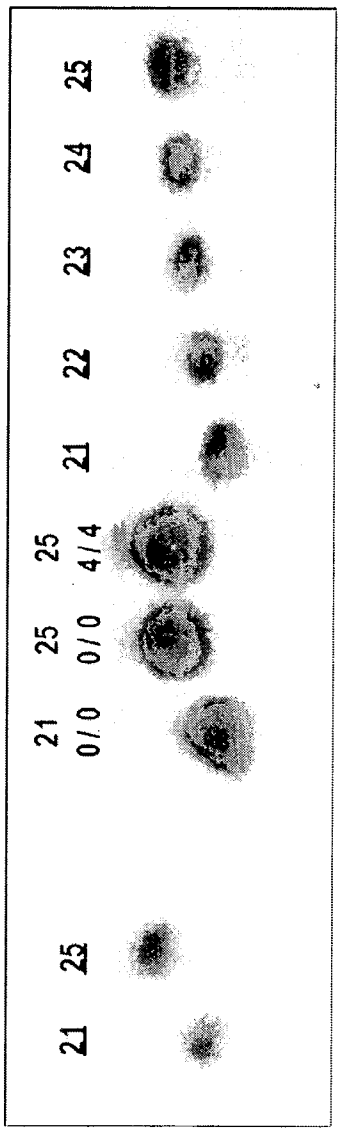


Figure 8B

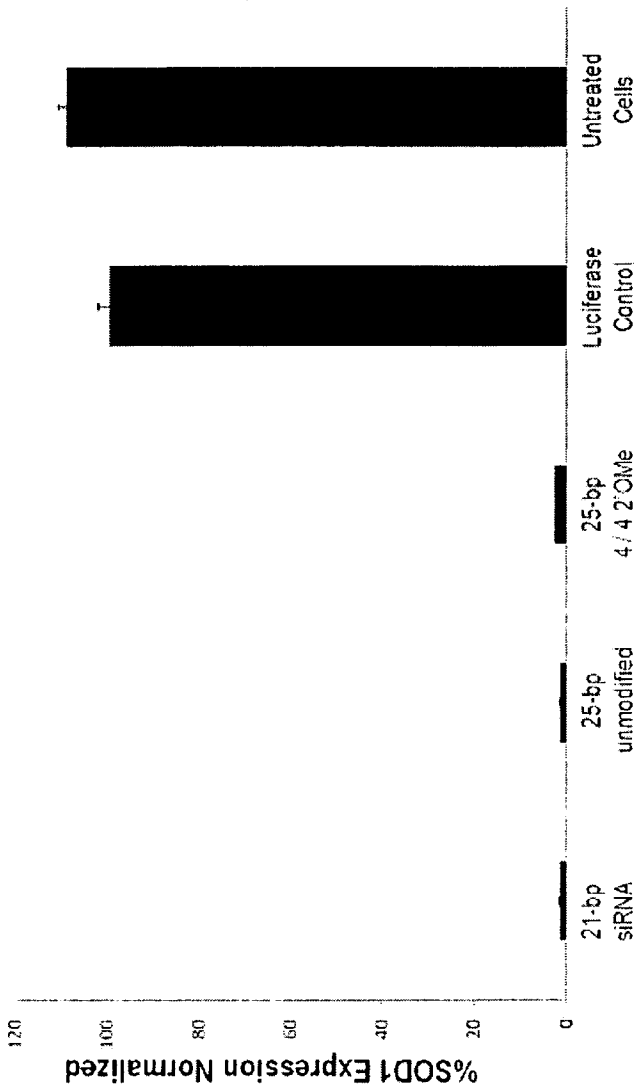


Figure 8C

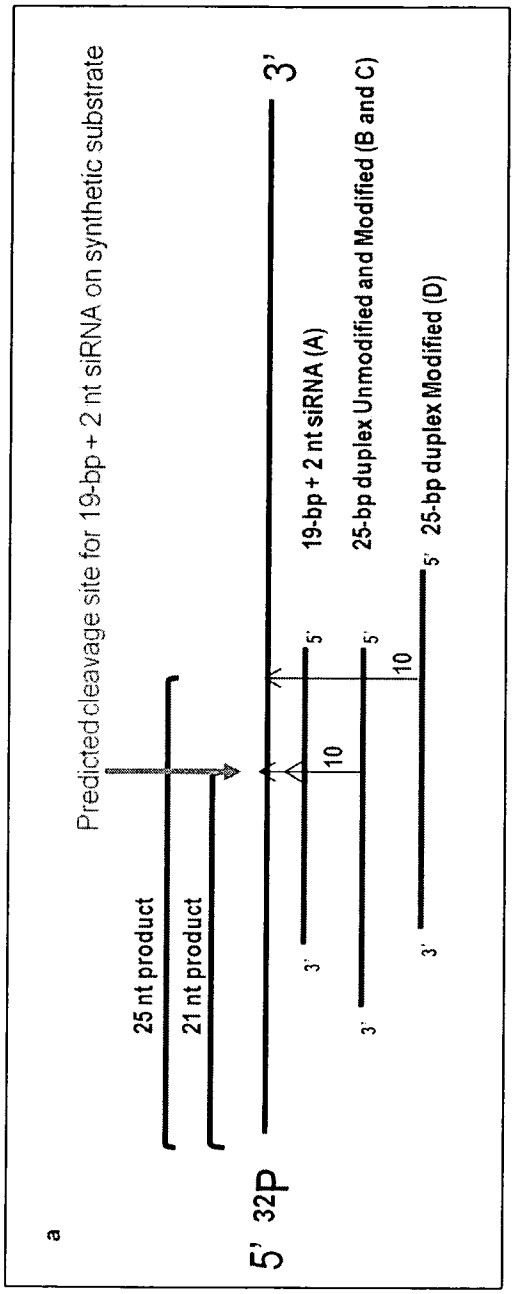


Figure 9A

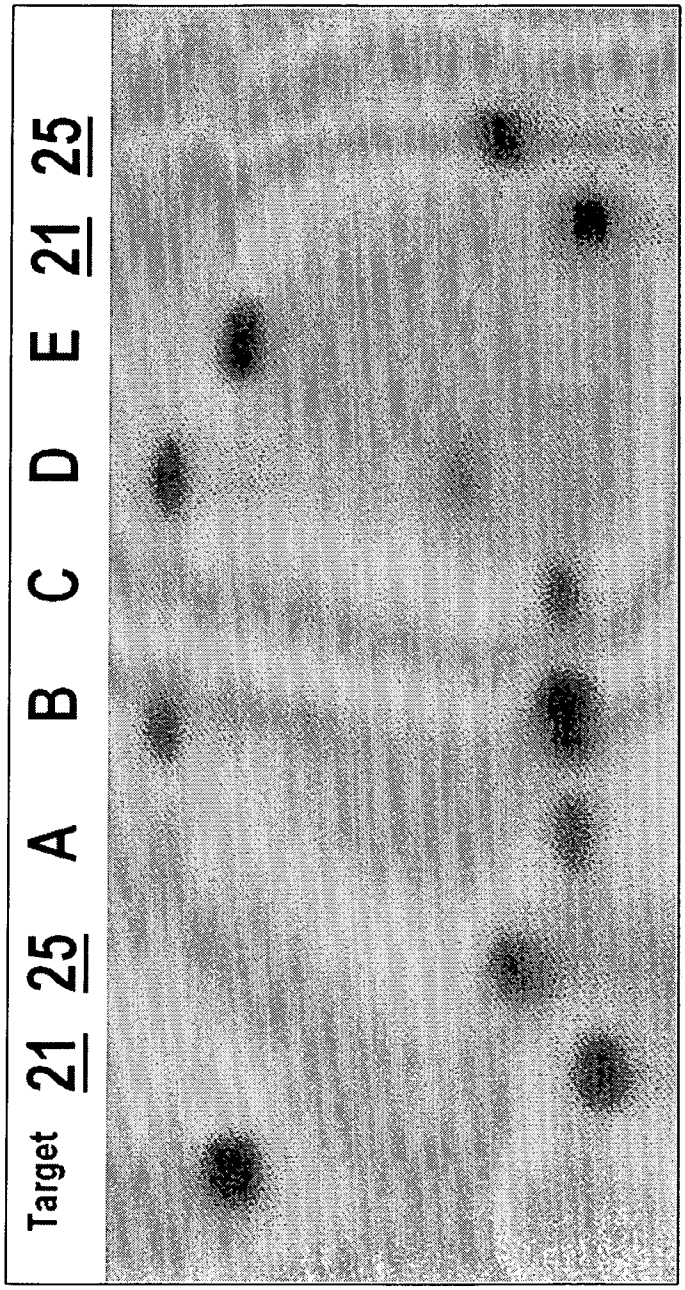
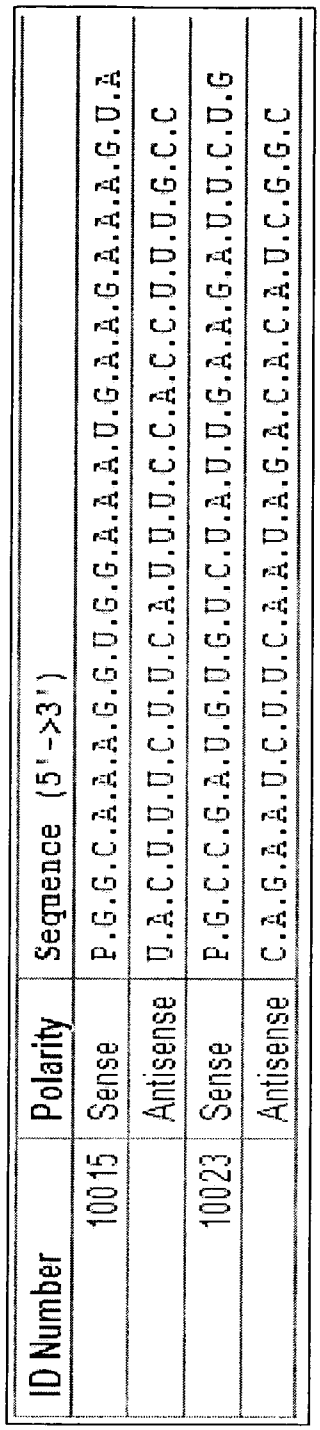


Figure 9B

Figure 10A



ID Number	Polarity	Sequence (5'->3')
10015	Sense	P.G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	Antisense	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
10023	Sense	P.G.C.C.G.A.U.G.U.G.U.C.U.A.U.U.G.A.A.G.A.U.U.C.U.G
	Antisense	C.A.G.A.A.U.C.U.U.C.A.A.U.A.G.A.C.A.C.A.U.C.G.G.C

Figure 10B

Sense (5'->3')	Chemistry ID		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
RxRNA (original)		001	Ome	Ome	Ome	Ome																		Ome	Ome	Ome	Ome
RxRNA with PS		002	Ome	Ome	Ome	Ome																		Ome	Ome	Ome	Ome
Complete 2'F on C and U		003	All C and U bases with 2'F																								
2'Ome with Central 2'F		004	Ome	Ome	Ome	Ome	All C and U bases with 2'F																	Ome	Ome	Ome	Ome
Alternating 2'Ome on ends		005		Ome		Ome		Ome		Ome												Ome		Ome		Ome	
Alternating 2'Ome on ends		006	Ome		Ome		Ome		Ome	Ome											Ome		Ome		Ome		Ome
2'F on ends with PS		007	All C and U bases with 2'F																			All C and U bases with 2'F					
Unmodified		008																									
2 and 2		009	Ome	Ome																						Ome	Ome
2 and 4		010	Ome	Ome																				Ome	Ome	Ome	Ome
2 and 8		019	Ome	Ome														Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
8 and 2		020	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome														Ome	Ome
4 and 6		021	Ome	Ome	Ome	Ome																Ome	Ome	Ome	Ome	Ome	Ome
4 and 8		022	Ome	Ome	Ome	Ome														Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
4 and 10		023	Ome	Ome	Ome	Ome												Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
4 and 12		024	Ome	Ome	Ome	Ome										Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
6 and 6		025	Ome	Ome	Ome	Ome	Ome	Ome														Ome	Ome	Ome	Ome	Ome	Ome
6 and 8		026	Ome	Ome	Ome	Ome	Ome	Ome												Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
6 and 10		027	Ome	Ome	Ome	Ome	Ome	Ome										Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
6 and 12		028	Ome	Ome	Ome	Ome	Ome	Ome								Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
8 and 6		029	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome												Ome	Ome	Ome	Ome	Ome	Ome
8 and 8		030	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome										Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
8 and 10		031	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome								Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
8 and 12		032	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome						Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
10 and 6		033	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome										Ome	Ome	Ome	Ome	Ome	Ome
10 and 8		034	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome								Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
10 and 10		035	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome						Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
10 and 12		036	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome				Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
12 and 6		037	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome								Ome	Ome	Ome	Ome	Ome	Ome
12 and 8		038	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome						Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
12 and 10		039	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome				Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
12 and 12		040	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome		Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
16 and 4		041	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome						Ome	Ome	Ome	Ome
Mismatch on SS base 24 (2 from 3' end), 4X 2'OME chemistry	MM001		Ome	Ome	Ome	Ome																		Ome	Ome	Ome	Ome
8 and 8 with MM	MM030		Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome										Ome	Ome	Ome	Ome	Ome	Ome	Ome	Ome
UCUCUU on 5' end SS	5UCU001		U Ome	C Ome	U Ome	C Ome	U	U																Ome	Ome	Ome	Ome
UCUCUU on 3' end SS	3UCU001		Ome	Ome	Ome	Ome																U	U	C Ome	U Ome	C Ome	U Ome

Antisense (3'->5'):		25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Unmodified	011																									
4 2'Ome on 3' end	012	Ome	Ome	Ome	Ome																					
(4) 2'Ome on 3' end with PS	013	Ome	Ome	Ome	Ome																					
All 2'F	014	All C and U Bases 2'F																								
(4) 2'Ome on 3' and rest 2'F	015	Ome	Ome	Ome	Ome	All C and U Bases 2'F																				
Alternating 2'Ome	016		Ome		Ome		Ome														Ome		Ome		Ome	
Alternating 2'Ome	017	Ome		Ome		Ome		Ome												Ome		Ome		Ome		Ome
Ends with 2'F, 3' end with 6 PS	018	All C and U Bases 2'F with PS after EVERY Base (6)																			All C and U Bases 2'F					
2'OME on nt2 of AS with no other modifications	042																								Ome	
AAGAGA on 3' end of AS	3AGAG	A	G	A	G	A	A																			
AAGAGA on 5' end of AS control for above	5AGAG																				A	A	G	A	G	A

Red = MM Green = Phosphothioate Backbone Linkage

WO 2009/102427 PCT/US2009/000852

Figure 11

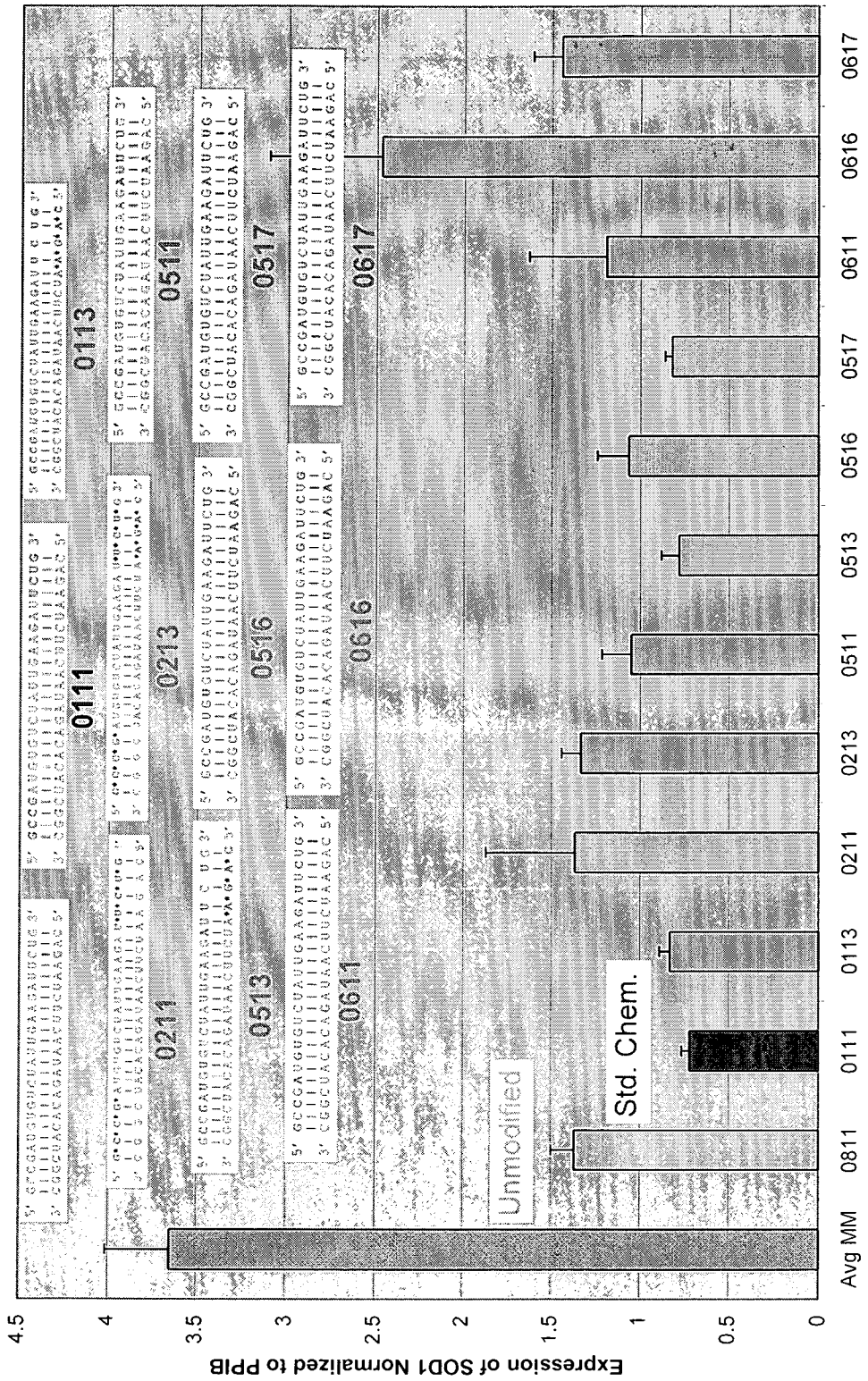
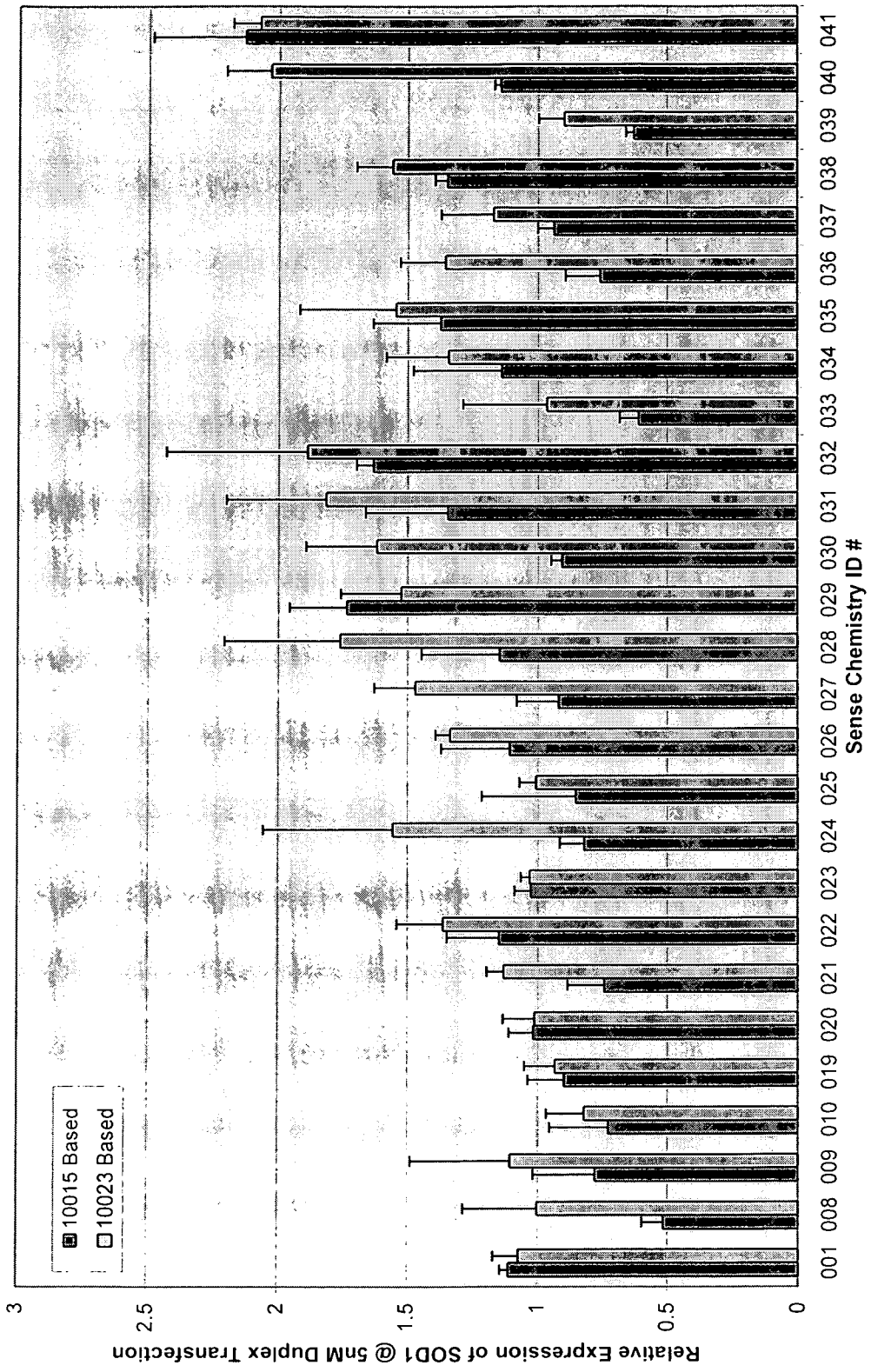

Selected 2'OMe Alternative Chemistries on duplex 10015 Test for Activity against SOD1
Expression of SOD1 Normalized to PPIB
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Avg MM
0811
0111
0113
0211
0213
0511
0513
0516
0517
0611
0616
0617
Std. Chem.
Unmodified
0111
0211
0113
0213
0511
0513
0516
0517
0611
0616
0617

Figure 12A


Extensive 2'OMe Testing
10015 Based
10023 Based
Relative Expression of SOD1 @ 5nM Duplex Transfection
0
0.5
1
1.5
2
2.5
3
001 008 009 010 019 020 021 022 023 024 025 026 027 028 029 030 031 032 033 034 035 036 037 038 039 040 041
Sense Chemistry ID #

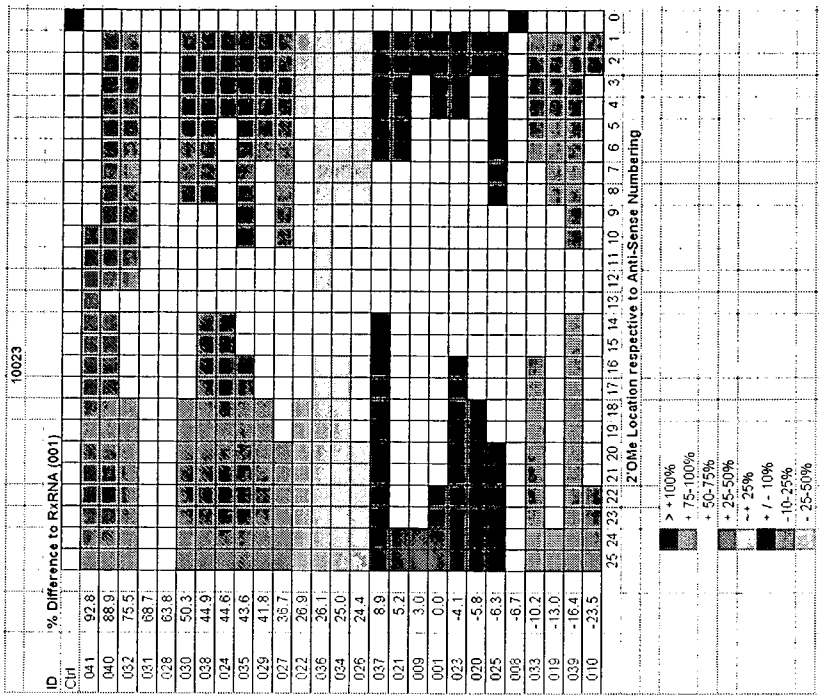


Figure 12C

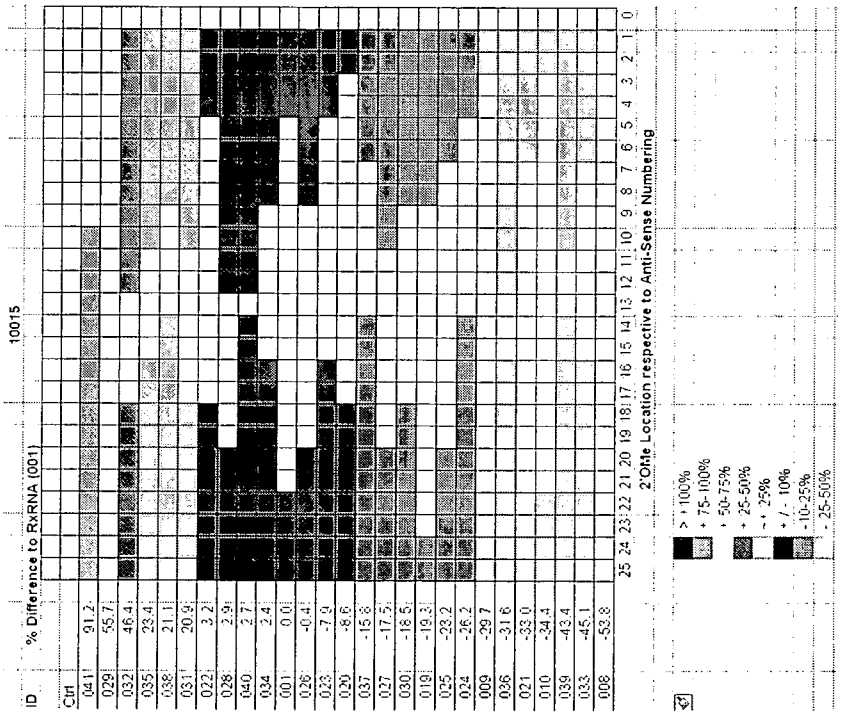


Figure 12B

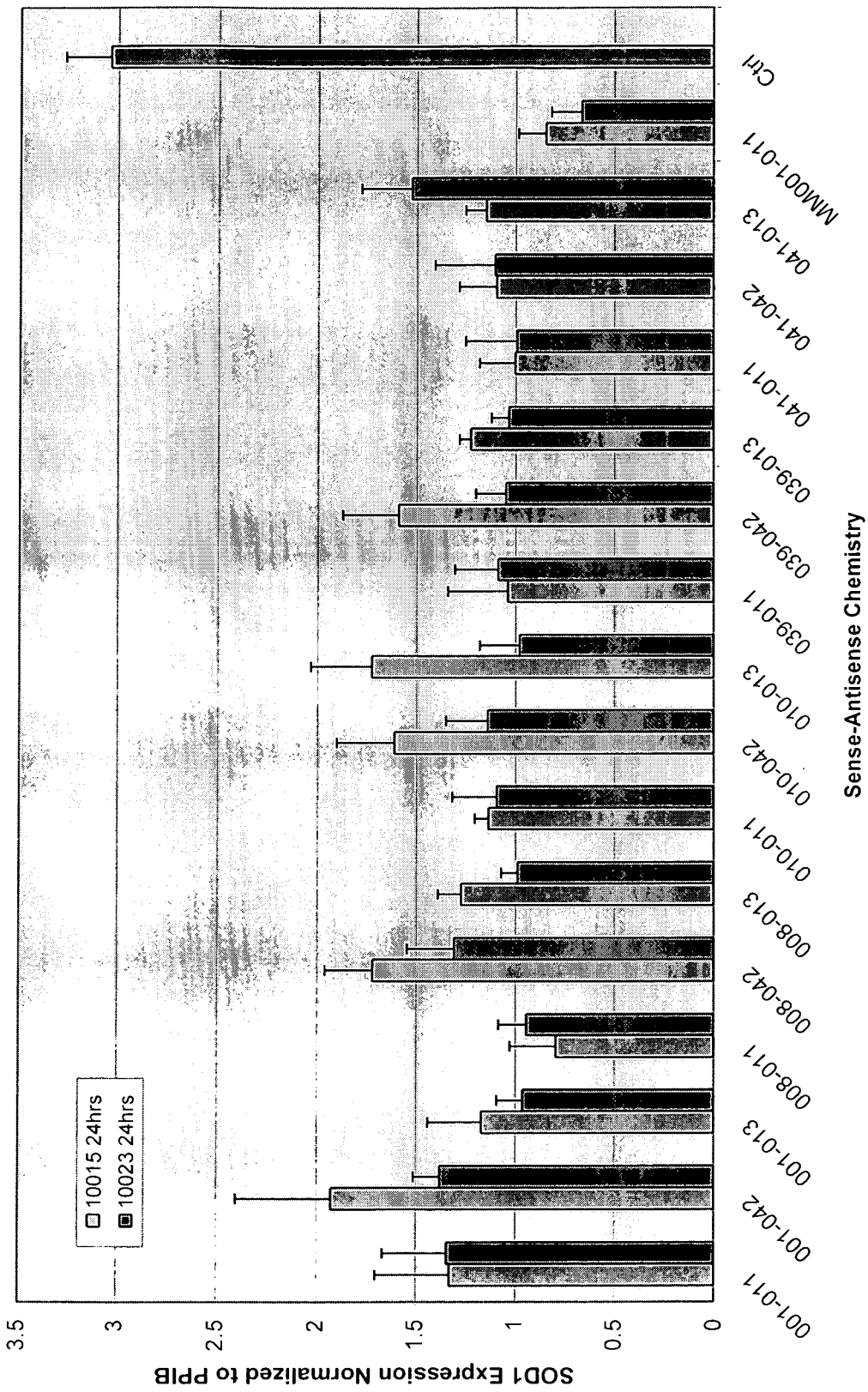

SOD1 Expression 24hrs Post-Trasnfection
10015 24hrs
10023 24hrs
SOD1 Expression Normalized to PPIB
3.5
3
2.5
2
1.5
1
0.5
0
001-011
001-042
001-013
008-011
008-042
008-013
010-011
010-042
010-013
039-011
039-042
039-013
041-011
041-042
041-013
MM001-011
Ctrl
Sense-Antisense Chemistry

Figure 13A

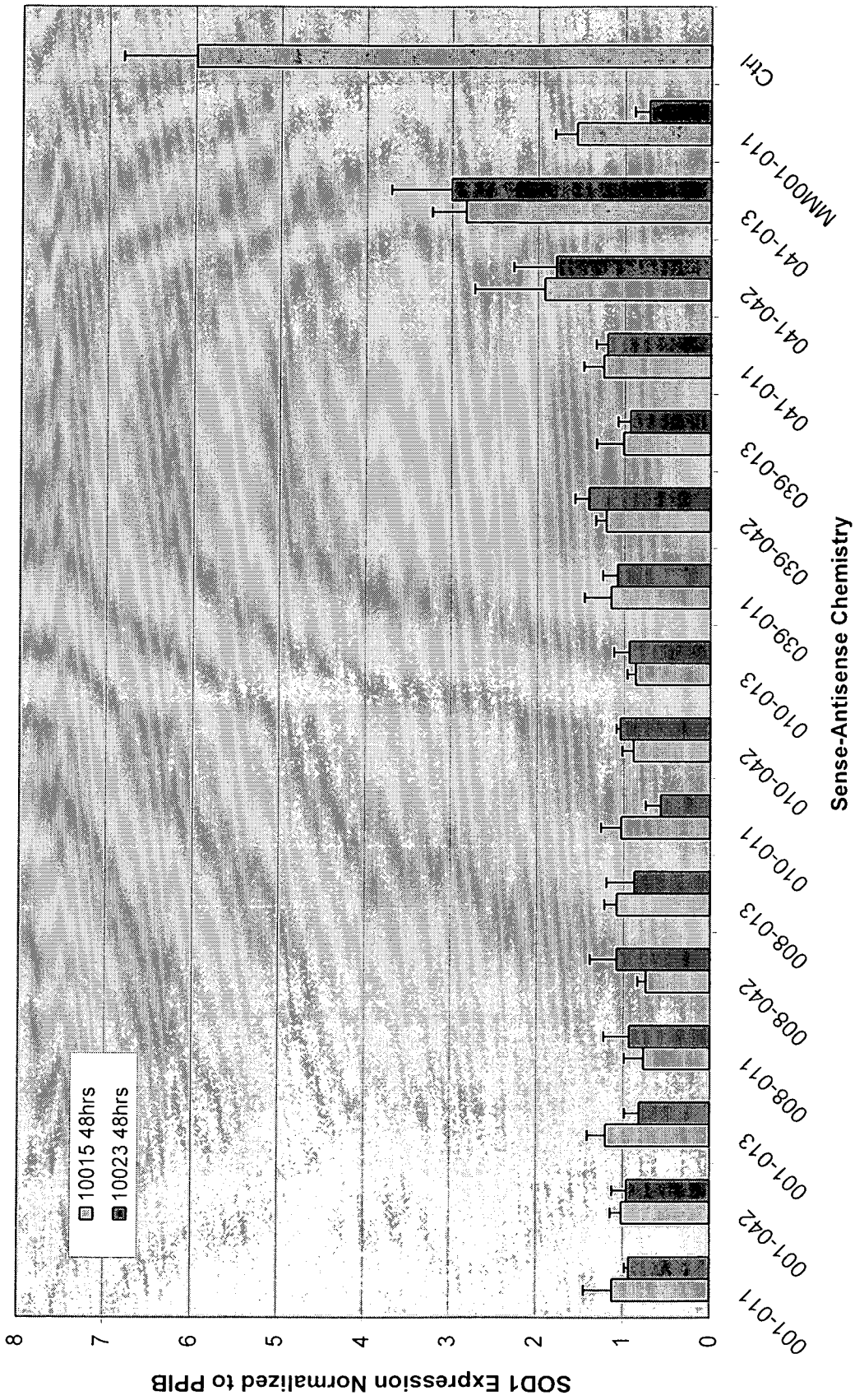

SOD1 Expression 48hrs Post-Trasnfection
10015 48hrs
10023 48hrs
SOD1 Expression Normalized to PPIB
0
1
2
3
4
5
6
7
8
001-011
001-042
001-013
008-011
008-042
008-013
010-011
010-042
010-013
039-011
039-042
039-013
041-011
041-042
041-013
MM001-011
Ctrl
Sense-Antisense Chemistry

Figure 13B

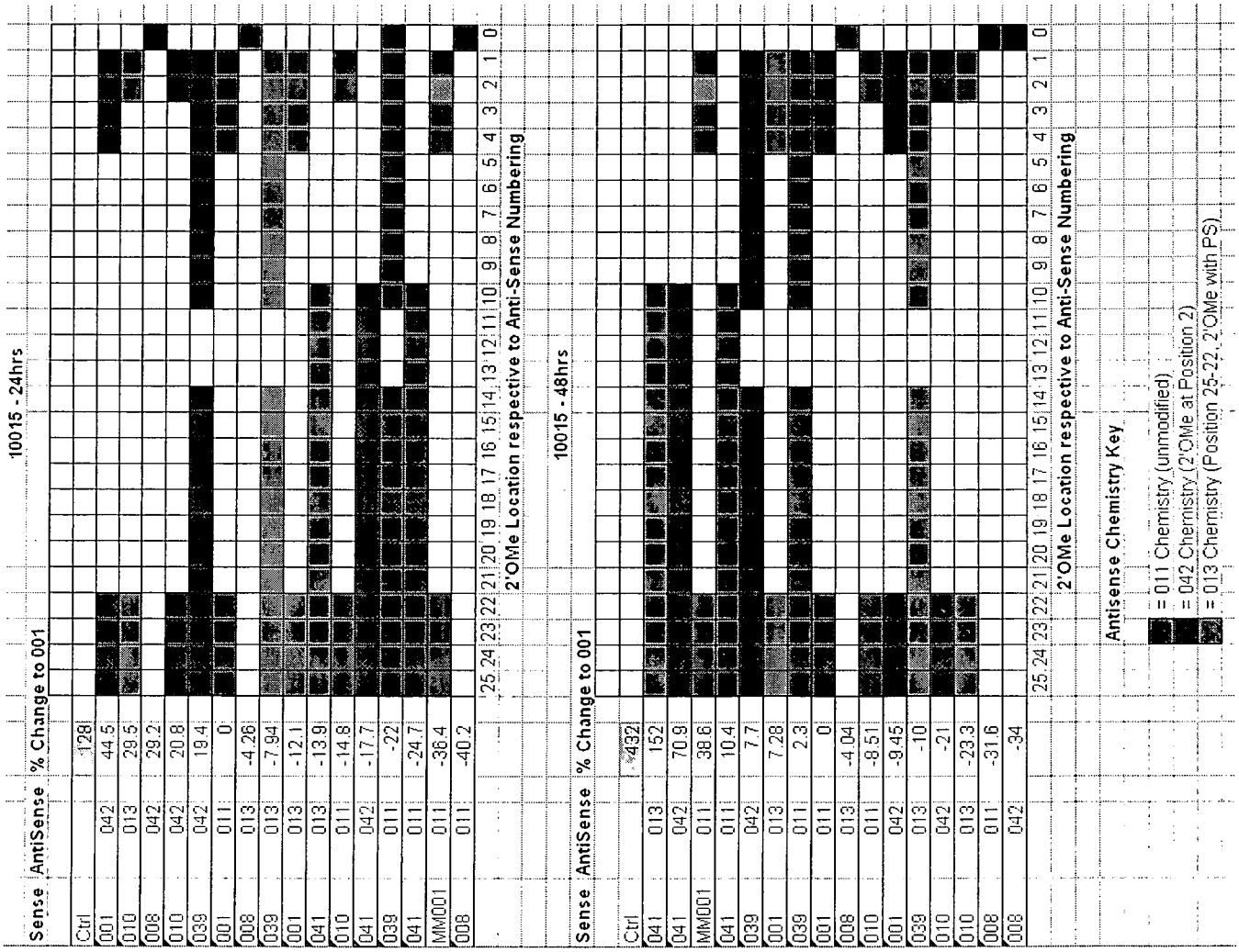

10015 - 24hrs
Sense
AntiSense
% Change to 001
Ctrl 128
001 042 44.5
010 013 29.5
008 042 29.2
010 042 20.8
039 042 19.4
001 011 0
008 013 -4.26
039 013 -7.94
001 013 -12.1
041 013 -13.9
010 011 -14.8
041 042 -17.7
039 011 -22
041 011 -24.7
MM001 011 -36.4
008 011 -40.2
25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
2'OMe Location respective to Anti-Sense Numbering
10015 - 48hrs
Sense
AntiSense
% Change to 001
Ctrl 432
041 013 152
041 042 70.9
MM001 011 38.6
041 011 10.4
039 042 7.7
001 013 7.28
039 011 2.3
001 011 0
008 013 -4.04
010 011 -8.51
001 042 -9.45
039 013 -10
010 042 -21
010 013 -23.3
008 011 -31.6
008 042 -34
25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
2'OMe Location respective to Anti-Sense Numbering
Antisense Chemistry Key
= 011 Chemistry (unmodified)
= 042 Chemistry (2'OMe at Position 2)
= 013 Chemistry (Position 25-22, 2'OMe with PS)

Figure 13C

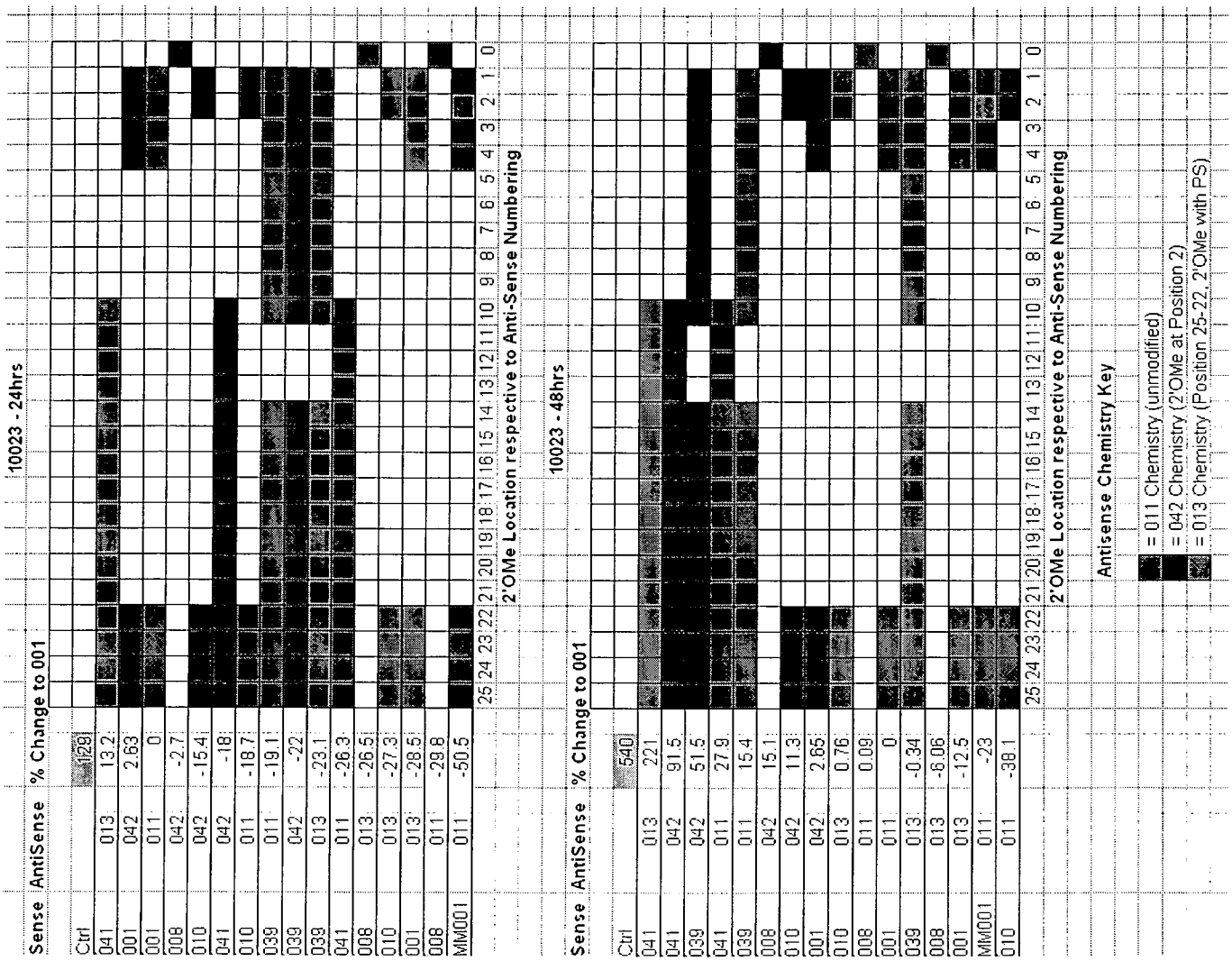

10023 - 24hrs
Sense AntiSense % Change to 001
Ctrl 129
041 013 13.2
001 042 2.63
001 011 0
008 042 -2.7
010 042 -15.4
041 042 -18
010 011 -18.7
039 011 -19.1
039 042 -22
039 013 -23.1
041 011 -26.3
008 013 -26.5
010 013 -27.3
001 013 -28.5
008 011 -29.8
MM001 011 -50.5
25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
2'OMe Location respective to Anti-Sense Numbering
10023 - 48hrs
Sense AntiSense % Change to 001
Ctrl 540
041 013 221
041 042 91.5
039 042 51.5
041 011 27.9
039 011 15.4
008 042 15.1
010 042 11.3
001 042 2.65
010 013 0.76
008 011 0.09
001 011 0
039 013 -0.34
008 013 -6.06
001 013 -12.5
MM001 011 -23
010 011 -38.1
25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
2'OMe Location respective to Anti-Sense Numbering
Antisense Chemistry Key
= 011 Chemistry (unmodified)
= 042 Chemistry (2'OMe at Position 2)
= 013 Chemistry (Position 25-22, 2'OMe with PS)

Figure 13D

Figure 14A

A Based on sequence 10023

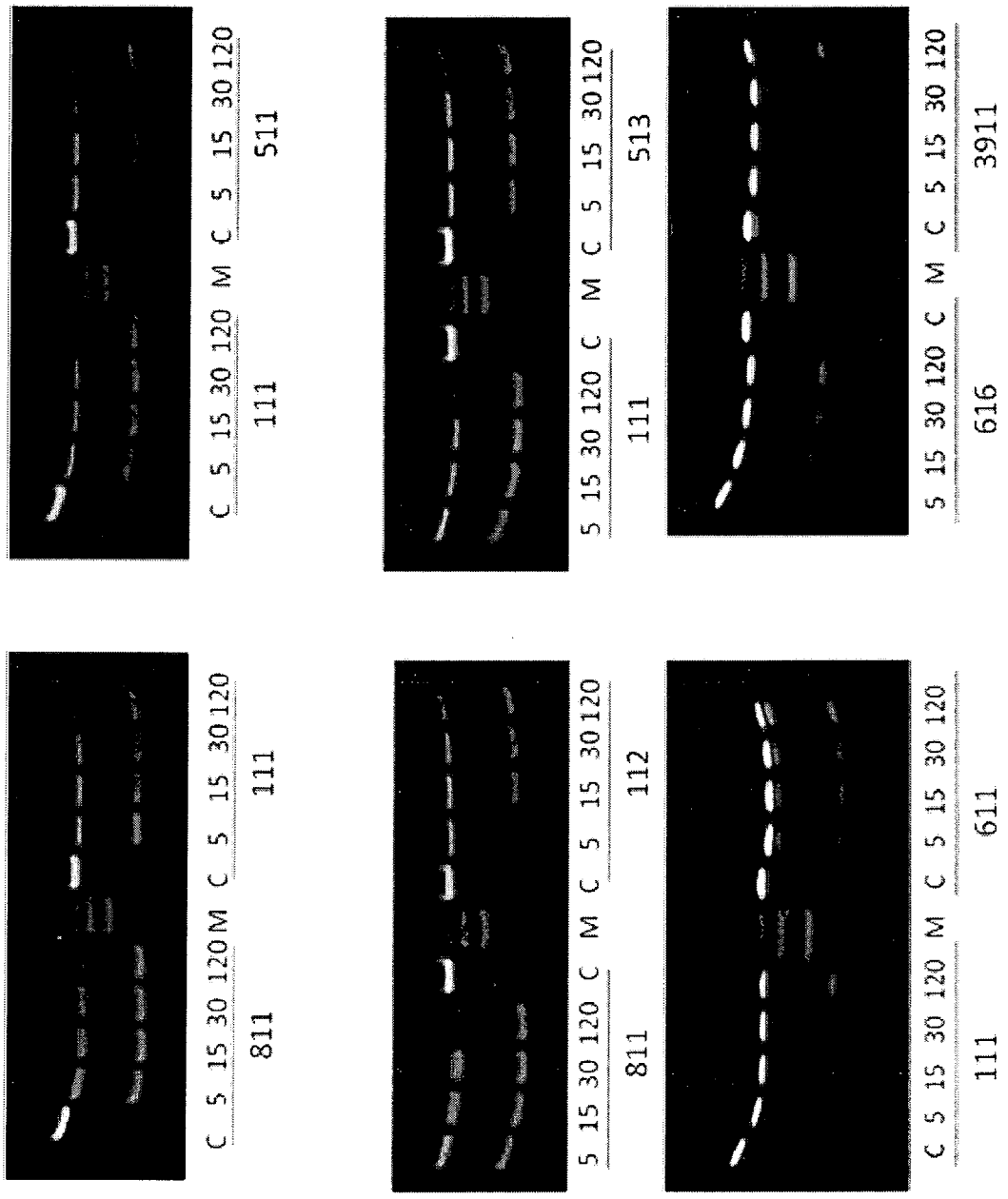


Figure 14B

B Based on sequence 10015

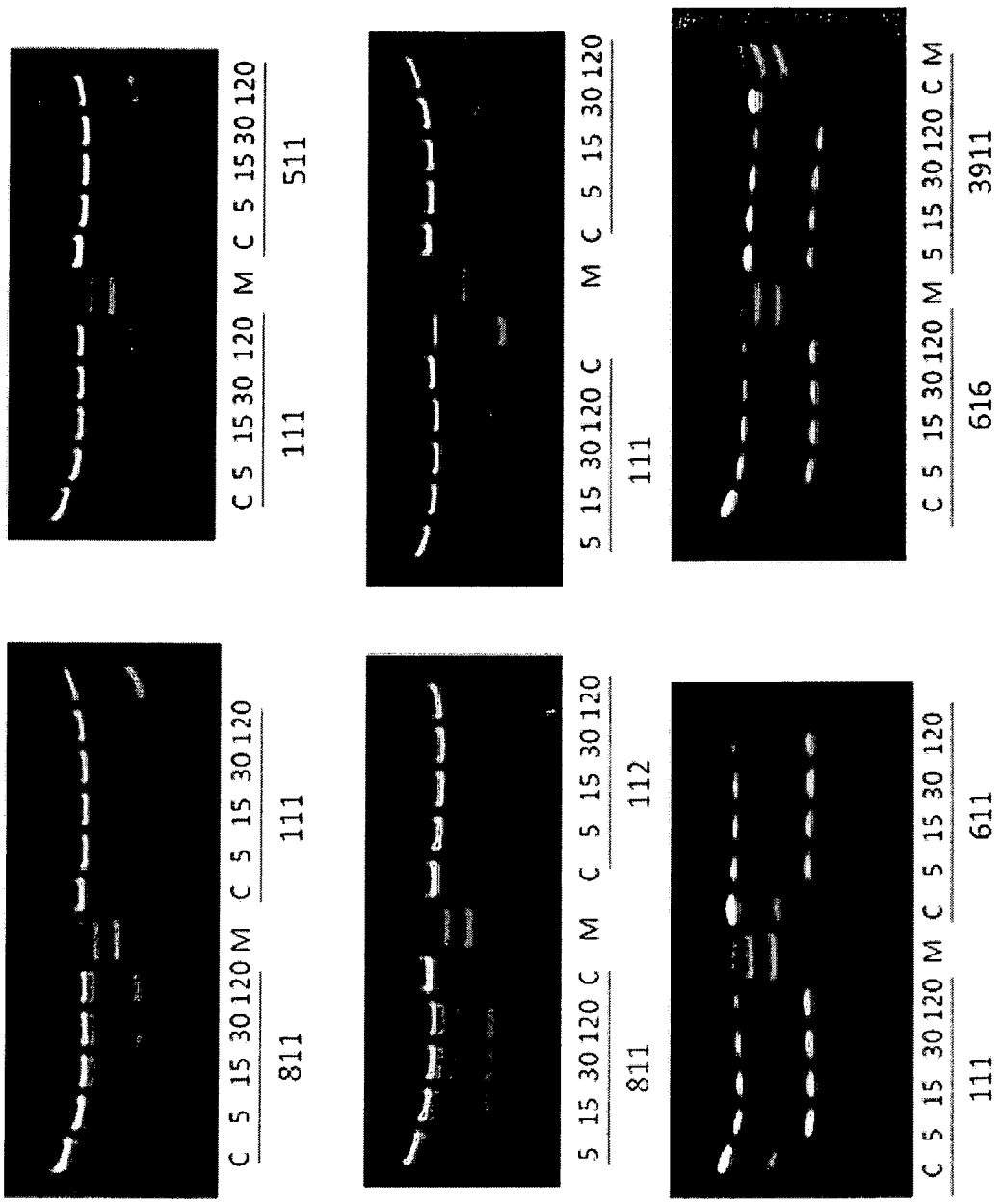


Figure 15A

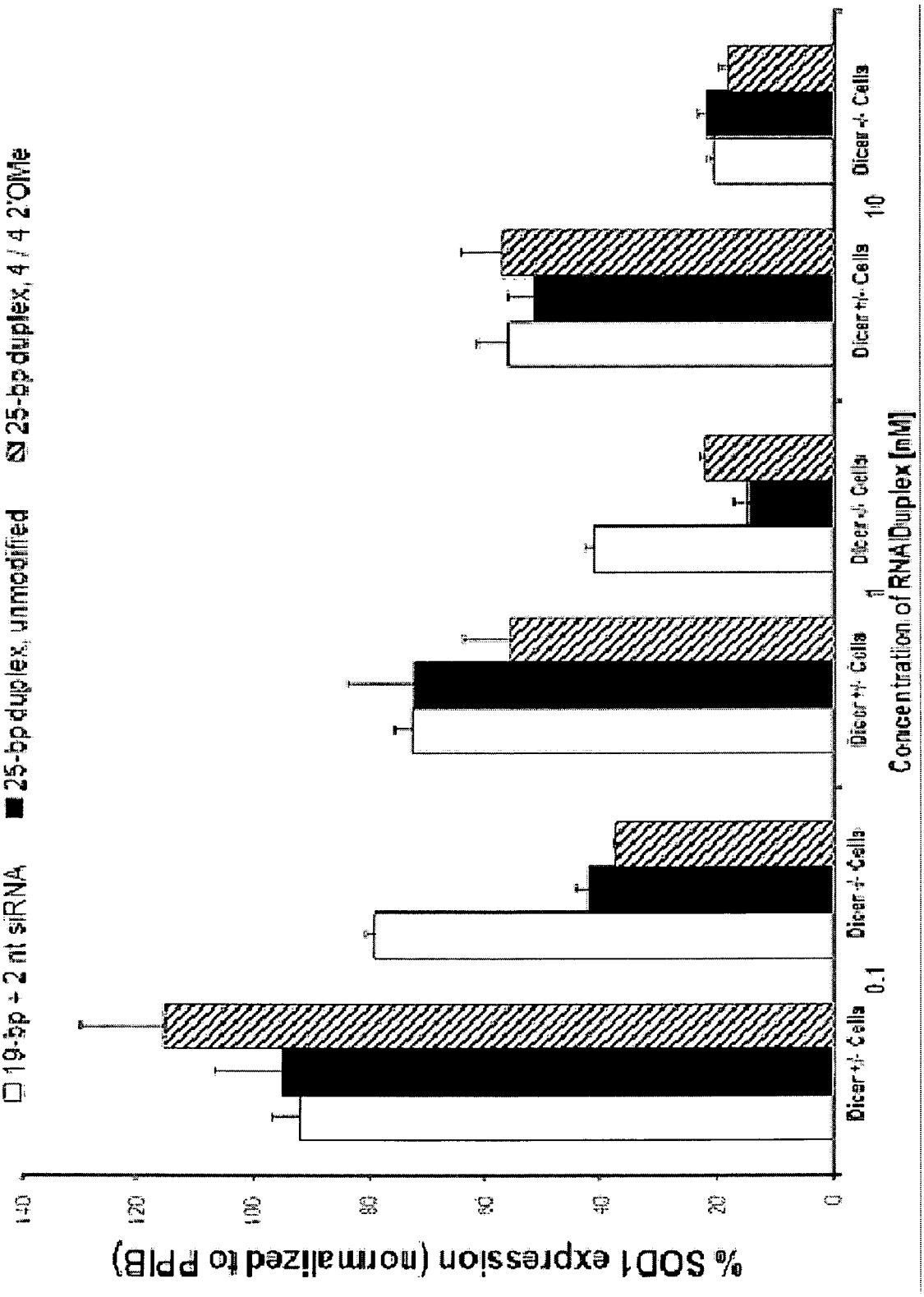

19-bp + 2 nt siRNA
25-bp duplex, unmodified
25-bp duplex, 4 / 4 2'OMe
% SOD1 expression (normalized to PPIB)
0
20
40
60
80
100
120
140
Dicer+/+ Cells
Dicer-/- Cells
0.1
1
10
Concentration of RNA Duplex [nM]

Figure 15B

Sequence Name	Polarity	Sequence (5'->3')
19-bp + 2 nt duplex (11892)	Sense	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U
	Antisense	G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A.U.U
(11893)	Sense	G.G.C.A.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.A.G.U.A
	Antisense	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C
(11897)	Sense	mG.mG.mC.mA.A.A.G.G.U.G.G.A.A.A.U.G.A.A.G.A.A.mA.mG.mU.mA
	Antisense	U.A.C.U.U.U.C.U.U.C.A.U.U.U.C.C.A.C.C.U.U.U.G.C.C

Figure 16A

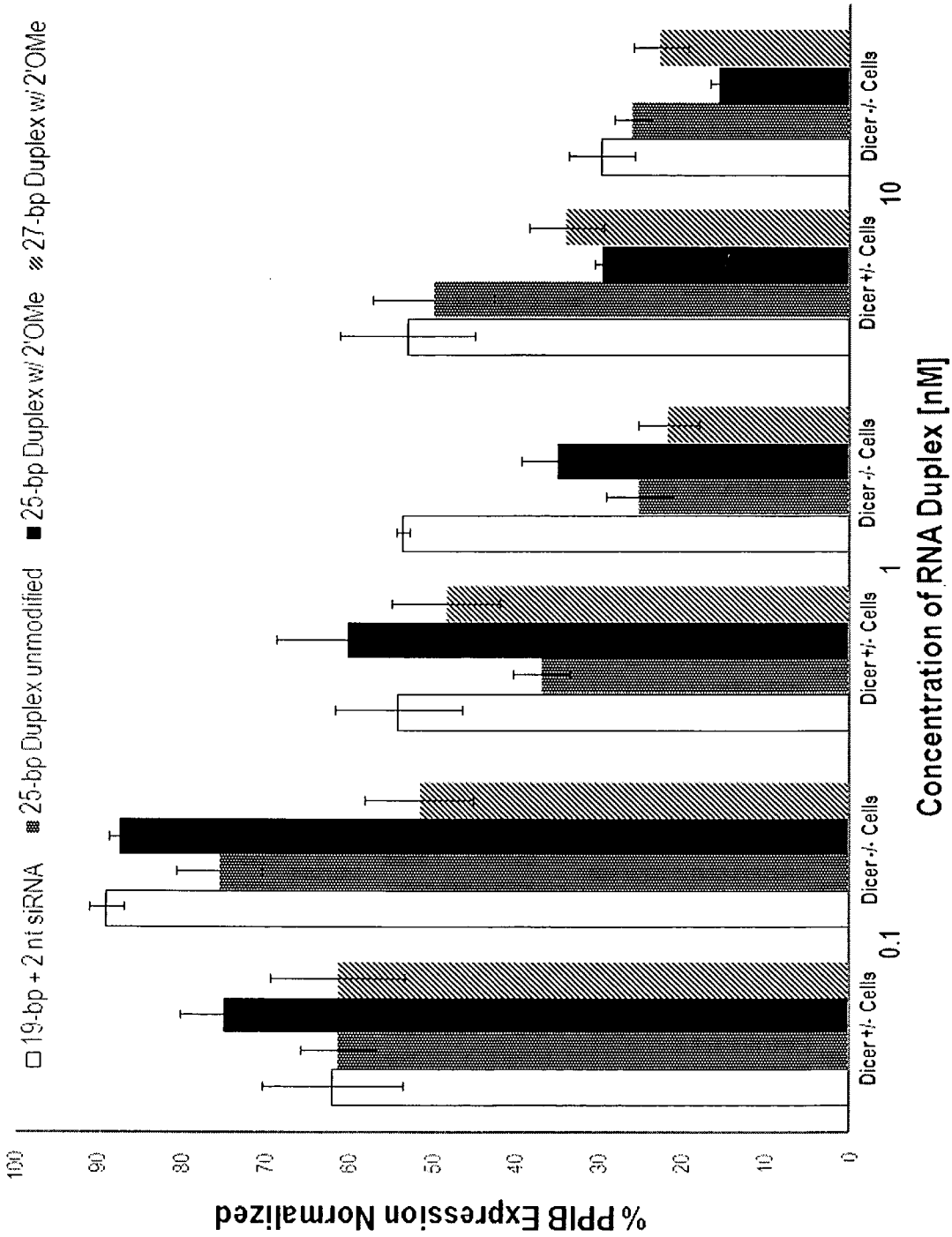

19-bp + 2 nt siRNA
25-bp Duplex unmodified
25-bp Duplex w/ 2'OMe
27-bp Duplex w/ 2'OMe
% PPIB Expression Normalized
0
10
20
30
40
50
60
70
80
90
100
Dicer +/- Cells
Dicer -/- Cells
0.1
1
10
Concentration of RNA Duplex [nM]

Figure 16B

Sequence Name	Polarity	Sequence (5'->3')
19-bp + 2 nt unmodified (10167)	Sense	G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.A.A.A.A.U.U
	Antisense	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.U.U
25-bp unmodified (11388)	Sense	C.U.C.U.U.C.G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.A.A.A.A
	Antisense	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.G.A.A.G.A.G
25-bp w/ 2'OMe (10460)	Sense	mC.mU.mC.mU.U.C.G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.mA.mA.mA.mA
	Antisense	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.G.A.A.G.A.G
27-bp w/ 2'OMe (10462)	Sense	mG.mU.mC.mU.C.U.U.C.G.G.A.A.A.G.A.C.U.G.U.U.C.C.A.mA.mA.mA.mA
	Antisense	U.U.U.U.U.G.G.A.A.C.A.G.U.C.U.U.U.C.C.G.A.A.G.A.G.A.C

Figure 17A

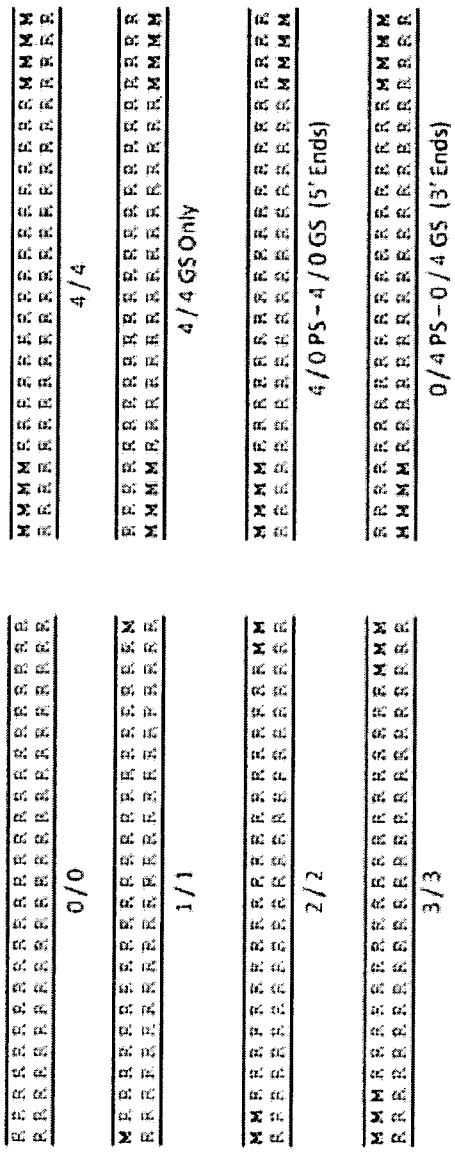
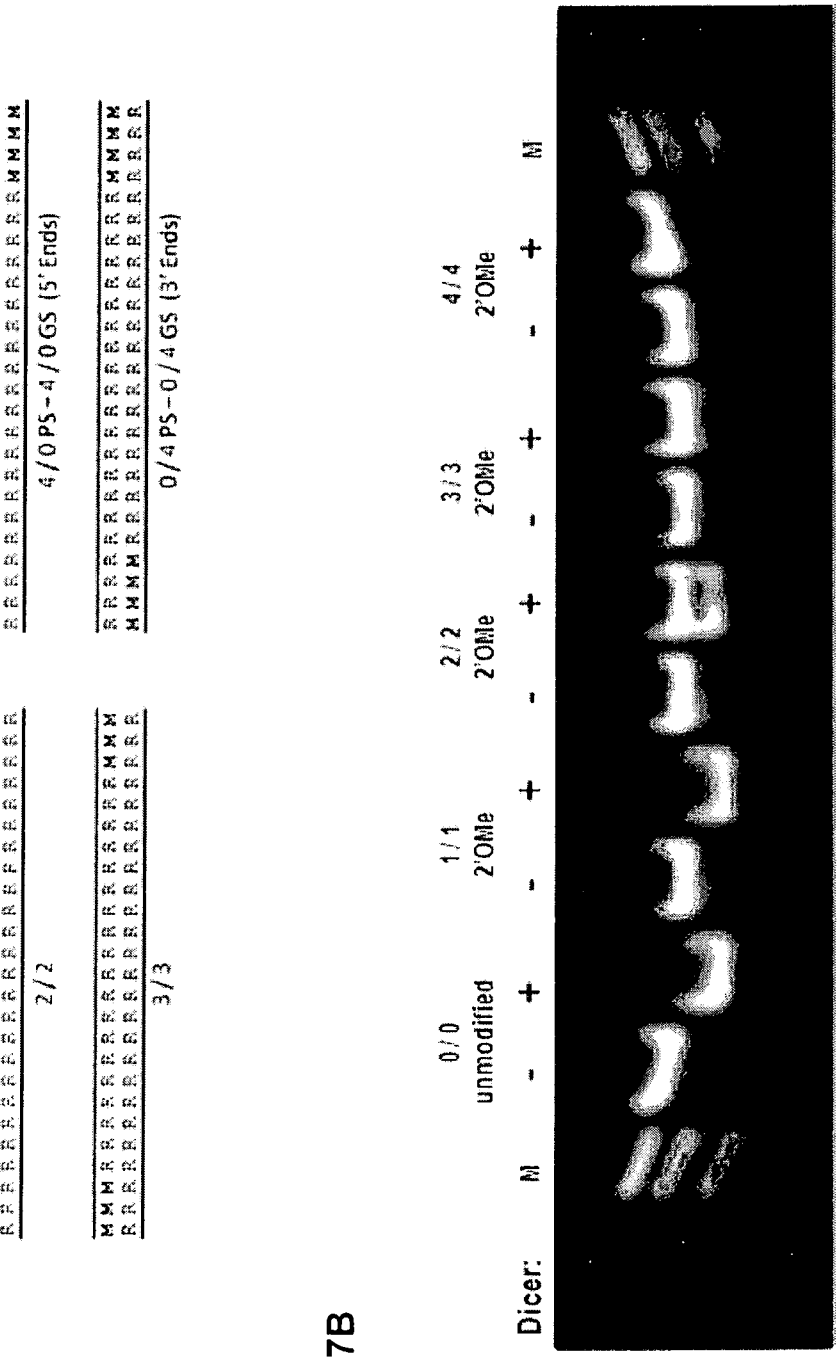


Figure 17B



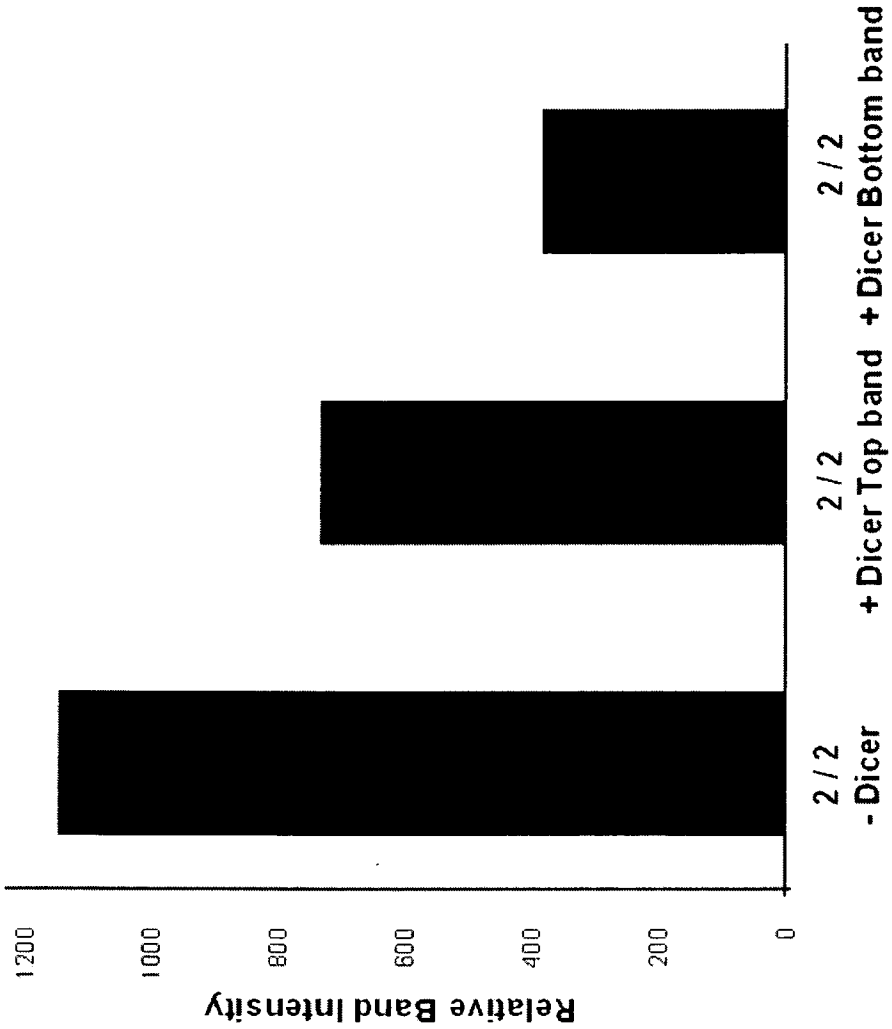

Relative Band Intensity
0
200
400
600
800
1000
1200
2 / 2
- Dicer
2 / 2
+ Dicer Top band
2 / 2
+ Dicer Bottom band

Figure 17C

Figure 17D

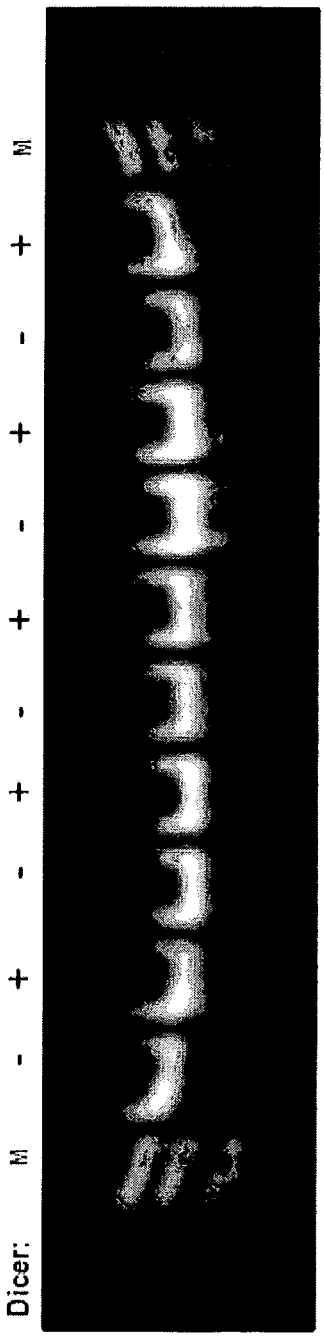


Figure 17E