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(54) Title: NUCLEIC ACID SILENCING AGENT-PROTEIN CONJUGATES AND USE THEREOF FOR TREATING HCV-RELATED DISORDERS

(57) Abstract: A method of using a complex or a molecule comprising a targeting moiety to deliver a second molecule effectively to HCV or to a cell being infected or having the potential to be infected by HCV, resulting in the prevention, immunization, inhibition or cure of HCV infection, or other beneficial effects related to HCV infection.



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**NUCLEIC ACID SILENCING AGENT-PROTEIN CONJUGATES AND USE
THEREOF FOR TREATING HCV-RELATED DISORDERS**

5 **BACKGROUND OF THE INVENTION**

Hepatitis C virus (HCV), the major cause of “non-A non-B hepatitis” (NANBH) before its identification, is a positive, single-stranded RNA virus of the *Flaviviridae* family. Its genome contains a single long open reading frame which encodes a
10 polyprotein of about three-thousand amino acid residues. (Choo *et al.* (1989) Science 244:359-362). The polyprotein is processed by host cell and viral proteases into three major structural proteins and several non-structural proteins necessary for viral replication. The nucleotide sequence of HCV is highly variable; the most divergent isolates sharing only 60% nucleotide sequence homology. Several different genotypes
15 of HCV with slightly different genomic sequences have been identified.

Isolates from all over the world have now been grouped into 6 main types, each with several subtypes, based on sequence data (Simmonds *et al.* (1995) Hepatology 21: 570-83). Genotypes 1-3 account for almost all infections in Europe, genotype 4 is prevalent in Egypt and Zaire, genotype 5 in South Africa and genotype 6 in Hong Kong.

20 The virus is transmitted primarily by blood and blood products. The majority of infected individuals have either received blood transfusions prior to 1990 (when screening of the blood supply for HCV was implemented) or have used intravenous drugs. Approximately 200 to 400 million people worldwide are chronically infected with HCV and of those infected about 2 to 5 million reside in the United States.

25 Chronic HCV infection occurs in approximately 80% of those infected and often leads to the development of cirrhosis and liver cancer.

Presently, the only therapy with any demonstrated efficacy against HCV-induced liver disease involves the use of alpha-interferon in combination with ribavirin, but this approach has achieved only limited success. The response rate of a chronically infected
30 HCV population to treatment with alpha-interferon and ribavirin in controlled clinical trials is reported as only 40-50% with 50% of these individuals relapsing when treatment is stopped. There is also evidence that alpha- interferon treatment is much less effective against some HCV genotypes than others.

Currently, there are no other licensed drugs indicated for treating HCV infection other than alpha-interferon and ribavirin. Accordingly, improved therapies for treating and preventing HCV infection are needed.

5 **SUMMARY OF THE INVENTION**

The present invention solves the foregoing problems by providing a method of using a complex or a molecule comprising a targeting moiety (e.g., a therapeutic targeting moiety) to deliver therapeutic RNA molecules effectively to HCV or to a cell being infected or having the potential to be infected by HCV, resulting in the
10 prevention, immunization, inhibition, cure, or other beneficial effects towards HCV infection.

The agents of the invention are not limited to those useful for the treatment of HCV, however. In its most general aspect, the invention includes any agent (e.g., any complex or conjugate) comprising a targeting moiety (e.g., a therapeutic targeting
15 moiety) and a nucleic acid molecule, wherein the agent delivers the nucleic acid molecule to a target cell of interest (e.g., a tumor cell, immune cell, or a cell infected with a virus other than HCV).

In certain embodiments, the agent is a conjugate formed by a nucleic acid and a protein (e.g., a therapeutic protein or non-therapeutic (i.e., carrier) protein). In some
20 embodiments, the protein is a binding protein. In certain embodiments, the binding protein binds (e.g., binds to a target molecule) and modulates the activity of said target molecule. In some embodiments, the binding protein in the conjugate is a fusion protein. One or more nucleic acid molecules (e.g., about 2, about 5, about 10, about 15, about 20, about 25, or about 30 nucleic acid molecules) may be conjugated to the
25 protein. The nucleic acid and protein may be covalently linked, e.g., via an intervening linker. In one preferred embodiment, the protein of the conjugate comprises a sulfhydryl moiety. Where the nucleic acid is a double stranded nucleic acid, the nucleic acid is preferably bound to the the protein via a terminal nucleotide of the sense strand, more preferably the 3' terminus. In some embodiments, the nucleic acid of the
30 conjugate comprises a carbonyl moiety at the 3' or 5' terminus. In preferred embodiments, the carbonyl moiety is present in a modified terminal ribonucleotide. In other preferred embodiments, the nucleic acid molecules are linked to modified lysine residues within the protein. Preferably, the nucleic acid molecules do not interfere with

the binding affinity of the binding protein when conjugated to the binding protein. For example, the nucleic acid molecules may be conjugated to residues located within the constant domains of an (e.g., within the Fc domain, e.g., hinge, CH2, and/or CH3 domains, or within the CH1 or CL domains of the Fv domain) or within portions of the variable region frameworks which are not critical for binding.

In certain embodiments, the protein and the nucleic acid are linked by a heterobifunctional crosslinker to form the conjugate. In certain embodiments, the heterobifunctional crosslinker links the sulfhydryl and carbonyl moieties to form the conjugate. In one embodiment, the crosslinker is bonded (e.g., via a hydrazide moiety) to the carbonyl moiety of the nucleic acid (e.g., a terminal ribonucleotide comprising a carbonyl group, e.g., an aldehyde-modified ribonucleotide) via a hydrazone bond. In another embodiment, the crosslinker is bonded to an amino moiety of the nucleic acid (e.g., a terminal nucleotide comprising an amino group, e.g., a terminal nucleotide comprising a primary amine (e.g., an amino alkyl phosphoester) at the 2' or 3' position). In another embodiment, the crosslinker (e.g., the maleimide moiety of a crosslinker) is covalently bonded to the sulfhydryl moiety of the protein (e.g., a modified lysine residue comprising a sulfhydryl moiety) via a thioester bond.

In one embodiment, the crosslinker comprises a maleimide moiety and a hydrazide moiety, linked by a spacer moiety. In one embodiment, the linker is a heterobifunctional crosslinker, selected from the group consisting of N-(ϵ -maleimidocaproic acid) hydrazide, N-(β -maleimidopropionic acid) hydrazide and N-(κ -maleimidoundecanoic acid) hydrazide, including EMCH, BPMH, KMUH and other linkers containing a maleimide and a hydrazide. In another embodiment, the heterobifunctional linker comprises a hydrozone bond formed by a SANH moiety (S-HyNic) (Solulink, <http://www.solulink.com/products/solulink-cross-linkers-s-1002-105.php>) and a SFB moiety. In preferred embodiments, the SFB moiety is covalently bonded to an amino moiety of the nucleic acid.

In certain embodiments, the agent of the invention comprises a protein which is a binding protein, e.g., an antigen binding protein, e.g., an antibody or fragment thereof, which binds to a target molecule target and thereby recruits the nucleic acid to the target molecule. In some embodiments, the binding protein is a human, humanized or chimeric antibody. In other embodiments, the binding protein is a fusion protein. In another embodiment, the binding protein is an antibody variant selected from the group

consisting of a multispecific antibody, a multivalent antibody, a nanobody and a single chain antibody.

In some embodiments, the binding protein in the conjugate is an antibody or antigen binding portion that specifically binds to one or more protein epitopes within HCV or a HCV-related hepacivirus. In one embodiment, the protein is an antibody or antigen binding portion that specifically binds to one or more epitopes within the E2 protein of HCV or a HCV-related hepacivirus. In one preferred embodiment, the protein in the conjugate is an antibody or antigen binding portion thereof that inhibits the ability of the virus to infect cells.

In some embodiments, the protein in the conjugate is a human, humanized or chimeric antibody. In one embodiment, the antibody competes therewith or binds other HCV-associated antigens. In one preferred embodiment, the antibody or antigen binding portion binds an epitope within amino acid residues 412-464, 412-423, or 413-420 of the E2 protein. In another embodiment, the binding protein is an antibody variant selected from the group consisting of a multispecific antibody, a multivalent antibody, a nanobody and a single chain antibody. In yet another embodiment, the conjugate binds to two or more HCV genotypes of an HCV E2 protein or fragment thereof. In a further embodiment, the conjugate binds an HCV E2 protein from a HCV virus of the 1a, 1b, 2b, 3a, 4a, 5, 5a, 6a, 6g or 6k genotypes, or combinations thereof. In some embodiments, the binding protein is a fusion protein.

In some embodiments, the protein, antibody or antigen binding portion thereof in the conjugate specifically binds to the E2 protein, or fragment thereof, of HCV with a K_D of at least about 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M or better.

In some embodiments, the antibody is one of the following human monoclonal antibodies: 95-2, 83-128, 95-2, 83-128, 95-14, 95-15, 95-18, 95-20, 95-21, 95-25, 95-26, 95-30, 95-38, 95-39, 95-42, 95-43, 95-48, 95-49, 95-52, 95-54, 95-58, and 95-62. In a preferred embodiment, the antibody is the 95-2 or the 83-128 human monoclonal antibody.

Some of the embodiments of the invention feature antibodies with a heavy chain variable region that is encoded from gene VH 3-33. In some embodiments, the light chain variable region of the antibody is encoded from gene V κ L6.

In some embodiments, the RNA in the conjugate inhibits replication of HCV. In one embodiment, the RNA in the conjugate is a target mRNA encoded by the genome of HCV.

In some embodiments, the nucleic acid in this conjugate is modified to comprise
5 a free carbonyl moiety at the 3' or 5' terminus. In one preferred embodiment, the nucleic acid is a nucleic acid silencing agent. In one embodiment, the protein and nucleic acid are linked by a heterobifunctional crosslinker linking the sulfhydryl and carbonyl moieties to form the RNA-protein conjugate.

In certain embodiments, the nucleic acid of an agent of the invention is a nucleic
10 acid with gene silencing activity (*e.g.*, a nucleic acid silencing agent). Exemplary nucleic acid silencing agents include DNA and RNA silencing agents. The nucleic acids employed in the invention are preferably modified to comprise one or more nucleotide analogs (*e.g.*, 2'OMe, LNA, and/or phosphorothiate modifications) which, for example, stabilize the nucleic acids from degradation *in vivo* and/or improve
15 delivery to a target antigen, target RNA, or target cell *in vivo*). The nucleic acids may be single-stranded (*e.g.*, ssRNA) or double-stranded (*e.g.*, dsRNA). In one embodiment, the agent RNA silencing agent comprises sense and antisense strands of 18-30 nucleotides in length. In another embodiment, the RNA silencing agent comprises sense and antisense strands of 21-23, 25-30, and preferably 27 nucleotides in length. In one
20 preferred embodiment, the RNA silencing agent comprises sense and antisense strands of 21-23 nucleotides in length. One or both ends of the dsRNA may comprise nucleotide 3' overhangs of one or more nucleotides (*e.g.*, dT or dTdT overhangs) or they may be blunt-ended. Where present, the 3' overhangs may comprise one or more modified nucleotides (*e.g.*, 2' OMe nucleotides). RNA silencing agents may be selected
25 from the group consisting of an siRNA, an miRNA, and an shRNA. In other embodiments, the nucleic acid is an inhibitor of RNA silencing (*e.g.*, a DNA or RNA antisense oligonucleotide with complementarity to a miRNA, *i.e.*, an antagomir). In preferred embodiments, the nucleic acid (*e.g.*, a dsRNA) comprises a terminal ribonucleotide (*e.g.*, at the 3' end of a sense strand). In some embodiments, the agent of
30 the invention is internalized by receptor-mediated endocytosis. In some embodiments, the nucleic acid of the agent is released by acid hydrolysis following internalization. In some embodiments, the nucleic acid of the agent is not released following internalization. In some embodiments, a nucleic acid of the agent is released via the loss

of the annealing of the nucleic acid to another nucleic acid of the agent. In some embodiments, a nucleic acid of the agent remains bound to the protein of the agent after the release of another nucleic acid of the agent. In one embodiment, the RNA exhibits greater than 50% silencing efficiency (e.g., 60%, 70%, 80%, 90% or more) of the target mRNA in a silencing assay relative to a control RNA.

In certain exemplary embodiments, the agents of the invention comprise a nucleic acid having a sequence that is substantially complementary to a target RNA (e.g., target mRNA or miRNA) associated with HCV to silence the target mRNA in a cell infected with the virus. In some embodiments, the nucleic acid inhibits replication of HCV. In some embodiments, the nucleic acid inhibits the ability of the virus to infect cells. In one embodiment, the nucleic acid targets an RNA encoded by the genome of HCV. In other embodiments, the nucleic acid targets the genome of HCV itself. In certain exemplary embodiments, the nucleic acid targets an mRNA encoding the E1 and/or E2 glycoprotein of HCV. In certain embodiments, the nucleic acid exhibits greater than 50% silencing efficiency of the target mRNA in a HCV infection assay relative to a control RNA. In one embodiment, the RNA in the conjugate inhibits HCV infection *in vitro*. In another embodiment, the RNA in the conjugate inhibits HCV replication *in vivo*. In one preferred embodiment, the RNA in the conjugate treats HCV-mediated disease *in vivo* in a mammal. In another embodiment, the RNA protects from or inhibits HCV-mediated hepatocyte pathology in a subject. In one embodiment, the conjugate is used to treat HCV-mediated pathology *in vivo* in a mammal.

In certain embodiments, the agents of the invention further comprise at least a second nucleic acid (e.g., a second siRNA) linked to the protein of the agent, e.g., by a heterobifunctional crosslinker. In certain embodiments, the second nucleic acid comprises a sequence that is substantially complementary to a different HCV-associated target mRNA than the first nucleic acid. The crosslinker linking the first nucleic acid to the protein and the crosslinker linking the second nucleic acid to the protein may be the same or different.

In some embodiments, the nucleic acid silencing agent-binding protein conjugate comprises a binding protein that specifically binds to one or more protein epitopes within a HCV or HCV-related hepacivirus and a nucleic acid silencing agent having a sequence that is substantially complementary to a target mRNA associated with HCV or a HCV-related hepacivirus to silence the target mRNA in a cell infected with the virus.

In one embodiment, the RNA silencing agent is selected from the group consisting of a siRNA, a miRNA, a shRNA and an antagomir. In one preferred embodiment, the protein and nucleic acid are linked by a heterobifunctional crosslinker to form the conjugate.

5 In another embodiment, the binding protein in the conjugate comprises a free sulfhydryl moiety, while the nucleic acid silencing agent is modified to contain a free carbonyl moiety at the 3' or 5' terminus, and the heterobifunctional crosslinker links the sulfhydryl and carbonyl moieties to form the RNA-protein. In one preferred embodiment, the nucleic acid silencing agent in the conjugate is a RNA silencing agent.

10 In some embodiments, the protein and the nucleic acid in the conjugate is linked together by a crosslinker. In one embodiment, the crosslinker comprises a maleimide moiety and a hydrazide moiety, linked by a spacer moiety. In another embodiment, a maleimide moiety of a crosslinker reacts with a free sulfhydryl group on the antibody, or antigen binding portion thereof, to form a thioester bond. In one embodiment, the free
15 sulfhydryl group on the antibody, or antigen binding portion thereof, is added to lysines via a thiolation agent. In one embodiment, the thiolation agent is 2-Iminothiolane (Traut's Reagent). In one embodiment, the linker is a heterobifunctional crosslinker, selected from the group consisting of N-(ϵ -maleimidocaproic acid) hydrazide, N-(β -maleimidopropionic acid) hydrazide and N-(κ -maleimidoundecanoic acid) hydrazide,
20 including EMCH, BPMH, KMHU and other linkers containing a maleimide and a hydrazide. In another embodiment, the hydrazide moiety of the crosslinker reacts with a carbonyl group on the RNA to form a hydrazone bond. In one embodiment, the carbonyl group on the 3' or 5' end of the RNA is added by chemical means. In one preferred embodiment, the carbonyl group on the 3' end of the RNA is formed by reaction with
25 sodium periodate. In one embodiment, the carbonyl group on the 5' is introduced by standard RNA synthesis techniques, e.g. chemically synthesized phosphoramidites. In another embodiment, the carbonyl group on the 5' is introduced by a chemical cross linker containing a carbonyl at one end and an NHS-ester at the other in which the NHS ester is reactive with an amine group at the 5' end of RNA generated by standard RNA
30 synthesis techniques.

 In some embodiments, the RNA is double stranded. In one embodiment, the RNA comprises sense and antisense strands of 18-30 nucleotides in length. In another embodiment, the RNA comprises sense and antisense strands of 21-23, 25-30, and

preferably 27 nucleotides in length. In one preferred embodiment, the RNA comprises sense and antisense strands of 21-23 nucleotides in length and contains 2 nucleotide 3' overhangs or blunt-ended. In one embodiment, the RNA comprises an antisense strand that is complimentary to target sequences in the mRNA encoding E2 protein from HCV isolates.

In some embodiments, the conjugate is internalized by receptor-mediated endocytosis. In one embodiment, the RNA silencing agent is released from the conjugate by acid hydrolysis. In one embodiment, the RNA exhibits greater than 50% silencing efficiency of the target mRNA in a silencing assay relative to a control RNA.

In one embodiment, the conjugate further comprises at least a second RNA linked to the protein, antibody or antigen binding portion by a heterobifunctional crosslinker, wherein the second RNA silencing agent has a sequence that is substantially complementary to a second HCV-associated target mRNA in the HCV-infected cell to mediate cleavage of the second target mRNA.

In one embodiment, the crosslinker in the conjugate linking the first RNA silencing agent and the crosslinker linking the second RNA silencing agent are the same. In another embodiment, the crosslinker linking the first RNA silencing agent and the crosslinker linking the second RNA silencing agent are different.

In one embodiment, the RNA in the conjugate inhibits HCV infection *in vitro*. In another embodiment, the RNA in the conjugate inhibits HCV replication *in vivo*. In one preferred embodiment, the RNA in the conjugate treats HCV-mediated disease *in vivo* in a mammal. In another embodiment, the RNA protects from or inhibits HCV-mediated hepatocyte pathology in a subject. In one embodiment, the conjugate is used to treat HCV-mediated pathology *in vivo* in a mammal.

The invention also includes a method of treating or preventing a HCV-associated disease or disorder in a subject comprising, administering to the subject the RNA-protein conjugate of any of the preceding claims in an amount effective to treat or prevent a symptom or disorder associated with HCV infection. In one embodiment, the conjugate is administered in combination with a second therapeutic agent. In one embodiment, the second agent is an antiviral agent. In another embodiment, said second therapeutic agent is administered sequentially with the conjugate. In one embodiment, said second therapeutic agent is administered prior to the administration of the

conjugate. In some embodiments, the subject is resistant to anti-viral therapy. In some embodiments, the subject has received a liver transplant.

This invention also features a pharmaceutical composition suitable for treating a HCV-mediated disease or disorder in a subject comprising the conjugate in a

5 pharmaceutically acceptable carrier. In one preferred embodiment, the composition further comprises a second therapeutic agent. In one embodiment, the conjugate in the composition is present in the composition in an amount that is less than 50% (*e.g.*, less than about 20%, about 10%, or about 5%) than the amount of the second therapeutic agent.

10 This invention also features a method of making an agent of the invention (*e.g.*, nucleic acid-binding protein conjugate). A method includes, for example, a method providing a binding protein comprising a free sulfhydryl group, a method providing a nucleic acid comprising a modified ribonucleotide at a 3' or 5' terminus of the nucleic acid, wherein the nucleic acid comprises a nucleic acid strand that is sufficiently
15 complementary to a target mRNA to silence expression of the target mRNA, and wherein the modified ribonucleotide comprises a modified carbonyl moiety, and a method combining the binding protein and the nucleic acid with a heterobifunctional linker under condition such that the binding protein and the nucleic acid are linked by the heterobifunctional crosslinker to form the nucleic acid-binding protein conjugate, etc. In some embodiments, the method includes, for example, (i) providing a binding
20 protein comprising a free sulfhydryl group, (ii) providing a nucleic acid comprising a modified ribonucleotide at a 3' or 5' terminus of the nucleic acid, wherein the nucleic acid comprises a nucleic acid strand that is sufficiently complementary to a target mRNA to silence expression of the target mRNA, and wherein the modified
25 ribonucleotide comprises a free carbonyl group (*e.g.*, an aldehyde-modified ribonucleotide), and (iii) combining the binding protein and the nucleic acid with a heterobifunctional linker under condition such that the binding protein and the nucleic acid are linked by the heterobifunctional crosslinker to form the nucleic acid-binding protein conjugate.

30 In one embodiment, the crosslinker comprises a maleimide moiety and a hydrazide moiety, linked by a spacer moiety. In one embodiment, a maleimide moiety of a crosslinker reacts with the free sulfhydryl group on the binding protein to form a thioester bond. In another embodiment, the free sulfhydryl group is added to a lysine

residue of the binding protein via a thiolation agent. In one preferred embodiment, the thiolation agent is 2-Iminothiolane (Traut's Reagent). In some embodiments, the heterobifunctional crosslinker is selected from the group consisting of N-(ε-maleimidocaproic acid) hydrazide, N-(β-maleimidopropionic acid) hydrazide and N-(κ-maleimidoundecanoic acid) hydrazide, including EMCH, BPMH, KMUH and other linkers containing a maleimide and a hydrazide. In one embodiment, the hydrazide moiety of the crosslinker reacts with the free carbonyl group of the nucleic acid to form a hydrazone bond. In one embodiment, the free carbonyl group is formed by oxidizing the modified ribonucleotide with sodium periodate. In one preferred embodiment, the modified ribonucleotide is at the 3' terminus of the nucleic acid. In one embodiment, the free carbonyl group is introduced by chemical synthesis with phosphoramidites. In another embodiment, the free carbonyl group is introduced by a chemical cross linker containing a carbonyl at one end and an NHS-ester at the other in which the NHS ester is reactive with an amine group at the 5' end of RNA generated by standard RNA synthesis techniques. In one preferred embodiment, the modified ribonucleotide is at the 5' terminus of the nucleic acid.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are graphs showing binding of human antibody 95-2 (Figure 1A) and human antibody 83-128 (Figure 1B) to soluble HCV genotype 1a E2-660 (triangles) and soluble HCV genotype 1b E2-661 (squares).

Figure 2A and 2B are graphs showing neutralization of infection of Hep3B cells with various HCV genotype pseudovirus in the presence of human antibodies 95-2, 83-128, and 17C7 (a control human mAb in these experiments).

Figure 3A and 3B are Western blots showing antibody binding to purified soluble mammalian expressed E2 protein (E2-661) from HCV genotype 1a and 1b subjected to reducing (Figure 3A) or non-reducing (Figure 3B) SDS-PAGE followed by transfer to PVDF membrane.

Figure 4 is a table showing the affinity of human antibodies 95-2 and 83-128 to HCV E2 412-423 epitope expressed as a bacterial fusion protein.

Figure 5 is a schematic representation of the succinimidyl 4-formylbenzoate (SFB) modification of the oligo with a N6 linker.

Figure 6 shows the modification of the oligonucleotide with SFB.

Figure 7 shows the comparison between the duplex oligo and the single strand
5 oligo in modifications with SFB.

Figure 8 shows that the oligo modification with SFB can be detected by the 2-HP method, while the antibody modification with succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH) can be detected by 4-NB method.

Figure 9 shows that 2-HP results are comparable with electrophoresis in
10 analyzing oligo modification with SFB.

Figure 10 shows the IC₅₀ of SFB modification of GFPz Stable 6 oligo

Figure 11 is a schematic representation of the formation of a hydrazone bond by conjugation of SFB-modified oligo and SANH-modified antibody.

Figure 12 is a schematic representation of the 2-day conjugation protocol.

Figure 13 is a schematic representation of the detection protocol of conjugates.
15

Figure 14 shows early non-optimized data for antibody-DNA/RNA duplex conjugation.

Figure 15 shows improved conjugation efficiency with fully modified DNA-SFB.

Figure 16 shows optimal conjugation with modified oligo.
20

Figure 17 shows optimization data for conjugation, detected by nucleic acid staining.

Figure 18 shows optimization data for conjugation, detected by protein staining.

Figure 19 shows the antibody-GFPz Stable 2 siRNA conjugation.

Figure 20 is a schematic representation of 3' oxidation of siRNA.
25

Figure 21 shows the optimization of oxidation reaction.

Figure 22 shows 3' oxidation of siRNA under varying pH conditions.

Figure 23 shows that 25 mM sodium periodate is sufficient to oxidize up to at least 100 uM siRNA.

Figure 24 is a schematic representation of reactions of oxidized siRNA with the linker, 3,3'-N-[e-Maleimidocaproic acid] hydrazide, trifluoroacetic acid salt (EMCH).
30

Figure 25 shows the reaction of oxidized siRNA with the EMCH linker.

Figure 26 shows the optimization of oxidation of siRNA and reaction with EMCH in a single tube.

Figure 27 is a schematic representation of HPLC purification of siRNA-EMCH conjugate.

5 **Figure 28** shows retention time for different component samples.

Figure 29 shows HPLC elution map of different components.

Figure 30 is a schematic representation of modification of lysine residues on the antibody by Traut's reagent.

10 **Figure 31** shows modification of lysine residues on the antibody with different amounts of Traut's reagent.

Figure 32 shows that antibody modification does not significantly alter its antigen recognition.

Figure 33 is a schematic representation of conjugation of siRNA to a human monoclonal antibody.

15 **Figure 34** shows the antibody-siRNA conjugation and detection on a native gel.

Figure 35 shows the detection of the antibody-siRNA conjugation using different staining methods.

Figure 36 shows the detection of the antibody-siRNA conjugation using isoelectric focusing (IEF) gel and staining.

20 **Figure 37** depicts the alignment of the heavy chain variable region of antibodies derived from clones 83-128 (SEQ ID NO:1), 95-2 (SEQ ID NO:3), 95-14 (SEQ ID NO:32), 95-38 (SEQ ID NO:33), 95-25 (SEQ ID NO:34), 95-42 (SEQ ID NO:35), 95-43 (SEQ ID NO:36), 95-49 (SEQ ID NO:37), 95-54 (SEQ ID NO:38), 95-58 (SEQ ID NO:39) and 95-62 (SEQ ID NO:40) and which are reactive against epitope 412-423 of
25 E2-660 HCV glycoprotein.

Figure 38 depicts the alignment of light chain variable region of antibodies derived from clones 83-128 (SEQ ID NO:2), 073-1 (SEQ ID NO:6), 95-2 (SEQ ID NO:4), 95-14 (SEQ ID NO:44) and 95-38 (SEQ ID NO:53).

30

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

In order to provide a clear understanding of the specification and claims, the following definitions are conveniently provided below.

As used herein, the term "RNA silencing" refers to a group of sequence-specific regulatory mechanisms (e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression) mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "target gene" is a gene whose expression is to be substantially inhibited or "silenced." This silencing can be achieved by RNA silencing, e.g. by cleaving the mRNA of the target gene or translational repression of the target gene. The term "non-target gene" is a gene whose expression is not to be substantially silenced. In one embodiment, the polynucleotide sequences of the target and non-target gene (e.g. mRNA encoded by the target and non-target genes) can differ by one or more nucleotides. In another embodiment, the target and non-target genes can differ by one or more polymorphisms. In another embodiment, the target and non-target genes can share less than 100% sequence identity. In another embodiment, the non-target gene may be a homolog (e.g. an ortholog or paralog) of the target gene.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g. the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include small (<50 b.p.), noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include siRNAs, miRNAs, siRNA-like duplexes, and dual-function oligonucleotides as well as precursors thereof. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

The term “nucleoside” refers to a molecule having a purine or pyrimidine base covalently linked to a ribose or deoxyribose sugar. Exemplary nucleosides include adenosine, guanosine, cytidine, uridine and thymidine. Additional exemplary nucleosides include inosine, 1-methyl inosine, pseudouridine, 5,6-dihydrouridine, ribothymidine, ²N-methylguanosine and ^{2,2}N,N-dimethylguanosine (also referred to as “rare” nucleosides). The term “nucleotide” refers to a nucleoside having one or more phosphate groups joined in ester linkages to the sugar moiety. Exemplary nucleotides include nucleoside monophosphates, diphosphates and triphosphates. The terms “polynucleotide” and “nucleic acid molecule” are used interchangeably herein and refer to a polymer of nucleotides joined together by a phosphodiester linkage between 5’ and 3’ carbon atoms.

The term “RNA” or “RNA molecule” or “ribonucleic acid molecule” refers to a polymer of ribonucleotides. The term “DNA” or “DNA molecule” or “deoxyribonucleic acid molecule” refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (*e.g.*, by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA can also be chemically synthesized. DNA and RNA can be single-stranded (*i.e.*, ssRNA and ssDNA, respectively) or multi-stranded (*e.g.*, double stranded, *i.e.*, dsRNA and dsDNA, respectively). “mRNA” or “messenger RNA” is single-stranded RNA that specifies the amino acid sequence of one or more polypeptide chains. This information is translated during protein synthesis when ribosomes bind to the mRNA.

As used herein, the term “rare nucleotide” refers to a naturally occurring nucleotide that occurs infrequently, including naturally occurring deoxyribonucleotides or ribonucleotides that occur infrequently, *e.g.*, a naturally occurring ribonucleotide that is not guanosine, adenosine, cytosine, or uridine. Examples of rare nucleotides include, but are not limited to, inosine, 1-methyl inosine, pseudouridine, 5,6-dihydrouridine, ribothymidine, ²N-methylguanosine and ^{2,2}N,N-dimethylguanosine.

The term “nucleotide analog” or “altered nucleotide” or “modified nucleotide” refers to a non-standard nucleotide, including non-naturally occurring ribonucleotides or deoxyribonucleotides. Preferred nucleotide analogs are modified at any position so as to alter certain chemical properties of the nucleotide yet retain the ability of the nucleotide analog to perform its intended function. Examples of preferred modified nucleotides include, but are not limited to, 2-amino-guanosine, 2-amino-adenosine, 2,6-diamino-

guanosine and 2,6-diamino-adenosine. Examples of positions of the nucleotide which may be derivatized include the 5 position, *e.g.*, 5-(2-amino)propyl uridine, 5-bromo uridine, 5-propyne uridine, 5-propenyl uridine, etc.; the 6 position, *e.g.*, 6-(2-amino)propyl uridine; the 8-position for adenosine and/or guanosines, *e.g.*, 8-bromo
5 guanosine, 8-chloro guanosine, 8-fluoroguanosine, etc. Nucleotide analogs also include deaza nucleotides, *e.g.*, 7-deaza-adenosine; O- and N-modified (*e.g.*, alkylated, *e.g.*, N6-methyl adenosine, or as otherwise known in the art) nucleotides; and other heterocyclically modified nucleotide analogs such as those described in Herdewijn, *Antisense Nucleic Acid Drug Dev.*, 2000 Aug. 10(4):297-310.

10 Nucleotide analogs may also comprise modifications to the sugar portion of the nucleotides. For example the 2' OH-group may be replaced by a group selected from H, OR, R, F, Cl, Br, I, SH, SR, NH₂, NHR, NR₂, COOR, or OR, wherein R is substituted or unsubstituted C₁–C₆ alkyl, alkenyl, alkynyl, aryl, etc. Other possible modifications include those described in U.S. Patent Nos. 5,858,988, and 6,291,438.

15 The phosphate group of the nucleotide may also be modified, *e.g.*, by substituting one or more of the oxygens of the phosphate group with sulfur (*e.g.*, phosphorothioates), or by making other substitutions which allow the nucleotide to perform its intended function such as described in, for example, Eckstein, *Antisense Nucleic Acid Drug Dev.* 2000 Apr. 10(2):117-21, Rusckowski *et al.* *Antisense Nucleic*
20 *Acid Drug Dev.* 2000 Oct. 10(5):333-45, Stein, *Antisense Nucleic Acid Drug Dev.* 2001 Oct. 11(5): 317-25, Vorobjev *et al.* *Antisense Nucleic Acid Drug Dev.* 2001 Apr. 11(2):77-85, and U.S. Patent No. 5,684,143. Certain of the above-referenced modifications (*e.g.*, phosphate group modifications) preferably decrease the rate of hydrolysis of, for example, polynucleotides comprising said analogs *in vivo* or *in vitro*.

25 The term “oligonucleotide” refers to a short polymer of nucleotides and/or nucleotide analogs. The term “RNA analog” refers to a polynucleotide (*e.g.*, a chemically synthesized polynucleotide) having at least one altered or modified nucleotide as compared to a corresponding unaltered or unmodified RNA but retaining the same or similar nature or function as the corresponding unaltered or unmodified
30 RNA. The oligonucleotides may be linked with linkages which result in a lower rate of hydrolysis of the RNA analog as compared to an RNA molecule with phosphodiester linkages. For example, the nucleotides of the analog may comprise methylenediol, ethylene diol, oxymethylthio, oxyethylthio, oxycarbonyloxy, phosphorodiamidate,

and/or phosphorothioate linkages. Exemplary RNA analogues include sugar- and/or backbone-modified ribonucleotides and/or deoxyribonucleotides. Such alterations or modifications can further include addition of non-nucleotide material, such as to the end(s) of the RNA or internally (at one or more nucleotides of the RNA). An RNA
5 analog need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNA silencing (*e.g.* RNA interference). In an exemplary embodiment, oligonucleotides comprise Locked Nucleic Acids (LNAs) or Peptide Nucleic Acids (PNAs).

As used herein, the terms “sufficient complementarity” or “sufficient degree of
10 complementarity” mean that the nucleic acid silencing agent (*e.g.*, RNA silencing agent) has a sequence (*e.g.* in the antisense strand, mRNA targeting moiety or miRNA recruiting moiety) which is sufficient to bind the desired target RNA, respectively, and to trigger the silencing of the target mRNA.

As used herein, the term “RNA interference” (“RNAi”) refers to a type of RNA
15 silencing which results in the selective intracellular degradation of a target RNA. RNAi occurs in cells naturally to remove foreign RNAs (*e.g.*, viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. Both RNAi and translational repression are mediated by RISC. Both RNAi and translational repression occur naturally or can
20 be initiated by the hand of man, for example, to silence the expression of target genes.

As used herein, the term “translational repression” refers to a selective inhibition of mRNA translation. Natural translational repression proceeds *via* miRNAs cleaved from shRNA precursors. Both RNAi and translational repression are mediated by RISC. Both RNAi and translational repression occur naturally or can be initiated by the
25 hand of man, for example, to silence the expression of target genes.

An RNA silencing agent having a strand which is “sequence sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference (RNAi)” means that the strand has a sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process.

As used herein, the term “small interfering RNA” (“siRNA”) (also referred to in
30 the art as “short interfering RNAs”) refers to an RNA (or RNA analog) comprising between about 10-50 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNA interference. Preferably, a siRNA comprises between about 15-30

nucleotides or nucleotide analogs, more preferably between about 16-25 nucleotides (or nucleotide analogs), even more preferably between about 18-23 nucleotides (or nucleotide analogs), and even more preferably between about 19-22 nucleotides (or nucleotide analogs) (*e.g.*, 19, 20, 21 or 22 nucleotides or nucleotide analogs). The term
5 “short” siRNA refers to a siRNA comprising ~21 nucleotides (or nucleotide analogs), for example, 19, 20, 21 or 22 nucleotides. The term “long” siRNA refers to a siRNA comprising ~24-25 nucleotides, for example, 23, 24, 25 or 26 nucleotides. Short siRNAs may, in some instances, include fewer than 19 nucleotides, *e.g.*, 16, 17 or 18 nucleotides, provided that the shorter siRNA retains the ability to mediate RNAi.
10 Likewise, long siRNAs may, in some instances, include more than 26 nucleotides, provided that the longer siRNA retains the ability to mediate RNAi absent further processing, *e.g.*, enzymatic processing, to a short siRNA.

As used herein, the term “microRNA” (“miRNA”), also referred to in the art as “small temporal RNAs” (“stRNAs”), refers to a small (10-50 nucleotide) RNA which
15 are genetically encoded (*e.g.* by viral, mammalian, or plant genomes) and are capable of directing or mediating RNA silencing. A “miRNA disorder” shall refer to a disease or disorder characterized by an aberrant expression or activity of a miRNA.

As used herein, the term “antisense strand” of an RNA silencing agent, *e.g.* an siRNA or RNAi agent, refers to a strand that is substantially complementary to a section
20 of about 10-50 nucleotides, *e.g.*, about 15-30, 16-25, 18-23 or 19-22 nucleotides of the mRNA of the gene targeted for silencing. The antisense strand or first strand has sequence sufficiently complementary to the desired target mRNA sequence to direct target-specific silencing, *e.g.*, complementarity sufficient to trigger the destruction of the desired target mRNA by the RNAi machinery or process (RNAi interference) or
25 complementarity sufficient to trigger translational repression of the desired target mRNA.

The term “sense strand” or “second strand” of an RNA silencing agent, *e.g.* a siRNA or RNAi agent, refers to a strand that is complementary to the antisense strand or first strand. Antisense and sense strands can also be referred to as first or second
30 strands, the first or second strand having complementarity to the target sequence and the respective second or first strand having complementarity to said first or second strand. miRNA duplex intermediates or siRNA-like duplexes include a RNA strand having sufficient complementarity to a section of about 10-50 nucleotides of the mRNA of the

gene targeted for silencing and another RNA strand having sufficient complementarity to form a duplex with the RNA strand that has complementarity with the mRNA.

As used herein, the term "guide strand" refers to a strand of an RNA silencing agent, e.g., an antisense strand of an siRNA duplex or siRNA sequence, that enters into
5 the RISC complex and directs cleavage of the target mRNA.

The term "engineered," as in an engineered RNA precursor, or an engineered nucleic acid molecule, indicates that the precursor or molecule is not found in nature, in that all or a portion of the nucleic acid sequence of the precursor or molecule is created or selected by man. Once created or selected, the sequence can be replicated, translated,
10 transcribed, or otherwise processed by mechanisms within a cell. Thus, an RNA precursor produced within a cell from a transgene that includes an engineered nucleic acid molecule is an engineered RNA precursor.

An "isolated nucleic acid molecule or sequence" is a nucleic acid molecule or sequence that is not immediately contiguous with both of the coding sequences with
15 which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA or RNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a
20 genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide sequence.

As used herein, the term "isolated RNA" (e.g., "isolated shRNA", "isolated siRNA", "isolated siRNA-like duplex", "isolated miRNA", "isolated gene silencing
25 agent", or "isolated RNAi agent") refers to RNA molecules which are substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "transgene" refers to any nucleic acid molecule, which
30 is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from the cell. Such a transgene may include a gene that is partly or entirely heterologous (*i.e.*, foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. The term "transgene" also means a

nucleic acid molecule that includes one or more selected nucleic acid sequences, *e.g.*, DNAs, that encode one or more engineered RNA precursors, to be expressed in a transgenic organism, *e.g.*, animal, which is partly or entirely heterologous, *i.e.*, foreign, to the transgenic animal, or homologous to an endogenous gene of the transgenic animal, but which is designed to be inserted into the animal's genome at a location which differs from that of the natural gene. A transgene includes one or more promoters and any other DNA, such as introns, necessary for expression of the selected nucleic acid sequence, all operably linked to the selected sequence, and may include an enhancer sequence.

10 The term “therapeutic moiety”, as used herein, refers to a moiety (*e.g.*, a biomolecule) capable of eliciting a therapeutic effect.

A gene “involved” in a disease or disorder includes a gene, the normal or aberrant expression or function of which effects or causes the disease or disorder or at least one symptom of said disease or disorder.

15 The terms “hepatitis C virus,” “HCV,” “non-A non-B hepatitis,” or “NANBH” are used interchangeably herein, and include any “genotype” or “subgenotype” (also termed “subtype”) of the virion, or portion thereof (*e.g.*, a portion of the E2 protein of genotype 1a of HCV), that is encoded by the RNA of hepatitis C virus or that occurs by natural allelic variation.

20 The term “HCV-mediated disorder,” as used herein, includes disease states and/or symptoms associated with HCV infection. In general, the term “HCV-mediated disorder” refers to any disorder, the onset, progression or the persistence of the symptoms of which requires the participation of HCV. Exemplary HCV-mediated disorders include, but are not limited to, for example, cirrhosis and liver cancer.

25 HCV is organized by a 5'-untranslated region that is followed by an open reading frame (ORF) that codes for about 3,010 amino acids. The ORF runs from nucleotide base pair 342 to 8,955 followed by another untranslated region at the 3' end. The amino acids are subdivided into ten proteins in the order from 5' to 3' as follows: C; E1; E2; NS1; NS2; NS3; NS4 (a and b); and NS5 (a and b). These proteins are formed from the cleavage of the larger polyprotein by both host and viral proteases. The C, E1, and E2 proteins are structural and the NS1-NS5 proteins are nonstructural proteins. The C region codes for the core nucleocapsid protein. E1 and E2 are glycosylated envelope proteins that coat the virus. NS2 may be a zinc metalloproteinase. NS3 is a helicase.

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NS4a functions as a serine protease cofactor involved in cleavage between NS4b and NS5a. NS5a is a serine phosphoprotein whose function is unknown. The NS5b region has both RNA-dependent RNA polymerase and terminal transferase activity.

There are about six (6) distinct genotypes (*e.g.*, genotypes 1, 2, 3, 4, 5, and 6) that are categorized by variations in the core protein and over 80 subgenotypes which exhibit further variation within each genotype, some of which include: 1a; 1b; 1c; 2a; 2b; 2c; 3a; 3b; 4a; 4b; 4c; 4d; 4e; 5a; and 6a.

The term “antibody” as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, “antigen-binding portion”) or single chain thereof. An “antibody” refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_H1, C_H2 and C_H3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system.

The term “human antibody” is an antibody that has variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies described herein may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*).

As used herein, the term “HCV” refers to the virion or portion thereof, for example, a protein portion, such as the HCV E1 or E2 glycoprotein that is encoded by the RNA of HCV, or a fragment or portion of an HCV gene product.

The term “anti-HCV antibody” is an antibody that interacts with (*e.g.*, binds to) a HCV or a protein, carbohydrate, lipid, or other component produced by or associated with HCV. A “HCV glycoprotein antibody” is an antibody that binds a glycoprotein of HCV or a fragment thereof. An anti-HCV or glycoprotein antibody may bind to an epitope, *e.g.*, a conformational epitope or a linear (non-conformational) epitope, or to a portion or fragment of the virus or component thereof. In some embodiments, an anti-HCV antibody is a neutralizing antibody. In some embodiments, an anti-HCV antibody is a binding antibody.

An anti-HCV antibody, or antigen-binding portion thereof, can be administered alone or in combination with a second agent. The subject can be a patient infected or suspected to be infected with HCV or having a symptom of HCV-mediated disease (*e.g.*, liver inflammation, jaundice, tender hepatomegaly, or loss of appetite). The treatment can be used to cure, heal, alleviate, relieve, alter, remedy, ameliorate, palliate, improve, or affect the infection and the disease associated with the infection, the symptoms of the disease, or a predisposition toward the disease. “Treatment” may be understood to mean the prophylaxis or prevention of a productive infection before the onset of illness.

An amount of an anti-HCV antibody effective to treat HCV infection, or a “therapeutically effective amount”, is an amount of the antibody that is effective, upon single or multiple dose administration to a subject, in inhibiting HCV infection, disease, or sequelae thereof, in a subject. A therapeutically effective amount of the antibody or antibody fragment may vary according to factors such as the disease state, severity of symptoms, degree of liver inflammation, HCV strain or isolate, route of infection or exposure to HCV, whether the route of infection or exposure is known, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion is outweighed by the therapeutically beneficial effects. The ability of an antibody to inhibit a measurable parameter can be evaluated in an animal model system predictive of efficacy in humans. Alternatively, this property of an antibody or antibody composition can be evaluated by examining the ability of the compound to modulate HCV/cell interactions, *e.g.*, binding, infection, virulence, and the like, by *in vitro* assays known to the skilled

practitioner. *In vitro* assays include binding assays, such as ELISA, and neutralization assays.

An amount of an antibody effective to prevent a disorder, or a “prophylactically effective amount,” of the antibody is an amount that is effective, upon single- or
5 multiple-dose administration to the subject, in preventing or delaying the occurrence of the onset or recurrence of the disorder, or inhibiting a symptom thereof. However, if longer time intervals of protection are desired, increased doses or more frequent doses can be administered.

The terms “antagonize”, “induce”, “inhibit”, “potentiate”, “elevate”, “increase”,
10 “decrease”, or the like, e.g., which denote quantitative differences between two states, refer to a difference, e.g., a statistically or clinically significant difference, between the two states.

The term “specific binding” or “specifically binds to” refers to the ability of an antibody to bind to its antigen (e.g., HCV, or a portion thereof), with an affinity of at
15 least 1×10^{-6} M, and/or bind to its antigen (e.g., HCV, or a portion thereof), with an affinity that is at least two-fold greater than its affinity for a nonspecific antigen.

An “antibody” is a protein including at least one or two, heavy (H) chain variable regions (abbreviated herein as VH), and at least one or two light (L) chain variable regions (abbreviated herein as VL). The VH and VL regions can be further
20 subdivided into regions of hypervariability, termed “complementarity determining regions” (CDRs), interspersed with regions that are more conserved, termed “framework regions” (FR). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E.A., et al. Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242,
25 1991, and Chothia, C. et al., J. Mol. Biol. 196:901-917, 1987, which are incorporated herein by reference). Preferably, each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

The VH or VL regions of the antibody can further include all or part of a heavy
30 or light chain constant region. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light

chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first
5 component (C1q) of the classical complement system. The term “antibody” includes intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda.

The term “immunoglobulin” refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized human
10 immunoglobulin genes include the kappa, lambda, alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, IgG4), delta (IgD), epsilon (IgE), and mu (IgM) constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin “light chains” (about 25 K_D and 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a kappa or
15 lambda constant region gene at the COOH-terminus. Full-length immunoglobulin “heavy chains” (about 50 K_D and 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g., gamma (encoding about 330 amino acids). The term “immunoglobulin” includes an immunoglobulin having: CDRs from a human or non-
20 human source. The framework of the immunoglobulin can be human, humanized, or non-human, e.g., a murine framework modified to decrease antigenicity in humans, or a synthetic framework, e.g., a consensus sequence. A mature immunoglobulin / antibody variable region is typically devoid of a leader sequence. Immunoglobulins / antibodies can be further distinguished by their constant regions into class (e.g., IgA, IgD, IgE, IgG, or IgM) and subclass or isotype (e.g., IgG1, IgG2, IgG3, or IgG4).
25

The term “antigen binding portion” of an antibody (or simply “antibody portion,” or “portion”), as used herein, refers to a portion of an antibody that specifically binds to an antigen (e.g., HCV or a component thereof, e.g., G glycoprotein), e.g., a molecule in which one or more immunoglobulin chains is not full length, but which
30 specifically binds to an antigen. Examples of binding portions encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the

hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., Nature 341:544-546, 1989), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) having sufficient
5 framework to specifically bind, e.g., an antigen binding portion of a variable region. An antigen binding portion of a light chain variable region and an antigen binding portion of a heavy chain variable region, e.g., the two domains of the Fv fragment, VL and VH, can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent
10 molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also encompassed within the term "antigen binding portion" of an antibody. These antibody portions are obtained using conventional techniques known to those with skill in the art, and the portions are screened for utility in the same
15 manner as are intact antibodies.

The term "monospecific antibody" refers to an antibody that displays a single binding specificity and affinity for a particular target, e.g., epitope. This term includes a "monoclonal antibody" or "monoclonal antibody composition," which as used herein refer to a preparation of antibodies or portions thereof with a single molecular
20 composition.

The term "recombinant" antibody, as used herein, refers to antibodies that are prepared, expressed, created, or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial antibody library, antibodies isolated from an
25 animal (e.g., a mouse) that is transgenic for human immunoglobulin genes or antibodies prepared, expressed, created, or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant antibodies include humanized, CDR grafted, chimeric, *in vitro* generated (e.g., by phage display) antibodies, and may optionally include constant regions derived from human
30 germline immunoglobulin sequences.

The term "substantially identical" (or "substantially homologous") refers to a first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (e.g., with a similar side chain, e.g., conserved amino acid substitutions)

amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities. In the case of antibodies, the second antibody has the same specificity and has at least 50% of the affinity of the first antibody.

5 Calculations of "homology" between two sequences are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is at
10 least 50% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid
15 "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

 The comparison of sequences and determination of percent homology between
20 two sequences can be accomplished using a mathematical algorithm. The percent homology between two amino acid sequences is determined using the Needleman and Wunsch, J. Mol. Biol. 48:444-453, 1970, algorithm which has been incorporated into the GAP program in the GCG software package, using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frame shift gap penalty of 5.

25 As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. 6.3.1-6.3.6, 1989, which is incorporated herein by reference. Aqueous and nonaqueous methods are
30 described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization conditions: 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for

low stringency conditions); 2) medium stringency hybridization conditions: 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; 3) high stringency hybridization conditions: 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and 4) very high stringency hybridization conditions: 0.5 M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C.

It is understood that the antibodies and antigen binding portions thereof described herein may have additional conservative or non-essential amino acid substitutions, which do not have a substantial effect on the polypeptide functions.

Whether or not a particular substitution will be tolerated, i.e., will not adversely affect desired biological properties, such as binding activity, can be determined as described in Bowie et al., Science, 247:1306-1310, 1990. A "conservative amino acid substitution" is one in which an amino acid residue is replaced with an amino acid residue having a similar side chain. 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., asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., glycine, 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).

A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of a polypeptide, such as a binding agent, e.g., an antibody, without substantially altering a biological activity, whereas an "essential" amino acid residue results in such a change.

Further information on antibodies for use in embodiments of the invention can be found in PCT/US08/01922, filed February 13, 2008, hereby incorporated by reference.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the present

specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

II. Nucleic Acid Silencing Agent – Binding Protein Conjugates

5 The present invention is based on the discovery, that a polypeptide or protein can be chemically conjugated to a nucleic acid silencing agent without substantially reducing the silencing activity of the nucleic acid silencing agent. Furthermore, such a conjugate can be used to effectively deliver the nucleic acid silencing agent into a cell which expresses a target RNA to which the nucleic acid silencing agent is
10 complementary. In exemplary embodiments, the target RNA accesses the endosomes of a cell. Without wishing to be bound in theory, the acidic environment of the endosome can facilitate release of the silencing agent from the conjugate in certain embodiments in order to silence the target RNA.

 It has been shown, for example, that a carbonyl group may be introduced into the
15 terminal ribonucleotide of a nucleic acid silencing agent and conjugated via a heterobifunctional linker to a sulfhydryl group in an antibody or other polypeptide.

 The conjugates of the invention have the following basic formula:



wherein

20 X is one or more nucleic acid silencing agents
 L is one or a linking moiety;
 n is a positive integer (e.g., 1, 2, 3, 4 or more); and
 Y is a polypeptide.

25 In certain embodiments, the nucleic acid silencing agent (X) is any one of the nucleic acid silencing agents described infra. Said nucleic acid silencing agents may be conjugated to any one of the polypeptides (“Y”) described infra.

 The linking moiety (“L”) may cross-link the polypeptide (Y) to one or more nucleic acid silencing agents (“X”) via covalent or non-covalent binding interactions.
30 For example, the linking moiety can be formed by a non-covalent binding interaction between a first functional group (e.g., a tag or domain) on the nucleic silencing agent and a second, complementary, functional group on the binding protein (e.g., a complementary tag or domain). In particularly preferred embodiments, however, the

linking moiety is formed by a more stable, covalent binding interaction between an amino acid present in the polypeptide and a nucleotide (e.g., a ribonucleotide) present in the nucleic acid silencing agent. For example, the linking moiety may comprise a heterobifunctional cross-linker which is bonded (e.g., covalently bonded) to both an amino acid in the polypeptide and a nucleotide in the nucleic acid silencing agent. For example, the linking moiety may comprise a cross-linker bonded to a free amine (e.g., a lysine ϵ -amine group or an N-terminal α -amine groups) or a carboxylate group (e.g., at the C-terminus, an aspartic acid, or a glutamic acid residues) in the polypeptide. In more preferred embodiments, the linking moiety comprises cross-linker bonded to a sulfhydryl group in the polypeptide. Such linking moieties may be formed by inter alia by NHS ester-maleimide-mediated conjugation, glutaraldehyde-mediated conjugation, or reductive-amination-mediated conjugation. NHS ester-maleimide-mediated conjugations may be formed, for example, by the reduction (e.g., with 2-mercaptoethylamine (MEA)) of disulfide groups (e.g., in the hinge region of an antibody). Alternatively, the polypeptide may be first treated with 2-iminothiolane (Traut's reagent) which reacts with amino groups in the antibody to introduce the sulfhydryl residues. Finally, in other embodiments, glutaraldehyde-mediated conjugation involves Schiff base formation with possible rearrangement to a stable product or through a Michael-type addition reaction.

The reductive-amination-mediated conjugations include, for example, the activation of antibodies with sodium periodate, conjugation of periodate-oxidized HRP (horseradish peroxidase) to antibodies, or conjugation of periodate-oxidized antibodies with amine or hydrazide derivatives. The conjugations using antibody fragments include, for example, preparation of F(ab')₂ fragments using pepsin, or preparation of Fab fragments using papain. Preparation of labeled antibodies involves, for example, fluorescently labeled antibodies, radiolabeled antibodies, or biotinylated antibodies. The antibody-toxin conjugation leads to immunotoxin conjugates. Toxins of many different types can be conjugated to monoclonal antibodies to create effective immunotoxins, including the proteins ricin from castor beans (*Ricinus communis*), abrin from *Abrus precatorius*, modeccin, gelonin from *Gelonium multiflorum* seeds, diphtheria toxin produced by *Corynebacterium diphtheriae*, pokeweed antiviral proteins (PAPs; three types: PAP, PAP II, and PAP-S) from *Phytolacca americana* seeds, cobra venom factor (CVF), *Pseudomonas* exotoxin, restrictocin from *Aspergillus restrictus*, momordin from

Momordica charantia seeds, and saporin from Saponaria officinalis seeds, as well as other ribosome-inactivating proteins (RIPs). Preparation of immunotoxin conjugates can be achieved from disulfide exchange reactions, amino- and sulfhydryl-reactive heterobifunctional cross-linkers, or reductive amination. The disulfide exchange reagents include, for example, pyridyldisulfide reagents (e.g. SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), SMPT (succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene), 3-(2-pyridyldithio)propionate, etc.). The amino- and sulfhydryl-reactive heterobifunctional cross-linkers include, for example, SIAB (N-succinimidyl(4-iodoacetyl)aminobenzoate), SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), or SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate). Reductive amination can be utilized to prepare immunotoxin, exemplified in the periodate oxidation of glycoproteins followed by reductive conjugation, or periodate-oxidized dextran as cross-linking agent. Antibodies may also be coupled through sulfhydryl residues to liposomes using liposomes containing PE groups derivatized with heterobifunctional cross-linkers such as SMCC, MBS, SMPB, SIAB, and SPDP. Antibodies also may be coupled through their amine groups using reductive amination to periodate-oxidized glycolipids. Antibodies or fragments of antibodies can be covalently linked to PEG (polyethylene glycols), which is a non-immunogenic polymer stabilizing these fragments *in vivo*. PEG can be linked to various sites on antibodies via lysine residue or cysteine residue that is located in the hinge region of the original intact IgG. Two Fabs can also be attached at one end of PEG or antibody-dextran conjugates. The most common activation methods for PEG include, for example, trichloro-s-triazine activation and coupling, NHS ester and NHS carbonate activation and coupling, carbodiimide coupling of carboxylate-PEG derivatives, or CDI (N',N'-Carbonyldiimidazole) activation and coupling. Antibodies can also be modified with activated dextran and the major methods for this modification include, for example, polyaldehyde activation and coupling, carbodiimide-mediated reaction to form an amide bond for carboxyl, amine, and hydrazide derivatives, epoxy activation and coupling, or cross-linking of sulfhydryl-reactive derivatives.

In one preferred embodiment, the therapeutic RNA interference agent (e.g. siRNAs, shRNAs, etc.) and the targeting antibody or fragment of the said antibody (e.g. Fab, F(ab')₂, scFv. etc.) of the invention can form conjugates through a binding or linking moiety ("L"), while still retaining the bio-activity of each. This conjugation is

able to improve stability of the antibody/fragment of the antibody and/or the RNA interference agent, target the RNA interference agent to a particular tissue or cell-type, or increase cell permeability of the antibody/fragment of the antibody and/or the RNA interference agent, e.g., by an endocytosis-dependent or -independent mechanism. In
5 other embodiments, this conjugation can also increase the interaction specificity and/or efficiency between the said antibody/fragment of the antibody and their target antigens/proteins.

In one embodiment, the binding or linking moiety ("L") can be a functional group(s) on the RNA molecule ("X") and/or the targeting molecule ("Y") to mediate the
10 non-covalent interaction between "X" and "Y". In one preferred embodiment, "L" is the nucleic acid binding domain of a protein selected from the group consisting of protamine, GCN4, Fox, Jun, TFIIS, FMRI, yeast protein HX, Vigillin, Mer1, bacterial polynucleotide phosphorylase, ribosomal protein S3, and heat shock protein. The binding or linking moiety ("L") can include biotin-avidin or streptavidin conjugates,
15 prepared by NHS ester-maleimide-mediated conjugation, periodate oxidation/reductive amination conjugation, glutaraldehyde conjugation, hydrazide activation, or modified by fluorescent labels (e.g. FITC (fluorescein isothiocyanate)), lissamine rhodamine B sulfonyl chloride, AMCA-NHS, or phycobiliproteins.

In one embodiment, the binding or linking moiety ("L") can be a binding
20 moiety(s) to mediate the non-covalent interaction between "X" and "Y". In one preferred embodiment, "L" is the nucleic acid binding domain of a protein selected from the group consisting of protamine, GCN4, Fox, Jun, TFIIS, FMRI, yeast protein HX, Vigillin, Mer1, bacterial polynucleotide phosphorylase, ribosomal protein S3, and heat shock protein. The binding or linking moiety ("L") can include biotin-avidin or
25 streptavidin conjugates, prepared by NHS ester-maleimide-mediated conjugation, periodate oxidation/reductive amination conjugation, glutaraldehyde conjugation, hydrazide activation, or modified by fluorescent labels (e.g. FITC (fluorescein isothiocyanate)), lissamine rhodamine B sulfonyl chloride, AMCA-NHS, or phycobiliproteins.

30 In one embodiment, the binding or linking moiety ("L") can be a functional group(s) on the RNA molecule ("X") and/or the targeting molecule ("Y") to mediate the covalent bond between "X" and "Y".

In one embodiment, the binding or linking moiety ("L") can be a cross-linking reagent or multiple reagents to mediate the covalent bond between "X" and "Y". In one preferred embodiment, the covalent linkage forms between the antibody/fragment of the antibody and the RNA interference agent through a cross-linker. A large number of different types of cross-linking reagents have been described and are known to those skilled in the art. In one embodiment, the cross-linker can be a liposome-based complex. In other embodiments, the cross-linker can be one of so-called zero-length cross-linking agents that bring about the direct formation of covalent bonds between existing amino acid side chain groups. The zero-length cross-linking agents can initiate the formation of at least three types of bonds: an amide linkage made by the condensation of a primary amine with a carboxylic acid, a phosphoramidate linkage made by the reaction of an organic phosphate group with a primary amine, and a secondary or tertiary amine linkage made by the reductive amination of a primary or secondary amine with an aldehyde group. The zero-length cross-linking agents can be, for example, carbodiimides, a Woodward's Reagent K (N-ethyl-3-phenylisoxazolium-3'-sulfonate), N',N'-Carbonyldiimidazole (CDI; Aldrich), or Schiff bases formed with or without reductive amination (or alkylation). Carbodiimides includes, for example, EDC (or EDAC; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) with or without supplement of sulfo-NHS (N-Hydroxysulfo-succinimide; Pierce), CMC (1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide, or its metho p-toluene sulfonate salt; Aldrich), or DIC (diisopropyl carbodiimide), or its by-products diisopropylurea and diisopropyl-N-acylurea). The use of water-soluble carbodiimides to bring about the formation of amide linkages between carboxyl groups of aspartate or glutamate and the ε-amino groups of lysine side chains have been illustrated in Uy and Wold, *Adu. Exp. Med. Biol.* 86A, 169-186. (1977); Weare and Reichert, *J. Biol. Chem.* 254, 6964-6971. (1979) and Willing et al., *J. Biol. Chem.* 264, 8499-8503 (1989). Disulfide bonds obtained from existing thiol groups would also, presumably, be considered zero-length cross-links (Korodi et al., *Biochemistry* 25, 6895-6900 (1986); Huston et al., *Biochemistry* 27, 8945-8952 (1988)). Such linkages appear to be formed only when the reacting groups are in close proximity.

In other embodiments, the cross-linking agents may be organized according to the type(s) of reactive groups, their side chain reactivity, their hydrophobicity or hydrophilicity, and the length or distance between the reactive groups; whether the two,

or in some cases more (as illustrated in Hiratsuka, Biochemistry 27, 4110-4114 (1988)) reactive groups are the same or different (i.e., "homobifunctional", "heterobifunctional" "trifunctional", or "multifunctional" reagents), whether the structure connecting the reactive groups is readily cleavable, and whether the groups are membrane permeable or impermeable, and according to various other criteria. A much more extensive list of cross-linking agents includes those presented in Ji, Methods Enzymol. 91, 580-609 (1983).

In some embodiments, homobifunctional linkers are used for bioconjugates. Most of these homobifunctional reagents are symmetrical in design with a carbon chain spacer connecting the two identical reactive ends. Like molecular rope, these reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Homobifunctional linkers include, for example, homobifunctional NHS (N-hydroxysuccinimide) esters, sulfo-NHS (N-hydroxysulfo-succinimide) esters, homobifunctional imidoesters, homobifunctional Sulfhydryl-reactive cross-linkers, difluorobenzene derivatives, homobifunctional photoreactive cross-linkers, homobifunctional aldehydes, bis-epoxides, homobifunctional hydrazides, bis-diazonium derivatives, or bis-alkylhalides. Homobifunctional NHS (N-hydroxysuccinimide) esters or sulfo-NHS (N-hydroxysulfo-succinimide) esters include, for example, Lomant's reagent or DSP (dithiobis(succinimidyl propionate) ; Pierce), DTSSP (dithiobis(sulfosuccinimidyl propionate; Pierce), DSS (disuccinimidyl suberate; Pierce), BS³ (bis(sulfosuccinimidyl)suberate; Pierce), DST (disuccinimidyl tartarate; Pierce), sulfo-DST (disulfosuccinimidyl tartarate; Pierce), BSOCOES (bis[2-(succinimidylloxycarbonyloxy)ethyl]sulfone; Pierce), sulfo-BSOCOES (bis[2-(sulfosuccinimidylloxycarbonyloxy)ethyl]sulfone; Pierce), EGS (ethylene glycolbis(succinimidylsuccinate; Pierce), sulfo-EGS (ethylene glycolbis(sulfosuccinimidylsuccinate; Pierce), DSG (disuccinimidyl glutarate; Pierce) or DSC (N,N'-disuccinimidyl carbonate; Aldrich). Homobifunctional imidoesters include, for example, DMA (dimethyl adipimidate; Pierce), DMP (dimethyl pimelimidate; Pierce), DMS (dimethyl suberimidate), or DTBP (dimethyl 3,3'-dithiobispropionimidate; Pierce). Homobifunctional Sulfhydryl-reactive cross-linkers include, for example, DPDPB (1,4-di-[3'-(2'-pyridyldithio)propionamido]butane), or BMH (bismaleimidohehexane; Pierce). Difluorobenzene derivatives include, for example, DFDNB (1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene; Pierce), or

DFDNPS (4,4'-difluoro-3,3'-dinitrophenylsulfone). Homobifunctional photoreactive cross-linkers include, for example, BASED (bis-[β -(4-azidosalicylamido)ethyl]disulfide; Pierce). Homobifunctional aldehydes include, for example, formaldehyde, or glutaraldehyde. Bis-exoxides include, for example, 1,4-butanediol diglycidyl ether. Homobifunctional hydrazides include, for example, adipic acid dihydrazide (Aldrich), or carbohydrazide. Bis-diazonium derivatives include, for example, diazotized o-Tolidine (or 3,3'-dimethylbenzidine), or bis-diazotized benzidine (or p-diaminodiphenyl).

In other embodiments, heterobifunctional linkers are preferred to form bioconjugates. These heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on the antibody/fragment of antibody and the RNA interference reagent of this invention. The cross-bridge or spacer that ties the two reactive ends together may also govern the overall hydrophilicity of the reagent, the reactivity of functional groups, and the immunogenic ability and stability of the conjugate. Some heterobifunctional linkers can contain cleavable groups within their cross-bridges. Heterobifunctional linkers include, for example, amine-reactive and sulfhydryl-reactive cross-linkers, carbonyl-reactive and sulfhydryl-reactive cross-linkers, amine-reactive and photoreactive cross-linkers, sulfhydryl-reactive and photoreactive cross-linkers, carbonyl-reactive and photoreactive cross-linkers, carboxylate-reactive and photoreactive cross-linkers, or arginine-reactive and photoreactive cross-linkers. Amine-reactive and sulfhydryl-reactive cross-linkers include, for example, SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate, including standard SPDP, a long-chain version designated LC-SPDP, and a water-soluble, sulfo-NHS form also containing an extended chain, called sulfo-LC-SPDP; all from Pierce), SMPT (succinimidyl-oxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene), sulfo-LC-SMPT (sulfosuccinimidyl-6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate), SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), sulfo-SMCC (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), sulfo-MBS (m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester), SIAB (N-succinimidyl(4-iodoacetyl)aminobenzoate), sulfo-SIAB (sulfosuccinimidyl(4-iodoacetyl)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate; Pierce), sulfo-SMPB (sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate; Pierce), GMBS (N-(γ -maleimidobutyryloxy)succinimide

ester; Pierce), sulfo-GMBS (N-(γ -maleimidobutyryloxy)sulfosuccinimide ester; Pierce), SIAX (succinimidyl 6-((iodoacetyl)amino)hexanoate; Molecular Probes), SIAXX (succinimidyl 6-(((4-iodoacetyl)amino)hexanoyl)amino)hexanoate; Molecular Probes), SIAC (succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate; Molecular Probes), SIACX (succinimidyl 6-(((4-iodoacetyl)amino)methyl)cyclohexane-1-carboxyl)amino)-hexanoate; Molecular Probes), or NPFA (p-nitrophenyl iodoacetate; Molecular Probes). Carbonyl-reactive and

sulfhydryl-reactive cross-linkers include, for example, MPBH (4-(4-N-maleimidophenyl)butyric acid hydrazide; Pierce), M₂C₂H (4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide; Pierce), or PDPH (3-(2-pyridyldithio)propionyl hydrazide; Pierce). Amine-reactive and photoreactive cross-linkers include, for example, NHS-ASA (N-hydroxysuccinimidyl-4-azidosalicylic acid; Pierce), sulfo-NHS-ASA, sulfo-NHS-LC-ASA, SASD (sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate; Pierce), HSAB (N-hydroxysuccinimidyl-4-azidobenzoate; Pierce), sulfo-HSAB (N-hydroxysulfosuccinimidyl-4-azidobenzoate; Pierce), SANPAH (N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate; Pierce), sulfo-SANPAH (sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate), ANB-NOS (N-5-azido-2-nitrobenzoyloxysuccinimide; Pierce), SAND (sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate; Pierce), SADP (N-succinimidyl-4-(4-azidophenyl)-1,3'-dithiopropionate; Pierce), sulfo-SADP, sulfo-SAPB (sulfosuccinimidyl 4-(p-azidophenyl)butyrate; Pierce), SAED (sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3'-dithiopropionate; Pierce), sulfo-SAMCA (sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate; Pierce), pNPDP (p-nitrophenyl diazopyruvate; Molecular Probes), or PNP-DTP (p-nitrophenyl-2-diazo-3,3,3-trifluoropropionate; Pierce). Sulfhydryl-reactive and photoreactive cross-linkers include, for example, ASIB (1-(p-azidosalicylamido)-4-(iodoacetamido)butane; Pierce), APDP (N-[4-(p-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide; Pierce), Benzophenone-4-iodoacetamide (Molecular Probes), or Benzophenone-4-maleimide (Molecular Probes). Carbonyl-reactive and photoreactive cross-linkers include, for example, ABH (p-azidobenzoyl hydrazide; Pierce). Carboxylate-reactive and photoreactive cross-linkers include, for example, ADBA (4-(p-azidosalicylamido)butylamine; Pierce). Arginine-

reactive and photoreactive cross-linkers include, for example, APG (p-azidophenyl glyoxal; Pierce).

In other embodiments, trifunctional cross-linkers are preferred to form bioconjugates. Trifunctional linkers possess three reactive or complexing groups per molecule and utilizes two reactive groups at two ends to couple with the antibody/fragment of the antibody and the RNA interference agent of this invention, while the third arm branching out with a reactive group to link with another molecule of the said antibody/fragment of the antibody, the RNA interference agent, or a third chemical or biological target. In one embodiment, trifunctional linkers are built from the amino acid L-lysine. Its three functional groups, α -carboxy, α -amino, and ϵ -amino, are derivatized independently to contain three arms. Each arm can be designed to terminate in a complexing group able to participate in a particular type of conjugation reaction. In another embodiment, trifunctional linkers are built from biocytin, the lysine derivative of biotin with its valeric acid side chain amide-bonded to the ϵ -amino group of the amino acid. Other trifunctional cross-linkers include, for example, 4-azido-2-nitrophenylbiocytin-4-nitrophenyl ester (ABNP) or sulfo-SBED (sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)hexanoamido]ethyl-1,3'-dithiopropionate). In one embodiment, one molecule of same RNA silencing agent of this invention may be linked with the third arm of the trifunctional linker. In another embodiment, one molecule of a different RNA silencing agent against the same target gene may be linked with the third arm of the trifunctional linker. In another embodiment, one molecule of a different RNA silencing agent against a different target gene may be linked with the third arm of the trifunctional linker. In another embodiment, one molecule of same antibody or fragment of the antibody of this invention may be linked with the third arm of the trifunctional linker. In another embodiment, one molecule of a different antibody or fragment of the antibody recognizing the same target antigen or protein may be linked with the third arm of the trifunctional linker. In another embodiment, one molecule of a different antibody or fragment of the antibody recognizing a different target antigen or protein may be linked with the third arm of the trifunctional linker. In other embodiments, one molecule of a moiety selected from the group of metal ions, nucleotides, amino acids, polyamines, peptides, peptide mimics, proteins, protein binding agents, toxins, antibiotics, lipids, lipophiles, steroids, terpenes, vitamins, carbohydrates, synthetic polymers, or organic compounds (e.g., a dye) may be linked

with the third arm of the trifunctional linker. Preferred moieties linked with the third arm of the trifunctional linker can improve transport, target-binding, or specificity properties and may also improve nuclease resistance of the resultant bioconjugate. The said moieties may be a cell or tissue targeting agent or to enhance cellular uptake by target cells.

In other embodiments, multifunctional cross-linkers are preferred to form bioconjugates. Multifunctional cross-linkers possess three or more reactive or complexing groups per molecule. Multifunctional cross-linkers include, for example, the polyaldehyde dextran or small organic molecules like trichloro-S-triazine (TsT). The dextran polymer contains adjacent hydroxyl groups on each glucose monomer. These diols may be oxidized with sodium periodate to cleave the associated carbon-carbon bonds and produce aldehydes. This procedure results in two aldehyde groups formed per glucose monomer, thus producing a highly reactive, multifunctional polymer able to couple with numerous amine-containing molecules (Bernstein et al., J. Natl. Cancer Inst. 60, 379-384, (1978)).

In a particular embodiment, a heterobifunctional linker is utilized to form a bioconjugate comprising of the antibody or fragment of the antibody and the RNA interference agent of this invention. In one embodiment, a heterobifunctional linker contains one or more thiol-reactive groups, including haloacetyl and alkyl halide derivatives, maleimides, aziridines, acryloyl derivatives, arylating agents, or thiol-disulfide exchange reagents (e.g. pyridyl disulfides, TNB-thiol, disulfide reductants, etc.). In a preferred embodiment, the heterobifunctional linker comprises of a maleimide moiety and a hydrazide moiety. The heterobifunctional linker can include but is not limited to N-(ϵ -maleimidocaproic acid) hydrazide (EMCH, with the formula as $C_{10}H_{16}N_3O_3Cl$), N-(β -maleimidopropionic acid) hydrazide (MPH, with the formula as $C_8H_{12}N_3O_3Cl$), N-(κ -maleimidoundecanoic acid) hydrazide (KMUH, with the formula as $C_{15}H_{25}N_3O_3$, and 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH). In one embodiment, the maleimide end of the heterobifunctional linker is linked to a thiolated antibody or fragment of the antibody of this invention through a thioether bond. In one embodiment, the thiol group is added by 2-Iminothiolane (Traut's Reagent) to a NH_2 side chain of a Lysine residue on a heavy chain of the antibody or fragment of the antibody of this invention. In another embodiment, the hydrazide end of the heterobifunctional linker is linked to the RNA interference agent of this invention. In

some embodiments, the heterobifunctional linker may contain cleavable reagents.

These cleavable reagents are typically built into the cross-bridge or reactive ends of a reagent using disulfides, glycol groups, diazo bonds, esters, sulfone groups, or acetal linkages. In one preferred embodiment, the cleavage element is built in as a pH-labile

5 hydrazone bond between the hydrazide end of the heterobifunctional linker and the aldehyde group on an alkylated RNA interference agent of this invention. In another preferred embodiment, the said hydrazone bond in the linker is stable in serum at pH7.4 but hydrolyzes at pH5.0 in the endosomes, leading to the release of the siRNA conjugated to the antibody.

10 Other chemical modifications include, for example, the diamine or bis-hydrazide modification, sulfhydryl modification, Biotin-labeling, enzyme conjugation, or fluorescent labeling. The diamine or bis-hydrazide modification results in the formation of an alkyl spacer arm terminating in a primary amine group or a hydrazide functional group, respectively, via bisulfite activation of cytosine, bromine activation of thymine, 15 guanine, and cytosine, or carbodiimide reaction with 5' phosphates of DNA (phosphoramidate formation). The sulfhydryl modification allows conjugation with sulfhydryl-reactive heterobifunctional cross-linkers and includes, for example, cystamine modification of 5' phosphate groups using EDC (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride; Pierce), SPDP (N-succinimidyl 3- 20 (2-pyridyldithio)propionate) modification of amines on nucleotides, or SATA (N-succinimidyl S-acetylthioacetate) modification of amines on nucleotides. Biotin-labeling includes, for example, the linkage of Biotin-LC-dUTP, photo-Biotin modification, reaction of NHS-LC-Biotin with diamine-modified nucleotide probes, Biotin-diazonium modification, reaction of Biotin-BMCC with sulfhydryl-modified 25 nucleotides, or Biotin-hydrazide modification of bisulfite-activated cytosine groups. Enzyme conjugations include, for example, alkaline phosphatase conjugation to cystamine-modified nucleotides using amine- and sulfhydryl-reactive heterobifunctional cross-linkers, alkaline phosphatase conjugation to diamine-modified nucleotides using DSS (disuccinimidyl suberate), enzyme conjugation to diamine-modified nucleotides 30 using PDITC, or conjugation of SFB-modified alkaline phosphatase to bis-hydrazide-modified oligonucleotides. The fluorescent labeling includes, for example, the conjugation of amine-reactive fluorescent probes to diamine-modified nucleotides, or conjugation of sulfhydryl-reactive fluorescent probes to sulfhydryl-modified

nucleotides. A few more examples of chemical modifications include the introduction of phosphorothioate linkages, boranophosphate linkage, 2'-O-methylation, 2'-fluoro nucleotides, 2'-O-(2-methoxyethyl) nucleotides, locked nucleic acid (LNA) nucleotides, or terminal modification on sense and/or antisense strand, including adding protein

5 transduction domain (PTDs), a Tat peptide containing the cationic peptide YGRKKRRQRRR, partial phosphorothioate linkages, 2'-O-methyl sugars, or cholesterol molecules. The methods to create new specific functional groups to the RNA molecules of this invention include, for example, the introduction of sulfhydryl residues (thiolation), carboxylate groups, primary amine groups, aldehyde residues, or

10 hydrazide functional groups. The introduction of sulfhydryl residues includes, for example, modification of amines with 2-iminothiolane (Traut's Reagent), modification of amines with SATA (N-succinimidyl S-acetylthioacetate), modification of amines with SATP (succinimidyl acetylthiopropionate), modification of amines with SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), modification of amines with SMPT

15 (succinimidyloxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene), modification of amines with N-acetylhomocysteinethiolactone, modification of amines with SAMSA (S-acetylmercaptosuccinic anhydride), modification of aldehydes or ketones with AMBH (2-acetamido-4-mercaptoputyric acid hydrazide), or modification of carboxylates or phosphates with cystamine. The introduction of carboxylate groups

20 includes, for example, the modification of amines with anhydrides, including succinic anhydride, glutaric anhydride, maleic anhydride, or citraconic anhydride, modification of sulfhydryls with iodoacetate, or modification of hydroxyls with chloroacetic acid. The introduction of primary amine groups includes, for example, the modification of carboxylates with diamines, modification of sulfhydryls with N-(β -

25 iodoethyl)trifluoroacetamide), modification of sulfhydryls with ethylenimine, modification of sulfhydryls with 2-bromoethylamine, modification of carbohydrates with diamines, modification of alkylphosphates with diamines, or modification of aldehydes with ammonia or diamines. The introduction of aldehyde residues includes, for example, the periodate oxidation of glycols and carbohydrates, oxidase modification

30 of sugar residues, modification of amines with NHS-aldehydes (SFB (succinimidyl p-formylbenzoate) and SFPA (succinimidyl p-formylphenoxyacetate), or modification of amines with glutaraldehyde. The introduction of hydrazide functional groups includes, for example, the modification of aldehydes with bis-hydrazide compounds, modification

of carboxylates with bis-hydrazide compounds, or modification of alkylphosphates with bis-hydrazide compounds. The conjugation can be accomplished by methods known in the art, e.g., using the methods of Lambert et al., Drug Deliv. Rev.:47(1), 99-112 (2001) (describes nucleic acids loaded to polyalkylcyanoacrylate (PACA) nanoparticles); Fattal
5 et al., J. Control Release 53(1-3):137-43 (1998) (describes nucleic acids bound to nanoparticles); Schwab et al., Ann. Oncol. 5 Suppl. 4:55-8 (1994) (describes nucleic acids linked to intercalating agents, hydrophobic groups, polycations or PACA nanoparticles); and Godard et al., Eur. J. Biochem. 232(2):404-10 (1995) (describes nucleic acids linked to nanoparticles).

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III. Nucleic Acid Silencing Agents

In certain aspects, the compositions and methods of the invention feature nucleic acid silencing agents (e.g., RNA or DNA silencing agents). In certain embodiments, the nucleic acid silencing agents are silencing agents having sufficient complementarity to a
15 target RNA (e.g., a HCV-related target mRNA or microRNA) to mediate gene silencing of the target RNA. In certain embodiments, gene silencing is such that the target gene level is reduced or decreased by at least 30% (e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or more). The nucleic acid silencing agents may be single or double stranded. For example, the nucleic acid silencing agents
20 may be RNA silencing agents comprising or consisting of an antisense RNA strand (or portions thereof) comprising a sequence with sufficient complementarity to the target mRNA to silence expression of a target mRNA via an RNA silencing mechanism (e.g. RNA interference (RNAi) or translational repression). In other embodiments, the nucleic acid silencing agent may be DNA silencing agents comprising or consisting of
25 an antisense strand comprising a sequence with sufficient complementarity to the target RNA to silence expression via an antisense mechanism (e.g., via RNase H mediated cleavage). In yet other embodiments, the nucleic acid silencing agents may be inhibitors of RNA silencing. For example, the nucleic acid silencing agents may comprise or consist of an antisense strand comprising a sequence with sufficient complementarity to
30 a small, non-coding RNA (e.g., a miRNA, pre-miRNA, pri-miRNA, rasi-RNA, or piRNA) to inhibit RNA silencing by the RNA (e.g., so-called "antagomiRs").

Candidate nucleic acid silencing agents are designed based on the sequence of a selected target site in a target RNA, e.g., a target mRNA or small non-coding RNA (e.g.,

a miRNA). The targeted sequence can be selected by performing, for example, a gene walk analysis of the target RNA. Overlapping, adjacent, or closely spaced candidate agents corresponding to all or some of the transcribed region can be generated and tested. Each of the silencing agents can be tested and evaluated for the ability to
5 mediate gene silencing. Alternatively, a target site can be selected using the method of Elbashir et al. (see e.g., Elbashir et al., *Methods*, 26: 199-213 (2002)). Using this method, the RNA (e.g., target mRNA) can be searched for regions that are likely to be free of translational or regulatory proteins (e.g., at least 100 bases downstream of the start codon). A sequence of about 10-50 contiguous nucleotides (e.g., about 18-30
10 contiguous nucleotides, about 20-25 contiguous nucleotides, or about 21-23 nucleotides, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides) may be selected. Preferably, the sequence has between 30-70% G/C content. In certain embodiments, sequences characterized by an AA (or NA) dinucleotide at their 5' terminus may be selected. In addition, a number of in silico
15 design programs are available to those of skill in the art including, for example, RFRCDDB-siRNA (Jiang et al., *Comput Methods Programs Biomed.* 2007 Sep;87(3):230-8), sIR (Shah et al., *BMC Bioinformatics.* 2007 May 31;8:178), and DQOR (Henschel et al., *Nucleic Acids Res.* 2004 Jul 1;32:W113-20).

The antisense (or guide) strand sequence employed in the nucleic acid silencing
20 agent may be designed based on the sequence of the selected target site in the target RNA. Preferably the antisense sequence is sufficiently complementary to target sequence such that the nucleic silencing agent can mediate silencing of the target RNA (e.g., via RNAi, translational repression, or other gene silencing mechanisms). For example, the antisense strand may have perfect complementarity (ie. 100%
25 complementarity) identity to the target site in a target RNA (e.g., a target mRNA). However, perfect complementarity is not required. Greater than 80% complementarity, e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% complementarity, between the antisense sequence and the target RNA sequence is preferred. In one embodiment, the
30 antisense sequence forms one or more mismatches (e.g., 1, 2, 3, 4, or 5 mismatched base pair(s)) with a target region. Moreover, antisense sequences with small insertions or deletions of 1 or 2 nucleotides (or nucleotide analogs) may also be effective for mediating gene silencing (e.g., via RNAi or translational repression).

Preferably the antisense sequence is sufficiently complementary to sequence of about 18-30 contiguous nucleotides, about 20-25 contiguous nucleotides, or about 21-23 nucleotides, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides, such that the silencing agent can mediate silencing of the target RNA (e.g., silencing of a target mRNA via RNAi or translational repression or silencing of a target microRNA or other small, non-coding RNA via RISC inhibition). For example, the antisense strand may have perfect complementarity (ie. 100% complementarity) identity to the target site. However, perfect complementarity is not required. Greater than 80% complementarity, e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% complementarity, between the antisense sequence and the target mRNA sequence is preferred. In one embodiment, the antisense sequence forms one or more mismatches (e.g., 1, 2, 3, 4, or 5 mismatched base pair(s)) with a target region. Moreover, antisense sequences with small insertions or deletions of 1 or 2 nucleotides (or nucleotide analogs) may also be effective for mediating silencing (e.g., via RNAi).

A nucleic acid silencing agent can be designed to specifically target and silence the sequence of a target RNA that is encoded by only one allele of a gene (i.e., an allele-specific target sequence). In certain embodiments, the silencing agent can be designed to have single-nucleotide specificity. For example, the silencing agent can be designed to specifically silence a heterozygous mutation (e.g., a point mutation) or a single-nucleotide polymorphism (SNP) found in a mutant allele (e.g., a disease-associated allele), but not a wild-type allele. Targeted SNPs may be located in the coding region of a target mRNA or in its untranslated regions (e.g., a 5' UTR). Silencing of the target RNA at these sites (e.g., via RNAi) should allow for allele-specific gene silencing and elimination of the corresponding mutant protein. Alternatively, where the target RNA is a small, non-coding RNA, the SNP may be located in the guide strand of the RNA (e.g., the guide strand of the miRNA) encoded by the mutant allele, but not the wild-type allele. Polymorphisms from other regions of the mutant gene are also suitable for targeting.

Allele-specific agents may be designed such that the antisense strand has a greater degree of complementarity for the target allele sequence than the non-target allele sequence. Sufficient complementarity may exist between the agent and the allele-specific target sequence in the targeted allele such that there are a greater number of

mismatches when the nucleic acid silencing agent is compared (e.g., aligned) to the corresponding sequence in the non-target allele (e.g., wild type allele or RNA sequence). For example, the nucleic acid silencing agent may have perfect complementarity with the target sequence in the desired allele and at least one mismatch with non-target allele sequence. The antisense strand sequence may be designed such that the extra mismatch with the non-target allele is essentially in the middle of the antisense strand. For example, the mismatch may be located at a nucleotide position that is 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 nucleotides (i.e., nucleotide position P6, P7, P8, P9, P10, P11, P12, P13, P14, P15 or P16) from the 5' end of the antisense or guide strand of an RNA silencing agent (e.g., an siRNA or miRNA). In certain preferred embodiments, the mismatch with the non-target allele is present at position P10 or P16. To enhance the ability of the nucleic acid silencing molecule to discriminate among the target and non-target allele, it may be desirable to design the nucleic acid silencing agent such that the one or more mismatches between the antisense strand and non-target allele form a purine:purine mismatch. The purine:purine pairing may be selected from among G:G, A:G, G:A and A:A pairings, with G:G being preferred. In other embodiments, a wobble base pairing may be employed instead of a mismatched based pairing.

Nucleic acid silencing agents may be chemically modified. For example, nucleic acid silencing agents may be substituted with at least one modified nucleotide analogue. Preferably, the nucleotide analogues are located at positions where the target-specific silencing activity, e.g., the RNAi mediating activity or translational repression activity, is not substantially effected, e.g., in a region at the 5'-end and/or the 3'-end of the molecule. Chemical modifications, even to a large number of nucleotides, may be made without interfering with the gene silencing activity of the nucleic acid silencing agent. Despite these modifications, one skilled in the art will recognize the molecule as being either an RNA or DNA molecule. For example, an RNA silencing agent will be recognized as RNA in that it will have "RNA-like" properties, i.e., it will possess the overall structural, chemical and physical properties of an RNA molecule, even though not exclusively of ribonucleotide-based content. For example, some or all of the nucleotide sugars can contain e.g., 2'OMe, or 2' fluoro in place of 2' hydroxyl. This deoxyribonucleotide-containing agent can still be expected to exhibit RNA-like properties. For example, a RNA silencing agent will be recognized as being a RNA molecule if has a sufficient number of nucleotide sugars in a C_{3'}-endo pucker

configuration (e.g., at least 50, 75, 80, 85, 90, or 95% of its sugars). Alternatively, the RNA silencing agent will have no more than 20, 10, 5, 4, 3, 2, or 1 sugar(s) which are not a C_{3'}-endo pucker structure. These limitations are particularly preferred in the antisense strand of an RNA silencing agent. Preferred 2'-modifications with C3'-endo sugar pucker include: 2'-OH, 2'-O-Me, 2'-O-methoxyethyl, 2'-O-aminopropyl, 2'-F, 2'-O-CH₂-CO-NHMe, 2'-O-CH₂-CH₂-O-CH₂-CH₂-N(Me)₂, and LNA. Regardless of the nature of the modification, it is preferred that DNA molecules, or any molecule in which more than 50, 60, or 70 % of the nucleotides in the molecule are deoxyribonucleotides, or modified deoxyribonucleotides which are deoxy at the 2' position, are excluded from the definition of RNA silencing agent.

Positions which are repeated within a nucleic acid silencing agent may be preferentially modified, e.g., a base, a phosphate moiety, or a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the RNA silencing agent but in many, and in fact in most, cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, e.g. at a position (e.g., a non-linking O position) on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. For example, a modification may occur only in the double stranded region of a double-stranded RNA silencing agent or may only occur in a single stranded region of an RNA silencing agent (e.g., a 5' overhang, a 3' overhang, the unpaired regions or regions of a hairpin structure (e.g., a loop region)). In other embodiments, both single-stranded and double-stranded regions are modified. A moiety can be conjugated to the 3' end, the 5' end, or at an internal position, or at a combination of these positions. In preferred embodiments, a moiety is conjugated to the 3' end of the antisense strand or sense strands. Similarly, where the nucleic acid silencing agent is double-stranded, a modification may be made only on the sense strand, only on the antisense strand, or on both strands. In some cases, the sense and antisense strand will have the same modifications or the same class of modifications, but in other cases the sense and antisense strand will have different modifications, e.g., in some cases it may be desirable to modify only one strand, e.g. the sense strand.

In certain embodiments, the nucleic acid silencing agents of the invention may be modified by the substitution of internal nucleotides with modified nucleotides. As

defined herein, an "internal" nucleotide is one occurring at any position other than the 5' end or 3' end of nucleic acid molecule, polynucleotide or oligonucleotide. An internal nucleotide can be within a single-stranded molecule or within a strand of a duplex or double-stranded molecule. In one embodiment, the sense strand and/or antisense strand is modified by the substitution of at least one internal nucleotide. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more internal nucleotides. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the internal nucleotides. In yet another embodiment, the sense strand and/or antisense strand is modified by the substitution of all of the internal nucleotides.

The nucleic acid silencing agents of the present invention can be modified to improve stability in serum or in growth medium for cell cultures. In order to enhance the stability, the 3'-residues may be stabilized against degradation, e.g., they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, pyrimidine nucleotides may be substituted by modified analogues, e.g., substitution of uridine by 2'-deoxythymidine.

Modifications can be made to stabilize an RNA silencing agent (e.g., against exonuclease or endonucleolytic degradation by cellular nucleases). In one embodiment, a stabilizing modification is a 2' modification. Exemplary 2' stabilizing modification include provision of a 2' OMe moiety (e.g., on a U in a sense or antisense strand, in a 3' overhang, or at the 3' end or terminus of a sense or antisense strand). In another embodiment, a stabilizing modification is a backbone modification. Exemplary backbone modifications include the replacement of an O or a P in the phosphate backbone with an S (e.g., a phosphorothioate backbone modification, e.g., on the U or the A or both) or the use of a methylated P in a 3' overhang. Other exemplary stabilizing modifications include replacement of the U with a C5 amino linker; replacement of an A with a G; modification with a 3' alkyl; and modification with an abasic pyrrolidine in a 3' overhang, e.g., at the 3' terminus. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications.

In some embodiments it is particularly preferred, e.g., to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, e.g., in a 5' or 3' overhang, or in both. E.g., it can be desirable to include one or more purine nucleotides (e.g., one or more deoxythymidines) in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, e.g., with a modification described herein. Modifications can include, e.g., the use of modifications at the 2' OH group of the ribose sugar, e.g., the use of deoxyribonucleotides, e.g., deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, e.g., phosphothioate modifications. Overhangs need not be homologous with the target sequence.

In other embodiments, RNA silencing agents may be modified with chemical moieties, for example, to enhance cellular uptake by target cells (e.g., neuronal cells). Thus, the invention includes RNA silencing agents which are conjugated or unconjugated (e.g., at its 3' terminus) to another moiety (e.g. a non-nucleic acid moiety such as a peptide), an organic compound (e.g., a dye), or the like. The conjugation can be accomplished by methods known in the art, e.g., using the methods of Lambert et al., Drug Deliv. Rev.:47(1), 99-112 (2001) (describes nucleic acids loaded to polyalkylcyanoacrylate (PACA) nanoparticles); Fattal et al., J. Control Release 53(1-3):137-43 (1998) (describes nucleic acids bound to nanoparticles); Schwab et al., Ann. Oncol. 5 Suppl. 4:55-8 (1994) (describes nucleic acids linked to intercalating agents, hydrophobic groups, polycations or PACA nanoparticles); and Godard et al., Eur. J. Biochem. 232(2):404-10 (1995) (describes nucleic acids linked to nanoparticles).

Chemical modifications may also be made to a double-stranded nucleic acid silencing agent in order to favor the ability of a particular strand to mediate gene silencing. For example, a double-stranded RNA silencing agent may be modified to favor entry of the antisense or guide strand into RISC such that undesirable, off-target silencing by the corresponding sense strand is reduced. It may be desirable to modify only the sense strand, e.g., to inactivate it, e.g., the sense strand can be modified in order to inactivate the sense strand and prevent its function as a guide strand. This can be accomplished by a modification which prevents 5'-phosphorylation of the sense strand, e.g., by modification with a 5'-O-methyl ribonucleotide (see Nykänen et al., (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be

used, e.g., simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate turning it into a phosphodiester linkage, though this may be less desirable as phosphodiesterases can cleave such a linkage and release a functional sRNA 5'-end.

5 In other embodiments, nucleic acid silencing agents may be substituted with a destabilizing nucleotide to enhance single nucleotide target discrimination (see US Application No. 11/698,689, filed January 25, 2007 which is incorporated herein by reference). Such a modification may be sufficient to abolish the specificity of the RNA silencing agent for a non-target mRNA (e.g. wild-type mRNA), without appreciably
10 affecting the specificity of the RNA silencing agent for a target mRNA (e.g. gain-of-function mutant mRNA). In preferred embodiments, the RNA silencing agents of the invention are modified by the introduction of at least one universal nucleotide in the antisense strand thereof. Universal nucleotides comprise base portions that are capable of base pairing indiscriminately with any of the four conventional nucleotide bases (e.g.
15 A,G,C,U). A universal nucleotide is preferred because it has relatively minor effect on the stability of the RNA duplex or the duplex formed by the guide strand of the RNA silencing agent and the target mRNA. Exemplary universal nucleotide include those having an inosine base portion or an inosine analog base portion selected from the group consisting of deoxyinosine (e.g. 2'-deoxyinosine), 7-deaza-2'-deoxyinosine, 2'-aza-2'-
20 deoxyinosine, PNA-inosine, morpholino-inosine, LNA-inosine, phosphoramidate-inosine, 2'-O-methoxyethyl-inosine, and 2'-OMe-inosine. In particularly preferred embodiments, the universal nucleotide is an inosine residue or a naturally occurring analog thereof.

 The RNA silencing agents of the invention are preferably modified by the
25 introduction of at least one destabilizing nucleotide within 5 nucleotides from a specificity-determining nucleotide (ie. the nucleotide which recognizes the disease-related polymorphism). For example, the destabilizing nucleotide may be introduced at a position that is within 5, 4, 3, 2, or 1 nucleotide(s) from a specificity-determining nucleotide. In exemplary embodiments, the destabilizing nucleotide is introduced at a
30 position which is 3 nucleotides from the specificity-determining nucleotide (ie. such that there are 2 stabilizing nucleotides between the destabilizing nucleotide and the specificity-determining nucleotide). In RNA silencing agents having two strands or strand portions (e.g. siRNAs and shRNAs), the destabilizing nucleotide may be

introduced in the strand or strand portion that does not contain the specificity-determining nucleotide. In preferred embodiments, the destabilizing nucleotide is introduced in the same strand or strand portion that contains the specificity-determining nucleotide.

5 In other embodiments, cross-linking can be employed to alter the pharmacokinetics of the RNA silencing agent, for example, to increase half-life in the body. Thus, the invention includes RNA silencing agents having two complementary strands of nucleic acid, wherein the two strands are crosslinked. The invention also includes RNA silencing agents which are conjugated or unconjugated (e.g., at its 3'
10 terminus) to another moiety (e.g. a non-nucleic acid moiety such as a peptide), an organic compound (e.g., a dye), or the like). Modifying siRNA derivatives in this way may improve cellular uptake or enhance cellular targeting activities of the resulting siRNA derivative as compared to the corresponding siRNA, are useful for tracing the siRNA derivative in the cell, or improve the stability of the siRNA derivative compared
15 to the corresponding siRNA.

Preferred nucleotide analogues include sugar- and/or backbone-modified ribonucleotides (i.e., include modifications to the phosphate-sugar backbone). For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In preferred backbone-modified
20 ribonucleotides the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g., of phosphothioate group. In preferred sugar-modified ribonucleotides, the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH₂, NHR, NR₂ or ON, wherein R is C₁-C₆ alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

25 In particular embodiments, the modifications are 2'-fluoro, 2'-amino and/or 2'-thio modifications. Particularly preferred modifications include 2'-fluoro-cytidine, 2'-fluoro-uridine, 2'-fluoro-adenosine, 2'-fluoro-guanosine, 2'-amino-cytidine, 2'-amino-uridine, 2'-amino-adenosine, 2'-amino-guanosine, 2,6-diaminopurine, 4-thio-uridine, and/or 5-amino-allyl-uridine. In a particular embodiment, the 2'-fluoro ribonucleotides
30 are every uridine and cytidine. Additional exemplary modifications include 5-bromo-uridine, 5-iodo-uridine, 5-methyl-cytidine, ribo-thymidine, 2-aminopurine, 2'-amino-butryl-pyrene-uridine, 5-fluoro-cytidine, and 5-fluoro-uridine. 2'-deoxy-nucleotides and 2'-Ome nucleotides can also be used within modified RNA-silencing agent moieties

of the instant invention. Additional modified residues include, for example, deoxy-abasic, inosine, N3-methyl-uridine, N6, N6-dimethyl-adenosine, pseudouridine, purine ribonucleoside and ribavirin. In a particularly preferred embodiment, the 2' moiety is a methyl group such that the linking moiety is a 2'-O-methyl oligonucleotide.

5 In an exemplary embodiment, the RNA silencing agent of the invention comprises Locked Nucleic Acids (LNAs). LNAs comprise sugar-modified nucleotides that resist nuclease activities (are highly stable) and possess single nucleotide discrimination for mRNA (Elmen et al., *Nucleic Acids Res.*, (2005), 33(1): 439-447; Braasch et al. (2003) *Biochemistry* 42:7967-7975, Petersen et al. (2003) *Trends*
10 *Biotechnol* 21:74-81). These molecules have 2'-O,4'-C-ethylene-bridged nucleic acids, with possible modifications such as 2'-deoxy-2''-fluorouridine. Moreover, LNAs increase the specificity of oligonucleotides by constraining the sugar moiety into the 3'-endo conformation, thereby preorganizing the nucleotide for base pairing and increasing the melting temperature of the oligonucleotide by as much as 10°C per base.

15 In another exemplary embodiment, the RNA silencing agent of the invention comprises Peptide Nucleic Acids (PNAs). PNAs comprise modified nucleotides in which the sugar-phosphate portion of the nucleotide is replaced with a neutral 2-amino ethylglycine moiety capable of forming a polyamide backbone which is highly resistant to nuclease digestion and imparts improved binding specificity to the molecule (Nielsen,
20 et al., *Science*, (2001), 254: 1497-1500).

 Also preferred are nucleobase-modified ribonucleotides, i.e., ribonucleotides, containing at least one non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase. Exemplary modified nucleobases include, but are not limited to, uridine
25 and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g., N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

30 Exemplary chemical modifications can include one or more of: (i) alteration, e.g., replacement, of a phosphate group in the phosphate backbone of an RNA molecule (e.g., replacement of the phosphate group with a siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide,

thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and/or methyleneoxymethylimino); (ii) alteration, e.g., replacement, of a constituent of the ribose sugar, e.g., of the 2' hydroxyl on the ribose sugar, or wholesale replacement of the ribose sugar with a structure other than ribose; (iii) modification or replacement of a naturally occurring nucleotide base; (iv) replacement or modification of the ribose-phosphate backbone with a surrogate backbone (e.g., a morpholino, cyclobutyl, pyrrolidine, or peptide nucleic acid (PNA) backbone); and (v) modification of the 3' end or 5' end or both ends of the RNA, e.g., removal, modification or replacement of a terminal phosphate group; (vi) conjugation of a moiety (e.g., to the 3' end of the RNA).

Other exemplary modifications include: (a) 2' modification, e.g., provision of a 2' OMe moiety on a U in a sense or antisense strand, but especially on a sense strand, or provision of a 2' OMe moiety in a 3' overhang, e.g., at the 3' terminus (3' terminus means at the 3' atom of the molecule or at the most 3' moiety, e.g., the most 3' P or 2' position, as indicated by the context); (b) modification of the backbone, e.g., with the replacement of an O with an S, in the phosphate backbone, e.g., the provision of a phosphorothioate modification, on the U or the A or both, especially on an antisense strand; e.g., with the replacement of a P with an S; (c) replacement of the U with a C5 amino linker; (d) replacement of an A with a G (sequence changes are preferred to be located on the sense strand and not the antisense strand); and (d) modification at the 2', 6', 7', or 8' position. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications. Yet other exemplary modifications include the use of a methylated P in a 3' overhang, e.g., at the 3' terminus; combination of a 2' modification, e.g., provision of a 2' O Me moiety and modification of the backbone, e.g., with the replacement of a P with an S, e.g., the provision of a phosphorothioate modification, or the use of a methylated P, in a 3' overhang, e.g., at the 3' terminus; modification with a 3' alkyl; modification with an abasic pyrrolidine in a 3' overhang, e.g., at the 3' terminus; modification with naproxen, ibuprofen, or other moieties which inhibit degradation at the 3' terminus.

Exemplary nucleic acid silencing agents include, but are not limited to, the following agents:

A. RNA Silencing Agents

i. siRNA Molecules

An exemplary siRNA molecule of the invention is a duplex consisting of a sense strand and complementary antisense or guide strand, the antisense strand comprises a
5 sequence (herein, “guide strand sequence” or “antisense strand sequence”) having sufficient complementary to an mRNA (e.g., a HCV-related mRNA) to mediate RNA silencing of the mRNA via an RNA silencing mechanism (e.g., RNAi). In certain embodiments, the antisense strand sequence may be perfectly complementary to the mRNA, although perfect complementarity to the mRNA is not required to mediate RNA
10 silencing. In certain embodiments, the antisense strand sequence has at least 80% sequence complementarity (e.g., 80%, 85%, 90%, 92%, 95%, or more sequence complementarity) to a corresponding sequence in a target mRNA. For example, the antisense sequence may form one or more mismatches (e.g., non-Watson Crick base pairs) with the target mRNA sequence (e.g., 1, 2, 3, 4, or 5 mismatches) and still
15 mediate RNA silencing of the target mRNA.

The antisense strand sequence may comprise about 10-50 contiguous nucleotides (or nucleotide analogs). More preferably, the antisense sequence may comprise about 18-30 contiguous nucleotides, about 20-25 contiguous nucleotides, or about 21-23
nucleotides, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,
20 or 35 nucleotides. In preferred embodiments, most or substantially all of the nucleotides of the antisense sequence of the siRNA molecule are ribonucleotides (or ribonucleotide analogs). In particularly preferred embodiments, at least 75% (more preferably 80%, 85%, 90%, 95%, or more) of the nucleotides in the antisense sequence are
ribonucleotides (or ribonucleotide analogs).

25 In certain embodiments, the antisense strand of the siRNA molecule consists of antisense strand sequence without any additional nucleotides. In other embodiments, the antisense strand comprises an antisense strand sequence with one or more additional nucleotides flanking either end of the sequence. These additional nucleotides (termed “flanking nucleotides”) may lack complementarity to target mRNA sequence and are not
30 necessary for mediating RNA silencing. In certain embodiments, the additional nucleotides are processed from the siRNA by cellular enzymes leaving the antisense strand sequence intact.

The siRNA may comprise a sense strand of siRNA may comprise a sequence of contiguous nucleotides that is perfectly complementary to, and the same length as, the antisense strand sequence of the antisense strand. However, sense strand sequence may, however, comprise greater or fewer number of nucleotides. For example, the sense strand sequence may comprise a small number of extra nucleotides (e.g., 1, 2, or 3) which form bulges in the duplexed siRNA molecule when the sense and antisense strands are aligned. In other embodiments, the sense strand sequence may form one or more mismatched base pairs (e.g., 1, 2, 3, 4, or 5 mismatches) with nucleotides in the antisense strand sequence.

Preferably, the siRNA molecule comprises strands having a total length from about 10-50 or more nucleotides, i.e., each strand comprises 10-50 nucleotides (or nucleotide analogs). More preferably, the strands of the siRNA molecule have a length from about 18-30, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides in each strand, wherein one of the strands is sufficiently complementary to a target mRNA. In certain preferred embodiments, the sense and antisense strands are each about 20-25 nucleotides in length (e.g., 21-23 nucleotides in length). In preferred embodiments, most or substantially all of the nucleotides of the siRNA molecule are ribonucleotides (or ribonucleotide analogs). In particularly preferred embodiments, at least 75% (more preferably 80%, 85%, 90%, 95%, or more) of the nucleotides are ribonucleotides (or ribonucleotide analogs).

The strands of an siRNA molecule may be aligned such that there are about 1-5 (e.g., 1, 2, or 3) bases at the end of the strands which do not align (i.e., for which no complementary bases occur in the opposing strand) such that an overhang of about 1-5 residues occurs at one or both ends of the duplex when strands are annealed. In preferred embodiments, the overhangs are present on one or both of the 3' ends of the duplex to form 3' overhangs. 5' overhangs are also contemplated. In other embodiments, the strands of the siRNA molecules are aligned such that one or both ends of the siRNA molecule are blunt-ended.

The total length of an siRNA molecule includes any overhangs which may be present. For example, where an siRNA molecule comprises a sense strand of 18 nucleotides and an antisense strand of 20 nucleotides, the total length of the siRNA molecule is considered to be 20 nucleotides. Preferably, the siRNA molecule has a total length from about 10-50 or more nucleotides (or nucleotide analogs). More preferably,

the siRNA molecule has a length from about 18 -30, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides (or nucleotide analogs). Preferably the siRNA molecule are of sufficiently short length that they do not elicit a PKR response.

However, longer RNA silencing agents may be useful, for example, in cell types
5 incapable of generating a PRK response or in situations where the PKR response has been downregulated or dampened by alternative means.

siRNA molecules employed in the invention preferably include a 5' phosphate group (e.g., a 5'-monophosphate) on the terminal nucleotide at the 5' end of the antisense strand. Accordingly, siRNA molecules may be synthesized to incorporate a 5'
10 phosphate on one or both 5' ends of the molecule, or they may be phosphorylated *in vitro* or *in vivo* by cellular enzymes.

siRNA (and other dsRNA silencing agents, such as shRNAs) may be designed to facilitate enhanced efficacy and specificity in mediating gene silencing (e.g., via RNAi). Several sequence criteria may be employed to select dsRNA silencing agent with
15 enhanced activity. Preferably, the silencing agent satisfies one or more of the following sequence criteria: (a) low internal stability at the 5' end of the antisense strand; (b) low propensity to form internal hairpins; (c) presence of adenosine (A) and/or absence at cytosine (C) at position 19 (P19) of the sense strand sequence; (d) presence of uridine (U) at position P10; (e) presence of adenosine (A) at position P3; and (f) absence of
20 guanosine (G) at position P13..

In certain embodiments, dsRNA silencing agents with enhanced sequence asymmetry are preferably selected (see International Publication No. WO 2005/001045, US Publication No. 2005-0181382 A1). For example, the RNA silencing agent may have fewer G:C base pairs at the 5' end of the first or antisense strand than the 5' end of
25 the sense strand. Alternatively, RNA silencing agent may contain a greater number of mismatched or wobble base pairs at the 5' end of antisense strand than the 5' end of the sense strand. The RNA silencing agent may modified such that its asymmetry is further enhanced. For example, the sequence of one or both 5'ends may be modified such there fewer G:C base pairs or a larger number of mismatches or wobbles at the 5' end of the
30 antisense strand than the 5' end of the sense strand. Such alterations favor entry of the antisense strand sequence of the RNA silencing agent into RISC in favor of the sense strand, such that the antisense strand preferentially guides cleavage or translational

repression of a target mRNA, and thus increasing or improving the efficiency of target cleavage and silencing.

In one embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there are fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the sense strand portion than between the 3' end of the first or antisense strand and the 5' end of the sense strand portion. In another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the sense strand portion. Preferably, the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C and U:U. In another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one wobble base pair, e.g., G:U, between the 5' end of the first or antisense strand and the 3' end of the sense strand portion. In another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one base pair comprising a rare nucleotide, e.g., inosine (I). Preferably, the base pair is selected from the group consisting of an I:A, I:U and I:C. In yet another embodiment, the the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one base pair comprising a modified nucleotide. In preferred embodiments, the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

To validate the effectiveness by which siRNAs cleave target mRNAs, the siRNA may be incubated with cDNA in a *Drosophila*-based *in vitro* mRNA expression system. Radiolabeled with ³²P, newly synthesized mRNAs are detected autoradiographically on an agarose gel. The presence of cleaved mRNA indicates mRNA nuclease activity. Suitable controls include omission of siRNA and use of wild-type cDNA. Alternatively, control siRNAs are selected having the same nucleotide composition as the selected siRNA, but without significant sequence complementarity to the appropriate target gene. Such negative controls can be designed by randomly scrambling the nucleotide sequence of the selected siRNA; a homology search can be performed to ensure that the negative control lacks homology to any other gene in the appropriate genome. In addition, negative control siRNAs can be designed by introducing one or more base mismatches into the sequence.

ii. miRNA Molecules

In other embodiments, the nucleic acid silencing agents are similar or identical in sequence to that of a miRNA. miRNAs are naturally-occurring noncoding RNAs of approximately 22 nucleotides which can regulate gene expression at the post transcriptional or translational level during plant and animal development. At least 5000 miRNAs have been identified to date and together they are thought to comprise a significant fraction (1-5%) of all predicted genes in the genome. Many natural miRNAs are clustered together in the introns of pre-mRNAs and can be identified in silico using homology-based searches (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) or computer algorithms (e.g. MiRScan, MiRSeeker) that predict the capability of a candidate miRNA gene to form the stem loop structure of a pri-mRNA (Grad et al., Mol. Cell., 2003; Lim et al., Genes Dev., 2003; Lim et al., Science, 2003; Lai EC et al., Genome Bio., 2003). An online registry provides a searchable database of all published miRNA sequences (The microRNA Registry at the Sanger Institute website; Griffiths-Jones S, Nuc. Acids Res., 2004). Exemplary miRNAs are also described in International PCT Publication No. WO 03/029459.

Naturally-occurring miRNAs are expressed by endogenous genes *in vivo* and are processed from a hairpin or stem-loop precursor (pre-miRNA or pri-miRNAs) by Dicer or other RNases (Lagos-Quintana et al., Science, 2001; Lau et al., Science, 2001; Lee and Ambros, Science, 2001; Lagos-Quintana et al., Curr. Biol., 2002; Mourelatos et al., Genes Dev., 2002; Reinhart et al., Science, 2002; Ambros et al., Curr. Biol., 2003; Brennecke et al., 2003; Lagos-Quintana et al., RNA, 2003; Lim et al., Genes Dev., 2003; Lim et al., Science, 2003). miRNAs can exist transiently *in vivo* as a double-stranded duplex but only one strand is taken up by the RISC complex to direct gene silencing. Certain miRNAs, e.g. plant miRNAs, have perfect or near-perfect complementarity to their target mRNAs and, hence, direct cleavage of the target mRNAs. Other miRNAs have less than perfect complementarity to their target mRNAs and, hence, direct translational repression of the target mRNAs. The degree of complementarity between an miRNA and its target mRNA is believed to determine its mechanism of action. For example, perfect or near-perfect complementarity between a miRNA and its target mRNA is predictive of a cleavage mechanism (Yekta et al.,

Science, 2004), whereas less than perfect complementarity is predictive of a translational repression mechanism.

In particular embodiments, the miRNA sequence has substantial sequence identity (i.e., 80% or greater sequence identity, e.g., 80%, 85%, 90%, 95% or 100% sequence identity) to that of a naturally-occurring miRNA sequence, the aberrant expression or activity of which is correlated with a miRNA disorder. Exemplary miRNAs whose expression or activity is correlated with disease include lin-4, let-7, miR-10, miR-15, miR-16, miR-122, miR-168, miR-175, miR-181, miR-196, miR-216, miR-217, miR-221, or miR-222 and their homologs. In yet other particular embodiments, the naturally-occurring miRNA sequence is derived from a viral miRNA, i.e., a viral miRNA expressed by a virus, e.g., HCV virus. Exemplary viral miRNAs include miR-155 (miR-K12-11) which is expressed by the Kaposi's Sarcoma associated Herpesvirus.

iii. siRNA-like Molecules

siRNA-like molecules of the invention have a sequence (i.e., have a strand having a sequence) that is "sufficiently complementary" to a heterozygous SNP of a target mRNA to direct gene silencing either by RNAi or translational repression. siRNA-like molecules are designed in the same way as siRNA molecules, but the degree of sequence identity between the sense strand and target RNA approximates that observed between an miRNA and its target. In general, as the degree of sequence identity between a miRNA sequence and the corresponding target gene sequence is decreased, the tendency to mediate post-transcriptional gene silencing by translational repression rather than RNAi is increased. Therefore, in an alternative embodiment, where post-transcriptional gene silencing by translational repression of the target gene is desired, the miRNA sequence has partial complementarity with the target gene sequence. In certain embodiments, the miRNA sequence has partial complementarity with one or more short sequences (complementarity sites) dispersed within the target mRNA (e.g. within the 3'-UTR of the target mRNA) (Hutvagner and Zamore, Science, 2002; Zeng et al., Mol. Cell, 2002; Zeng et al., RNA, 2003; Doench et al., Genes & Dev., 2003). Since the mechanism of translational repression is cooperative, multiple complementarity sites (e.g., 2, 3, 4, 5, or 6) may be targeted in certain embodiments.

The capacity of a siRNA-like duplex to mediate RNAi or translational repression may be predicted by the distribution of non-identical nucleotides between the target gene sequence and the nucleotide sequence of the silencing agent at the site of complementarity. In one embodiment, where gene silencing by translational repression is desired, at least one non-identical nucleotide is present in the central portion of the complementarity site so that duplex formed by the msiRNA guide strand and the target mRNA contains a central "bulge" (Doench JG et al., Genes & Dev., 2003). In another embodiment 2, 3, 4, 5, or 6 contiguous or non-contiguous non-identical nucleotides are introduced. The non-identical nucleotide may be selected such that it forms a wobble base pair (e.g., G:U) or a mismatched base pair (G:A, C:A, C:U, G:G, A:A, C:C, U:U). In a further preferred embodiment, the "bulge" is centered at nucleotide positions 12 and 13 from the 5' end of the msiRNA molecule.

iv. Short Hairpin RNA (shRNA) Molecules

Short hairpin RNAs, or engineered RNA precursors, are artificial constructs based on these naturally occurring pre-miRNAs, but which are engineered to deliver desired RNA silencing agents (e.g., siRNAs of the invention). By substituting the stem sequences of the pre-msiRNA with sequence complementary to the target mRNA, a shRNA molecule is formed. The shRNA is processed by the entire gene silencing pathway of the cell, thereby efficiently mediating RNAi.

The requisite elements of a shRNA molecule include a first portion and a second portion, having sufficient complementarity to anneal or hybridize to form a duplex or double-stranded stem portion. The two portions need not be fully or perfectly complementary. The first and second "stem" portions are connected by a portion having a sequence that has insufficient sequence complementarity to anneal or hybridize to other portions of the shRNA. This latter portion is referred to as a "loop" portion in the shRNA molecule. The shRNA molecules are processed to generate siRNAs. shRNAs can also include one or more bulges, i.e., extra nucleotides that create a small nucleotide "loop" in a portion of the stem, for example a one-, two- or three-nucleotide loop. The stem portions can be the same length, or one portion can include an overhang of, for example, 1-5 nucleotides. The overhanging nucleotides can include, for example, uracils (Us), e.g., all Us. Such Us are notably encoded by thymidines (Ts) in the shRNA-encoding DNA which signal the termination of transcription.

In shRNAs, or engineered precursor RNAs, of the instant invention, one portion of the duplex stem is a nucleic acid sequence that is complementary (or anti-sense) to the heterozygous SNP. Preferably, one strand of the stem portion of the shRNA is sufficiently complementary (e.g., antisense) to a target RNA (e.g., mRNA) sequence to
5 mediate degradation or cleavage of said target RNA via RNA interference (RNAi). Thus, engineered RNA precursors include a duplex stem with two portions and a loop connecting the two stem portions. The antisense portion can be on the 5' or 3' end of the stem. The stem portions of a shRNA are preferably about 15 to about 50 nucleotides in length. Preferably the two stem portions are about 18 or 19 to about 21, 22, 23, 24, 25,
10 30, 35, 37, 38, 39, or 40 or more nucleotides in length. In preferred embodiments, the length of the stem portions should be 21 nucleotides or greater. When used in mammalian cells, the length of the stem portions should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway. In non-mammalian cells, the stem can be longer than 30 nucleotides. In fact, the stem can
15 include much larger sections complementary to the target mRNA (up to, and including the entire mRNA). In fact, a stem portion can include much larger sections complementary to the target mRNA (up to, and including the entire mRNA).

The two portions of the duplex stem must be sufficiently complementary to hybridize to form the duplex stem. Thus, the two portions can be, but need not be, fully
20 or perfectly complementary. In addition, the two stem portions can be the same length, or one portion can include an overhang of 1, 2, 3, or 4 nucleotides. The overhanging nucleotides can include, for example, uracils (Us), e.g., all Us. The loop in the shRNAs or engineered RNA precursors may differ from natural pre-miRNA sequences by modifying the loop sequence to increase or decrease the number of paired nucleotides,
25 or replacing all or part of the loop sequence with a tetraloop or other loop sequences. Thus, the loop in the shRNAs or engineered RNA precursors can be 2, 3, 4, 5, 6, 7, 8, 9, or more, e.g., 15 or 20, or more nucleotides in length.

The loop in the shRNAs or engineered RNA precursors may differ from natural pre-miRNA sequences by modifying the loop sequence to increase or decrease the
30 number of paired nucleotides, or replacing all or part of the loop sequence with a tetraloop or other loop sequences. Thus, the loop portion in the shRNA can be about 2 to about 20 nucleotides in length, i.e., about 2, 3, 4, 5, 6, 7, 8, 9, or more, e.g., 15 or 20, or more nucleotides in length. A preferred loop consists of or comprises a "tetraloop"

sequences. Exemplary tetraloop sequences include, but are not limited to, the sequences GNRA, where N is any nucleotide and R is a purine nucleotide, GGGG, and UUUU.

In certain embodiments, shRNAs of the invention include the sequences of a desired siRNA molecule described supra. In other embodiments, the sequence of the antisense portion of a shRNA can be designed essentially as described above or generally by selecting an 18, 19, 20, 21 nucleotide, or longer, sequence from within the target RNA (e.g., SOD1 or htt mRNA), for example, from a region 100 to 200 or 300 nucleotides upstream or downstream of the start of translation. In general, the sequence can be selected from any portion of the target RNA (e.g., mRNA) including the 5' UTR (untranslated region), coding sequence, or 3' UTR, provided said portion is distant from the site of the gain-of-function mutation. This sequence can optionally follow immediately after a region of the target gene containing two adjacent AA nucleotides. The last two nucleotides of the nucleotide sequence can be selected to be UU. This 21 or so nucleotide sequence is used to create one portion of a duplex stem in the shRNA. This sequence can replace a stem portion of a wild-type pre-msiRNA sequence, e.g., enzymatically, or is included in a complete sequence that is synthesized. For example, one can synthesize DNA oligonucleotides that encode the entire stem-loop engineered RNA precursor, or that encode just the portion to be inserted into the duplex stem of the precursor, and using restriction enzymes to build the engineered RNA precursor construct, e.g., from a wild-type pre-msiRNA.

Engineered RNA precursors include in the duplex stem the 21-22 or so nucleotide sequences of the siRNA or siRNA-like duplex desired to be produced *in vivo*. Thus, the stem portion of the engineered RNA precursor includes at least 18 or 19 nucleotide pairs corresponding to the sequence of an exonic portion of the gene whose expression is to be reduced or inhibited. The two 3' nucleotides flanking this region of the stem are chosen so as to maximize the production of the siRNA from the engineered RNA precursor and to maximize the efficacy of the resulting siRNA in targeting the corresponding mRNA for translational repression or destruction by RNAi *in vivo* and *in vitro*.

30

v. Other RNA Silencing Agents

Embodiments of the invention feature a wide variety of possible nucleic acid-based and non-nucleic acid-based silencing agents. In some embodiments of the

invention featuring nucleic acid-based silencing agents, the activity of a silencing agent may belong to one category of activity ascribed to such agents. In some embodiments of the invention, the activity of a silencing agent may belong to two or more categories of activity ascribed to such agents and/or to nucleic acids. Some types of RNA

5 silencing agents for use in preferred embodiments of the invention include RNAi, siRNA, miRNA, rasiRNA, shRNA, smRNA, dsRNA, tncRNA, piRNA.

RNA silencing agents for use with particular embodiments of the invention may have activity belonging to the categories of agents described above or may have activity ascribed to other categories of RNA agent. In some embodiments, the particular activity
10 of a silencing agent may not be characterized.

B. DNA Silencing Agents

i. Antisense Oligonucleotides

In other embodiments, a nucleic acid molecule employed in a composition of the
15 invention is a nucleic acid molecule other than an RNA silencing agent. In certain embodiments, said nucleic acid molecules may comprise any of the chemical modifications discussed *supra*.

In one embodiment, a nucleic acid molecule employed in the invention is an antisense nucleic acid molecule that is complementary to a target mRNA or to a portion
20 of said mRNA, or a recombinant expression vector encoding said antisense nucleic acid molecule. Antisense nucleic acid molecules are generally single-stranded DNA, RNA, or DNA/RNA molecules which may comprise one or more nucleotide analogs. The use of antisense nucleic acids to downregulate the expression of a particular protein in a cell is well known in the art (see *e.g.*, Weintraub, H. *et al.*, Antisense RNA as a molecular
25 tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986; Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule comprises a nucleotide
30 sequence that is complementary to the target mRNA sequence and accordingly is capable of hydrogen bonding to the mRNA. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the

coding region and an untranslated region (*e.g.*, at the junction of the 5' untranslated region and the coding region). Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon in the 3' untranslated region of an mRNA.

5 Given the known nucleotide sequence of a target mRNA, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of an mRNA, but more preferably is antisense to only a portion of the coding or noncoding region of an mRNA. For example, the antisense oligonucleotide can be
10 complementary to the region surrounding the translation start site of a target mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 100, 500, 1000 nucleotides or more in length. In some embodiments, the antisense oligonucleotide may be as long as, or longer than, the length of the mRNA that is targeted.

15 An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical
20 stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-
25 carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-
30 mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (*v*), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (*v*), 5-methyl-2-thiouracil, 3-(3-

amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. To inhibit expression in cells, one or more antisense oligonucleotides can be used.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which all or a portion of a cDNA has been subcloned in an antisense orientation (*i.e.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which direct constitutive, tissue specific or inducible expression of antisense RNA. The antisense expression vector is prepared according to standard recombinant DNA methods for constructing recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus.

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix.

a. Inhibitors of RNA silencing

In one particular embodiment, antisense oligonucleotides may be employed which are complementary to one or more of the RNA silencing agents (*e.g.*, miRNA molecules) described *supra*. Said anti-miRNA oligonucleotides may be DNA or RNA oligonucleotides, or they may be comprised of both ribonucleotide and deoxyribonucleotides or analogs thereof. In preferred embodiments, said anti-miRNA oligonucleotides comprise one or more (*e.g.*, substantially all) 2'-O-methyl ribonucleotides. Such molecules are potent and irreversible inhibitors of miRNA-mediated silencing and are therefore useful for modulating RNA silencing both *in vitro* and *in vivo*. *In vivo* methodologies are useful for both general RNA silencing

modulatory purposes as well as in therapeutic applications in which RNA silencing modulation (*e.g.*, inhibition) is desirable. For example, insulin secretion has been shown to be regulated by at least one miRNA (Poy *et al.* 2004), and a role for miRNAs has also been implicated in spinal muscular atrophy (SMA; Mourelatos *et al.* 2002).

5

ii. α -anomeric nucleic acid molecules

In yet another embodiment, a nucleic acid molecule employed in the invention is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids Res.* 15:6625-6641). Such a nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

10

iii. repeat-associated siRNA (rasiRNA) molecules

Unlike siRNAs, which derive from both the sense and antisense strands of their dsRNA precursors, rasiRNAs arise mainly from the antisense strand. rasiRNAs are involved in the shutdown of expression of transposable elements, histone modification and DNA methylation modification. They function via partly overlapping components of the siRNA pathway (See, *e.g.*, Vagin, V.V. *et al.*, (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 313:320–324).

20

iv. tiny non-coding RNA (tncRNA) molecules

This is a class of short 20–22-nt RNAs described in *C. elegans*. Unlike many miRNAs, the sequences of tncRNAs are not commonly conserved among related species. Although their functions are unknown, many are complementary to mRNA and might target them for degradation (See, *e.g.*, Shrivastava N. and Srivastava A. (2008). RNA interference: An emerging generation of biologicals. *Biotechnol. J.* 3:339–353).

25

v. piwi-interacting RNA (piRNA) molecules

piRNA is a class of small RNA molecules that is expressed uniquely in mammalian testes and forms RNA-protein complexes with Piwi proteins. These piRNA complexes have been linked to transcriptional gene silencing of retrotransposons and

30

other genetic elements in germ-line cells, particularly those in spermatogenesis. Purification of these complexes has revealed that these oligonucleotides are approximately 29–30 nt long. They are distinct in size from miRNA and are associated with distinct protein complexes. It remains unclear how piRNAs are generated, but their biogenesis pathway is distinct from miRNA and siRNA (See, *e.g.*, Carthew R.W. (2006). A New RNA Dimension to Genome Control. *Science* 313:305-306).

IV. Anti-HCV Nucleic Acid Silencing Agents

Hepatitis C virus (HCV), formerly known as non-A, non-B hepatitis, was first identified in 1988. HCV infection is the second leading cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, affecting more than 170 million people worldwide. As a small (50 nm in size) and enveloped RNA virus in the genus *Hepacivirus* and the family *Flaviviridae*, HCV contains a single positive-strand RNA genome that consists of about 9,600 nucleotides that encode a single polyprotein composed of 3,010 amino acid residues.

The present invention includes a method of using a complex or a molecule comprising a targeting moiety to deliver therapeutic RNA molecules effectively to Hepatitis C virus or to a cell being infected or having the potential to be infected by Hepatitis C virus, resulting in the prevention, immunization, inhibition, cure, or other beneficial effects towards Hepatitis C virus infection. In some embodiments, the therapeutic RNA molecules are RNA interference agents (*e.g.*, siRNAs, shRNAs, miRNA, smRNA, rasiRNA, tncRNA, piRNA, etc.), with or without modifications. In one preferred embodiment, the therapeutic RNA molecules are designed to antagonize one or more elements promoting or positively affecting the infection cycle of Hepatitis C virus, which includes, but is not limited to, the stability and activity of Hepatitis C virus itself, the virus entry into a cell, the replication and translation of viral RNAs and proteins in a cell, the up-regulation of host proteins in the infection cycle of HCV, the virus packaging and exit from a cell, the transport and spread of Hepatitis C virus in a mammal, etc. In another embodiment, the therapeutic RNA molecules are designed to promote or positively affect one or more elements inhibiting or negatively affecting the infection cycle of Hepatitis C virus. These elements include, but are not limited to, the cellular defense systems and immune systems (apoptosis, necrosis, autophagy, macrophagy, interferons, chemokines, cytokines, etc.).

Accordingly, in one embodiment, the invention provides a method of RNA interference in a cell, which is or is not infected by Hepatitis C virus. In another embodiment, the RNA interference is performed in an animal, preferably a mammal, most preferably a human, which is or is not infected by Hepatitis C virus. In some
5 embodiments, the RNA interference agents are designed to recognize the genomic RNA sequence, or a RNA sequence complementary to the genomic RNA sequence, of Hepatitis C virus. In one preferred embodiment, the RNA interference agent is designed to recognize the mRNA sequence, or a RNA sequence complementary to the mRNA sequence, encoding each of viral proteins, *i.e.*, C (a.k.a. core protein), E1, E2, NS1
10 (a.k.a. P7), NS2, NS3, NS4A, NS4B, NS5A and NS5B. In another embodiment, the RNA interference agent is designed to recognize the mRNA sequences, or RNA sequences complementary to the mRNA sequences, encoding multiple viral proteins selected from the above groups. In other embodiments, the RNA interference agents are designed to recognize the mRNA sequences, or RNA sequences complementary to the
15 mRNA sequences, encoding the borders or junctions on the various viral proteins, selected from the above groups, after their cleavage from the single 3010 amino acid-containing viral polyprotein. In some other embodiments, the RNA interference agents are designed to recognize the mRNA sequences, or RNA sequences complementary to the mRNA sequences, in the overlapping reading frames, *e.g.*, +1, +2, +3, -1, -2, or -3.
20 One example of these gene products is ARFP (alternative reading frame protein) or F (frameshift) protein, comprising up to 160 amino acids. In one embodiment, the RNA interference agents recognize each or multiple of untranslated regions (UTR) at the 5' and 3' ends of the HCV genomic RNA, which may fine-regulate expression of the virus genes. In another embodiment, the RNA interference agents recognize a RNA sequence
25 complementary to each or multiple of untranslated regions (UTR) at the 5' and 3' ends of the HCV genomic RNA. In one preferred embodiment, the RNA interference agents recognize the mRNA sequence, or a RNA sequences complementary to the mRNA sequence, representing the ribosome binding site in the 5' UTR of Hepatitis C virus. In one embodiment, the RNA interference agent recognized the mRNA sequence, or a
30 RNA sequence complementary to the mRNA sequence, of viral proteases, *e.g.*, NS2-3 and NS3-4A, which are able to cleave the single viral polyprotein into individual proteins. In another embodiment, the RNA interference agent recognized the mRNA sequence, or a RNA sequence complementary to the mRNA sequence of host

endoplasmic reticulum (ER) signal peptidase(s) (*e.g.*, see Reed K.E. and Rice C.M.

“Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties.” *Curr Top Microbiol Immunol* 2000; 242: 55-84), which are able to cleave

the single viral polyprotein into individual proteins. In some embodiments, the RNA

5 interference agents recognize the regions of viral genes that are important for the functional protein-protein interactions between the said viral products and others viral or host proteins. These interactions include, for example, the heterodimers between E1 and E2 proteins, the hexamers of P7 proteins, NS5A dimers, the interaction between C and E1/E2, etc.

10 In some embodiments, the RNA interference agents are designed to antagonize the virus entry process. In one embodiment, the RNA interference agents recognize the mRNA sequence, or a RNA sequence complimentary to the mRNA sequence, of HCV envelope glycoproteins E1 and E2. In one preferred embodiment, the RNA interference agents recognize the hypervariable regions (HVR) in E1 and/or E2. In one embodiment,

15 the RNA interference agents recognize the receptors for HCV particles on the surfaces of host cells, *e.g.*, hepatocytes, lymphocytes, monocytes, etc. These receptors, and other host or viral cofactors facilitating the virus entry into the cell, include ubiquitous receptors/co-factors and specific receptors/co-factors, for example, the tetraspanin CD81, the scavenger receptor class B type I (SR-BI), the tight junction protein claudin-1

20 (CLDN1), the mannose binding C-type lectins L-SIGN and DC-SIGN, the asialoglycoprotein receptor, heparan-sulfate proteoglycans, and low-density lipoprotein receptor (LDLR). In one preferred embodiment, the RNA interference agents are designed to down-regulate the expressions of ubiquitous or specific receptors/co-factors. These co-factors further include, for example, lipoproteins, high density lipoprotein

25 (HDL), etc. In some embodiments, the RNA interference agents antagonize the mechanisms (*e.g.*, host tropism) of HCV infection and entry of host cells, including binding of Hepatitis C virus to cellular receptors, the following endocytosis and then the release of viral nucleocapsid or other proteins into the cytoplasm of the cell. In some

30 embodiments, the RNA interference agents recognize members of the cellular endocytosis machinery, *e.g.*, the clathrin network, which facilitates the Hepatitis C virus entry into the cell. In some embodiments, the RNA interference agents antagonize the release of single virus particles from endosomal compartments into the cytoplasm, which is associated with membrane fusion and virus uncoating.

In line with the extreme competition between viral and host mRNAs within the cell with respect to components of the translational machinery, Hepatitis C virus have developed mechanisms to utilize certain eukaryotic translation initiation factors (eIFs), and thus hijack the translational machinery for their own profit. This invention also includes methods to design RNA interference agents to antagonize the virus replication and translation process in infected cells. In one embodiment, the RNA interference agent is designed to recognize the 5' UTR of the HCV genome, preferably one or multiple domain sequences of domains I–IV in the 5' UTR. In one preferred embodiment, the RNA interference agent recognizes the IRE sequence on the HCV genome. The IRE sequence comprises nearly the entire 5' UTR of the HCV genome and can also include part of the first coding sequence, *e.g.*, the first 12 to 30 nt. In some embodiments, the RNA interference agents are designed to recognize members of the host replication machinery facilitating the replication of HCV. In one embodiment, the RNA interference agent down-regulates the expression of these host proteins. In another embodiment, the RNA interference agent antagonizes the interaction of these host proteins with HCV viral genome, preferably the viral IRE sequence. These host proteins include, for example, the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA–eIF2–GTP ternary complex. Other cellular factors such as La autoantigen, heterogeneous ribonucleoprotein L, poly-C binding protein, and pyrimidine tract-binding protein, also bind to the IRES element and modulate viral translation. In another embodiment, the RNA interference agent recognizes the 3' UTR of the HCV genome, preferably one or multiple domains of a variable region of about 40 nt, a variable length poly(U/UC) tract, and a highly conserved, 98-nt 3' terminal segment (3'X) that putatively forms three stem-loop structures.

IRES-mediated translation of the HCV ORF yields a polyprotein precursor that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins. In one embodiment of the present invention, the RNA interference agent is designed to recognize the host signal peptidases that process the viral polyprotein. In one preferred embodiment, the RNA interference agent down-regulates the expression of these host signal peptidases. In another embodiment of the present invention, the RNA interference agent is designed to recognize the viral proteases that process the viral polyprotein, *e.g.*, the NS2–3 protease, which spans NS2

and the N-terminal domain of NS3, and the NS3-4A protease, which contains NS4A as a cofactor of the NS3 serine protease. In one preferred embodiment, the RNA interference agent down-regulates the expression of these viral proteases. In another embodiment, the RNA interference agent is designed to recognize the sequence of these host and/or viral proteases to antagonize their interaction with HCV genome. In other embodiments of this invention, the RNA interference agent is designed to antagonize the maturation and/or post-translational regulation of viral proteins. In one embodiment, the RNA interference agent is designed to antagonize the expression or activity of host machinery responsible for HCV protein maturation and/or post-translational modifications. In one preferred embodiment, the RNA interference agent is designed to antagonize the expression or activity of host proteins in ubiquitin-proteasome pathway. These host proteins may include, for example, E6-AP and other HECT-domain E3 ligases, the proteasome activator PA28 γ , which binds to HCV C protein, etc.

In some embodiments of this invention, the RNA interference agents are designed to antagonize the HCV RNA replication. HCV is assumed to replicate its genome through the synthesis of a full-length negative-strand RNA. Positive-strand RNA is then produced from the negative-strand template; it is several-fold more abundant than the negative-stranded RNA and is utilized for translation, replication, and packaging into progeny viruses. In one embodiment, the RNA interference agent is complementary to and recognizes the positive-strand RNA of HCV. In another embodiment, the RNA interference agent is complementary to and recognizes the negative-strand RNA of HCV. In some embodiments, the RNA interference agents recognize and antagonize viral or host factors important for conferring proper RNA replication. These viral or host factors may include, for example, the NS5B, NS3, NS4B, NS5A viral proteins, host proteins interacting with these viral proteins, *e.g.*, human vesicle-associated membrane protein associated proteins (hVAP-A and -B), FBL2, cyclophilin B and other components in the replication complexes (RCs).

In some embodiments, the RNA interference agents antagonize the localization of the viral RNA or proteins within cellular structures. In one preferred embodiment, the RNA interference agent antagonizes the transportation process and host transportation machinery for transporting HCV RNA or proteins into the ER system. In another embodiment, the RNA interference agent antagonizes the transportation process and host transportation machinery for transporting HCV RNA or proteins out of the ER

system and into the Golgi fraction. In other embodiments, the RNA interference agents antagonize the transportation process and host transportation machinery for transporting HCV RNA or proteins out of the Golgi fraction.

The present invention includes methods to design RNA interference agents to
5 antagonize the assembly and budding process to release mature Hepatitis C virus out of the infected cell. In one embodiment, the RNA interference agents recognize the mRNA sequence, or a RNA sequence complimentary to the mRNA sequence, of HCV envelope glycoproteins E1 and E2. In one preferred embodiment, the RNA interference agents recognize the hypervariable regions (HVR) in E1 and/or E2. In another
10 embodiment, the RNA interference agent down-regulates the expression of viral proteins facilitating the formation of the nonenveloped nucleocapsids. These viral proteins include, for example, the C proteins. In one embodiment, the RNA interference agent is designed to recognize the sequences of viral and host proteins, as exemplified above. These sequences are crucial for the interaction between these proteins. In other
15 embodiments, the RNA interference agents are designed to antagonize the formation of enveloped virions. In one embodiment, the RNA interference agent down-regulates the expression of viral C and/or E1/E2 proteins. In another embodiment, the RNA interference agent is designed to the regions of sequences of these proteins for their interactions. In other embodiments, the RNA interference agents antagonize the
20 expression of viral proteins or host proteins capable of inducing cellular membrane curvature and budding off virus particles.

In some embodiments, the RNA interference agents antagonize the induction of cellular gene expression by Hepatitis C virus. In one embodiment, the RNA interference agents antagonize the expression of gene products that are specific to
25 hepatocyte or have a selective effect on hepatocyte functions. In one preferred embodiment, the gene products include growth factors, for example, FGF-4 (fibroblast growth factor homologous factor 4) and neuroleukin (a neurotropic growth factor). In one embodiment, the gene products include cell-cycle-related proteins, for example, Cyclin D1, CDK4, CCNG2, BZAP45, etc. In another embodiment, the gene products
30 include cell proliferation-related proteins, for example, SSR1, ANXA2, S100A10, PTMA, proteins in Mitogen-Activated Protein Kinase (MAPK) and NF- κ B signaling pathways, etc. In one preferred embodiment, the gene products include CXC (or α -chemokines) and CC (or β -chemokines) chemokine ligands. In another embodiment,

the host gene products include non-coding microRNAs (miRNAs) associated with the carcinogenic process of liver cancers resulted from HCV infection (for example, see Murakami Y. *et al.*, "Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 2006; 25: 2537-45;

5 Gramantieri L., *et al.*, "Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* 2007; 67: 6092-9). These miRNAs may include, for example, the let 7 family miRNAs targeting the Ras oncogenes, microRNA 21 (miR-21) targeting the PTEN tumor suppressor gene and miR-122 targeting the cyclin G1 cell-cycle regulator, etc. In other embodiments, the

10 RNA interference agents are designed to recognize and antagonize the expression and/or activity of other gene products resulting from Hepatitis C virus infection. These gene products may have functions in cell damage and repair, replication and spread of Hepatitis C virus, cell activators, etc (for example, see Tan A. *et al.*, Viral hepatocarcinogenesis: from infection to cancer. *Liver International* 2008; 175-188).

15 These gene products include, for example, p53, PTEN, DDB1, Hepatitis B X-interacting protein (HBXIP), Hepatitis B virus X-associated protein (HBXAP), FLICE inhibitory protein (c-FLIP), 14-3-3, Peroxisome proliferators-activated receptor γ (PPAR- γ), RNA polymerase II subunit 5 (RPB5)/transcription factor IIB (TFIIB), TATA-binding protein (TBP), TFIIH, CREB, CREB-binding protein (CBP)/p300, androgen receptor (AR),

20 retinoid X receptor (RXR), activating signal cointegrator 2 (ASC-2), Jun activation domain-binding protein 1 (Jab1), HVDAC3, proteasome subunits, PSMA7/PSMC1, Skp2, Serine/threonine protein phosphatase PP2C- α , lymphotoxin- β receptor (LT- β R), Sp110b (a repressor for retinoic acid receptor α (RAR- α)), heterogeneous nuclear ribonucleoprotein K (hnRNP K), CAP-Rf (a putative RNA helicase), 14-3-3 epsilon

25 protein, PKR (RNA-activated protein kinase), B23/YY1/P300 complex, p21Waf1/Cip1/Sdi1, intermediate microfilament cytokeratin proteins, PAK-2, IL-6, IGFII, TNF- α , IL-1 β , STAT3, p16INK4a, SOCS-1, APC, GSTP1, SFRP1, RUNX3, RASSF1, p73, IGFBP3, E-Cad, DNA methyltransferase genes (DNMTs), Dicer and other components in the host RNA interference machinery, Wnt1, β -catenin,

30 components in the Wnt/ β -catenin signaling pathway, components in the TGF β signaling pathway, products of oncogenes like the c-myc, c-jun/fos or Ras signaling pathway, components in the Rb signaling pathway, RNA polymerases II and III, cathepsin S, oligoadenylate synthetase (OAS), GARG49/IRG2, lymphocyte antigen-6A (Ly-6A),

macrophage activation gene-2 (Mpa2), early growth response gene1 (Egr1), pyrimidine 5'-nucleotidase (P5N), apolipoprotein D (ApoD), interferon regulatory factor 7 (IRF7), interferon-inducible protein 1 (LRG-47/Ifi 1), LRG21, interferon-inducible GTPase (IIGP1), interferon- γ -induced GTPase (IGTP), T-cell-specific GTPase (TGTP/Mg21),
 5 interferon-activated gene 202B (Ifi202b), chemokine (C-X-C) motif ligand 10 (Cxc1 10), histocompatibility 2, D region locus 1 (H2-D1), granzyme B, lysophospholipase (Lyp1a1), cyclophilin c-associated protein (CyC-AP), Fc fragment of IgG, low-affinity IIIa (Fcgr3a), glucocorticoid attenuated response gene 16 (GARG16), glucocorticoid attenuated response gene 39 (GARG39), viral hemorrhagic septicemia virus (VHSV)-
 10 induced gene 1, tumor protein, translationally controlled (Tpt1), solute carrier family 25, member 3 (Slc25a3), Olfactomedin 1 (Olfm1), Internexin neuronal intermediate filament protein (Ina/NF-66), Dystonin (Dst/Bpag1), *Mus musculus* double minute (Mdm2), Guanylate-binding protein 5 (Gbp5), GAPDH, EBI-1 ligand chemokine, mouse MHC class I H2-D-b, NADH-ubiquinone oxidoreductase, genes encoding
 15 mitochondrial tRNA, postsynaptic density protein (PSD-95), ribosomal protein L26, virus-like retro-transposon 30 (VL-30), replication-dependent histone H2A.1, eyes absent homolog 2, early growth response protein 3, major prion protein precursor, programmed cell death 2, Trombomodulin, vesicle-associated membrane protein 1 (VAMP1), N-cadherin, DNA-binding protein SMBP2, Semaphorin G, heat shock
 20 protein 90 (HSP-90), glial cells missing gene homolog, insulin-like growth factor I receptor, Sp4 zinc finger transcription factor, transcription termination factor 1, Laminin receptor 1, Bcl-xL, GM-CSF receptor, interleukin-5 receptor alpha subunit, Frizzled homolog 6, guanine nucleotide binding protein, Calmodulin, CDC10, replacement histone H3.3 gene, GTP binding protein Gz, Metallothionein II, L1 retrotransposon and
 25 Signal Transducers and Activators of Transcription protein 1 (STAT1), etc.

The ability to escape from cellular antiviral responses, in particular the type I interferon (IFN) system, is crucial for all viruses. This invention includes methods to design RNA interference agents to antagonize Hepatitis C virus as an IFN antagonist.

The present invention also includes methods to design RNA interference agents
 30 to antagonize the anti-apoptotic or pro-apoptotic activity of Hepatitis C virus. In one embodiment, the RNA interference agents antagonize the expression of Fas, FasL or other components in the Fas-FasL signaling pathway induced by Hepatitis C virus infection. In one preferred embodiment, the RNA interference agents antagonize the

expression and/or activity of cellular anti-apoptotic molecules, which include, for example, FADD, procaspase-8, FLIP, BH3-only proteins (such as Bid, tBid, etc.), Bax, Bak, Bcl-2 family proteins, Bcl-xL, cytochrome c, Apaf-1, caspase-9, Smac/DIABLO, inhibitors of apoptotic proteins (IAPs) proteins, procaspase-3, ICAD, etc.

5 HCV may circulate in various forms in the sera of infected hosts, for example, as (1) free mature virions, (2) virions bound to low-density lipoproteins and very low density lipoproteins, (3) virions bound to immunoglobulins, and (4) nonenveloped nucleocapsids, which exhibit physicochemical and antigenic properties. The present invention includes methods to design RNA interference agents to antagonize the
10 transport and spread process of Hepatitis C virus *in vivo*. In one preferred embodiment, the RNA interference agents down-regulated the expression of the host proteins facilitating the circulation and spread of HCV. These host proteins include, but are not limited to, low-density lipoproteins, very low density lipoproteins, immunoglobulins, etc. In some embodiments, the RNA interference agents antagonize viral proteins to
15 disrupt the balance of replication and transcription of Hepatitis C virus. In another embodiment, the RNA interference agents antagonize the cell mobility stimulated by viral infection, which helps to spread Hepatitis C virus.

A. Anti-HCV RNA Silencing Agents

20 In certain embodiments, the invention relates RNA silencing agent (e.g., an siRNA or other dsRNA agents) which is complementary to at least a portion of a 5'-untranslated region (5'-UTR) of HCV. In a preferred embodiment, the nucleotide sequence is within a highly conserved region of the 5'-UTR. In some embodiments of the invention, an RNA silencing agent is complementary to at least a portion of a 3'-
25 untranslated region (3'-UTR) of HCV. In a preferred embodiment, the nucleotide sequence is within a highly conserved region of the 3'-UTR. In a particular embodiment, the complementary RNA strand comprises the nucleotide sequence of ggaccuuucacagcuagccg uga and the sense RNA strand comprises the nucleotide sequence of acggcuagcugugaaagguccgu. Additional exemplary anti-HCV RNA
30 silencing agents are described in US Patent No. 7,348,314, filed March 7, 2003, International PCT Publication No. WO 03/070750, filed February 20, 2003; International PCT Publication No. WO 05/028650, filed September 15, 2004; International PCT Publication No. WO 05/107816, filed November 17, 2005;

International PCT Publication No. WO 06/031901, filed September 12, 2005; International PCT Publication No. WO 06/039656, filed September 30, 2005; and International PCT Publication No. WO 07/076328, filed December 18, 2006; each of which is incorporated by reference herein in its entirety.

5

B. Anti-HCV Antisense Oligonucleotides

In certain embodiments, the antisense oligonucleotide targets miR-122 such that hepatitis C virus (HCV) infection is inhibited. MicroRNA 122 (miR-122) is specifically expressed in liver cells and has been identified as an endogenous host gene or host factor required for viral infection by HCV. miR-122 interacts directly with a specific sequence in the 5' non-coding sequence region of the HCV genome leading to increased abundance of autonomously replicating Hepatitis C viral RNAs (see Jopling et al., *Science*, (2005), 309(5740):1577-81). Thus, in certain embodiments, the antisense oligonucleotide (e.g., an "AntagomiR") is sufficiently complementary to miR-122 to inhibit the viral replication function of miR-122. In one exemplary embodiment, the antisense oligonucleotide comprises the sequence ACAAACACCAUUGUCACACUCCA. Additional exemplary anti-HCV antisense oligonucleotides are described in US Patent No. 7,307,067, filed May 3, 2005, which is incorporated herein by reference in its entirety.

20

V. Binding Proteins

As noted above, certain embodiments of the invention feature a targeting moiety that can deliver a therapeutic payload to a virus (e.g., an HCV or HCV-like virus) or a cell (e.g., a virally infected cell or a cell that has the capacity to be infected by a virus). Particular embodiments feature binding proteins, including antibodies, antibody fragments, molecules with regions, domains or portions derived from antibodies, and non-antibody binding proteins, that can function as a targeting moiety.

25

A. Antibody Fragments Antibody Mimetics, and Binding Polypeptides

30

The instant invention is not limited to traditional antibodies and may be practiced through the use of binding proteins or polypeptides, antibody fragments and antibody mimetics. As detailed below, a wide variety of binding polypeptide, antibody fragment and antibody mimetic technologies have now been developed and are widely

known in the art. While a number of these technologies, such as domain antibodies, Nanobodies, and UniBodies make use of fragments of, or other modifications to, traditional antibody structures, there are also alternative technologies, such as Affibodies, DARPins, Anticalins, Avimers, and Versabodies that employ binding
5 structures that, while they mimic traditional antibody binding, are generated from and function via distinct mechanisms.

Domain Antibodies (dAbs) are the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies. Domain Antibodies have a molecular weight of
10 approximately 13 kDa. Domantis has developed a series of large and highly functional libraries of fully human VH and VL dAbs (more than ten billion different sequences in each library), and uses these libraries to select dAbs that are specific to therapeutic targets. In contrast to many conventional antibodies, Domain Antibodies are well expressed in bacterial, yeast, and mammalian cell systems. Further details of domain
15 antibodies and methods of production thereof may be obtained by reference to US Patent 6,291,158; 6,582,915; 6,593,081; 6,172,197; 6,696,245; US Serial No. 2004/0110941; European patent application No. 1433846 and European Patents 0368684 & 0616640; WO05/035572, WO04/101790, WO04/081026, WO04/058821, WO04/003019 and WO03/002609, each of which is herein incorporated by reference in
20 its entirety.

Nanobodies are antibody-derived therapeutic proteins that contain the unique structural and functional properties of naturally-occurring heavy-chain antibodies. These heavy-chain antibodies contain a single variable domain (VHH) and two constant domains (CH2 and CH3). Importantly, the cloned and isolated VHH domain is a
25 perfectly stable polypeptide harbouring the full antigen-binding capacity of the original heavy-chain antibody. Nanobodies have a high homology with the VH domains of human antibodies and can be further humanized without any loss of activity. Importantly, Nanobodies have a low immunogenic potential, which has been confirmed in primate studies with Nanobody lead compounds.

30 Nanobodies combine the advantages of conventional antibodies with important features of small molecule drugs. Like conventional antibodies, Nanobodies show high target specificity, high affinity for their target and low inherent toxicity. However, like small molecule drugs they can inhibit enzymes and readily access receptor clefts.

Furthermore, Nanobodies are extremely stable, can be administered by means other than injection (see e.g. WO 04/041867, which is herein incorporated by reference in its entirety) and are easy to manufacture. Other advantages of Nanobodies include recognizing uncommon or hidden epitopes as a result of their small size, binding into
5 cavities or active sites of protein targets with high affinity and selectivity due to their unique 3-dimensional, drug format flexibility, tailoring of half-life and ease and speed of drug discovery.

Nanobodies are encoded by single genes and are efficiently produced in almost all prokaryotic and eukaryotic hosts e.g. *E. coli* (see e.g. US 6,765,087, which is herein
10 incorporated by reference in its entirety), molds (for example *Aspergillus* or *Trichoderma*) and yeast (for example *Saccharomyces*, *Kluyveromyces*, *Hansenula* or *Pichia*) (see e.g. US 6,838,254, which is herein incorporated by reference in its entirety). The production process is scalable and multi-kilogram quantities of Nanobodies have been produced. Because Nanobodies exhibit a superior stability compared with
15 conventional antibodies, they can be formulated as a long shelf-life, ready-to-use solution.

The Nanoclone method (see e.g. WO 06/079372, which is herein incorporated by reference in its entirety) is a proprietary method for generating Nanobodies against a desired target, based on automated high-throughout selection of B-cells and could be
20 used in the context of the instant invention.

UniBodies are another antibody fragment technology, however this one is based upon the removal of the hinge region of IgG4 antibodies. The deletion of the hinge region results in a molecule that is essentially half the size of traditional IgG4 antibodies and has a univalent binding region rather than the bivalent binding region of IgG4
25 antibodies. It is also well known that IgG4 antibodies are inert and thus do not interact with the immune system, which may be advantageous for the treatment of diseases where an immune response is not desired, and this advantage is passed onto UniBodies. For example, UniBodies may function to inhibit or silence, but not kill, the cells to which they are bound. Additionally, UniBody binding to cancer cells do not stimulate
30 them to proliferate. Furthermore, because UniBodies are about half the size of traditional IgG4 antibodies, they may show better distribution over larger solid tumors with potentially advantageous efficacy. UniBodies are cleared from the body at a similar rate to whole IgG4 antibodies and are able to bind with a similar affinity for their

antigens as whole antibodies. Further details of UniBodies may be obtained by reference to patent WO2007/059782, which is herein incorporated by reference in its entirety.

Diabodies are bivalent, bispecific molecules in which V_H and V_L domains are expressed on a single polypeptide chain, connected by a linker that is too short to allow for pairing between the two domains on the same chain. The V_H and V_L domains pair with complementary domains of another chain, thereby creating two antigen binding sites (see e.g., Holliger *et al.*, 1993 Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak *et al.*, 1994 Structure 2:1121-1123). Diabodies can be produced by expressing two polypeptide chains with either the structure $V_{HA}-V_{LB}$ and $V_{HB}-V_{LA}$ (V_H-V_L configuration), or $V_{LA}-V_{HB}$ and $V_{LB}-V_{HA}$ (V_L-V_H configuration) within the same cell. Most of them can be expressed in soluble form in bacteria.

Single chain diabodies (scDb) are produced by connecting the two diabody-forming polypeptide chains with linker of approximately 15 amino acid residues (see Holliger and Winter, 1997 Cancer Immunol. Immunother., 45(3-4):128-30; Wu *et al.*, 1996 Immunotechnology, 2(1):21-36). scDb can be expressed in bacteria in soluble, active monomeric forms (see Holliger and Winter, 1997 Cancer Immunol. Immunother., 45(34): 128-30; Wu *et al.*, 1996 Immunotechnology, 2(1):21-36; Pluckthun and Pack, 1997 Immunotechnology, 3(2): 83-105; Ridgway *et al.*, 1996 Protein Eng., 9(7):617-21).

A diabody can be fused to Fc to generate a "di-diabody" (see Lu *et al.*, 2004 J. Biol. Chem., 279(4):2856-65).

The invention further provides binding molecules that exhibit functional properties of antibodies but derive their framework and antigen binding portions from other polypeptides (e.g., polypeptides other than those encoded by antibody genes or generated by the recombination of antibody genes *in vivo*). The antigen binding domains (e.g., anti-HCV epitope binding domains) of these binding molecules are generated through a directed evolution process. See U.S. Pat. No. 7,115,396, hereby incorporated in its entirety by reference. Molecules that have an overall fold similar to that of a variable domain of an antibody (an "immunoglobulin-like" fold) are appropriate scaffold proteins. Scaffold proteins suitable for deriving antigen binding molecules include fibronectin or a fibronectin dimer, tenascin, N-cadherin, E-cadherin, ICAM, titin, GCSF-receptor, cytokine receptor, glycosidase inhibitor, antibiotic

chromoprotein, myelin membrane adhesion molecule P0, CD8, CD4, CD2, class I MHC, T-cell antigen receptor, CD1, C2 and I-set domains of VCAM-1, I-set immunoglobulin domain of myosin-binding protein C, I-set immunoglobulin domain of myosin-binding protein H, I-set immunoglobulin domain of telokin, NCAM, twitchin, neuroglian, growth hormone receptor, erythropoietin receptor, prolactin receptor, interferon-gamma receptor, β -galactosidase/glucuronidase, β -glucuronidase, transglutaminase, T-cell antigen receptor, superoxide dismutase, tissue factor domain, cytochrome F, green fluorescent protein, GroEL, and thaumatin.

The antigen binding domain (*e.g.*, the immunoglobulin-like fold) of the non-antibody binding molecule can have a molecular mass less than 10 kD or greater than 7.5 kD (*e.g.*, a molecular mass between 7.5-10 kD). The protein used to derive the antigen binding domain is a naturally occurring mammalian protein (*e.g.*, a human protein), and the antigen binding domain includes up to 50% (*e.g.*, up to 34%, 25%, 20%, or 15%), mutated amino acids as compared to the immunoglobulin-like fold of the protein from which it is derived. The domain having the immunoglobulin-like fold generally consists of 50-150 amino acids (*e.g.*, 40-60 amino acids).

To generate non-antibody binding molecules, a library of clones is created in which sequences in regions of the scaffold protein that form antigen binding surfaces (*e.g.*, regions analogous in position and structure to CDRs of an antibody variable domain immunoglobulin fold) are randomized. Library clones are tested for specific binding to the antigen of interest (*e.g.*, an epitope or antigen from HCV) and for other functions (*e.g.*, inhibition of biological activity of HCV). Selected clones can be used as the basis for further randomization and selection to produce derivatives of higher affinity for the antigen.

High affinity binding molecules are generated, for example, using the tenth module of fibronectin III ($^{10}\text{Fn3}$) as the scaffold. A library is constructed for each of three CDR-like loops of $^{10}\text{Fn3}$ at residues 23-29, 52-55, and 78-87. To construct each library, DNA segments encoding sequence overlapping each CDR-like region are randomized by oligonucleotide synthesis. Techniques for producing selectable $^{10}\text{Fn3}$ libraries are described in U.S. Pat. Nos. 6,818,418 and 7,115,396; Roberts and Szostak, 1997 Proc. Natl. Acad. Sci USA 94:12297; U.S. Pat. No. 6,261,804; U.S. Pat. No. 6,258,558; and Szostak et al. WO98/31700.

Non-antibody binding molecules can be produced as dimers or multimers to increase avidity for the target antigen. For example, the antigen binding domain is expressed as a fusion with a constant region (Fc) of an antibody that forms Fc-Fc dimers. See, *e.g.*, U.S. Pat. No. 7,115,396.

5 Affibody molecules represent a new class of affinity proteins based on a 58-amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. This three helix bundle domain has been used as a scaffold for the construction of combinatorial phagemid libraries, from which Affibody variants that target the desired molecules can be selected using phage display technology (Nord
10 K, Gunneriusson E, Ringdahl J, Stahl S, Uhlen M, Nygren PA, Binding proteins selected from combinatorial libraries of an α -helical bacterial receptor domain, Nat Biotechnol 1997;15:772-7. Ronmark J, Gronlund H, Uhlen M, Nygren PA, Human immunoglobulin A (IgA)-specific ligands from combinatorial engineering of protein A, Eur J Biochem 2002;269:2647-55.). The simple, robust structure of Affibody molecules
15 in combination with their low molecular weight (6 kDa), make them suitable for a wide variety of applications, for instance, as detection reagents (Ronmark J, Hansson M, Nguyen T, et al, Construction and characterization of affibody-Fc chimeras produced in Escherichia coli, J Immunol Methods 2002;261:199-211) and to inhibit receptor interactions (Sandstrom K, Xu Z, Forsberg G, Nygren PA, Inhibition of the CD28-CD80
20 co-stimulation signal by a CD28-binding Affibody ligand developed by combinatorial protein engineering, Protein Eng 2003;16:691-7). Further details of Affibodies and methods of production thereof may be obtained by reference to US Patent No 5831012 which is herein incorporated by reference in its entirety. Labelled Affibodies may also be useful in imaging applications for determining abundance of isoforms.

25 DARPins (Designed Ankyrin Repeat Proteins) are one example of an antibody mimetic DRP (Designed Repeat Protein) technology that has been developed to exploit the binding abilities of non-antibody polypeptides. Repeat proteins such as ankyrin or leucine-rich repeat proteins, are ubiquitous binding molecules, which occur, unlike antibodies, intra- and extracellularly. Their unique modular architecture features
30 repeating structural units (repeats), which stack together to form elongated repeat domains displaying variable and modular target-binding surfaces. Based on this modularity, combinatorial libraries of polypeptides with highly diversified binding specificities can be generated. This strategy includes the consensus design of self-

compatible repeats displaying variable surface residues and their random assembly into repeat domains.

DARPin can be produced in bacterial expression systems at very high yields and they belong to the most stable proteins known. Highly specific, high-affinity
5 DARPins to a broad range of target proteins, including human receptors, cytokines, kinases, human proteases, viruses and membrane proteins, have been selected. DARPins having affinities in the single-digit nanomolar to picomolar range can be obtained.

DARPins have been used in a wide range of applications, including ELISA,
10 sandwich ELISA, flow cytometric analysis (FACS), immunohistochemistry (IHC), chip applications, affinity purification or Western blotting. DARPins also proved to be highly active in the intracellular compartment for example as intracellular marker proteins fused to green fluorescent protein (GFP). DARPins were further used to inhibit viral entry with IC₅₀ in the pM range. DARPins are not only ideal to block protein-
15 protein interactions, but also to inhibit enzymes. Proteases, kinases and transporters have been successfully inhibited, most often an allosteric inhibition mode. Very fast and specific enrichments on the tumor and very favorable tumor to blood ratios make DARPins well suited for *in vivo* diagnostics or therapeutic approaches.

Additional information regarding DARPins and other DRP technologies can be
20 found in US Patent Application Publication No. 2004/0132028 and International Patent Application Publication No. WO 02/20565, both of which are hereby incorporated by reference in their entirety.

Anticalins are an additional antibody mimetic technology, however in this case the binding specificity is derived from lipocalins, a family of low molecular weight
25 proteins that are naturally and abundantly expressed in human tissues and body fluids. Lipocalins have evolved to perform a range of functions *in vivo* associated with the physiological transport and storage of chemically sensitive or insoluble compounds. Lipocalins have a robust intrinsic structure comprising a highly conserved β -barrel which supports four loops at one terminus of the protein. These loops form the entrance
30 to a binding pocket and conformational differences in this part of the molecule account for the variation in binding specificity between individual lipocalins.

While the overall structure of hypervariable loops supported by a conserved β -sheet framework is reminiscent of immunoglobulins, lipocalins differ considerably from

antibodies in terms of size, being composed of a single polypeptide chain of 160-180 amino acids which is marginally larger than a single immunoglobulin domain.

Lipocalins are cloned and their loops are subjected to engineering in order to create Anticalins. Libraries of structurally diverse Anticalins have been generated and
5 Anticalin display allows the selection and screening of binding function, followed by the expression and production of soluble protein for further analysis in prokaryotic or eukaryotic systems. Studies have successfully demonstrated that Anticalins can be developed that are specific for virtually any human target protein can be isolated and binding affinities in the nanomolar or higher range can be obtained.

10 Anticalins can also be formatted as dual targeting proteins, so-called Duocalins. A Duocalin binds two separate therapeutic targets in one easily produced monomeric protein using standard manufacturing processes while retaining target specificity and affinity regardless of the structural orientation of its two binding domains.

Modulation of multiple targets through a single molecule is particularly
15 advantageous in diseases known to involve more than a single causative factor. Moreover, bi- or multivalent binding formats such as Duocalins have significant potential in targeting cell surface molecules in disease, mediating agonistic effects on signal transduction pathways or inducing enhanced internalization effects via binding and clustering of cell surface receptors. Furthermore, the high intrinsic stability of
20 Duocalins is comparable to monomeric Anticalins, offering flexible formulation and delivery potential for Duocalins.

Additional information regarding Anticalins can be found in US Patent No. 7,250,297 and International Patent Application Publication No. WO 99/16873, both of which are hereby incorporated by reference in their entirety.

25 Another antibody mimetic technology useful in the context of the instant invention are Avimers. Avimers are evolved from a large family of human extracellular receptor domains by *in vitro* exon shuffling and phage display, generating multidomain proteins with binding and inhibitory properties. Linking multiple independent binding domains has been shown to create avidity and results in improved affinity and
30 specificity compared with conventional single-epitope binding proteins. Other potential advantages include simple and efficient production of multitarget-specific molecules in *Escherichia coli*, improved thermostability and resistance to proteases. Avimers with sub-nanomolar affinities have been obtained against a variety of targets. Additional

information regarding Avimers can be found in US Patent Application Publication Nos. 2006/0286603, 2006/0234299, 2006/0223114, 2006/0177831, 2006/0008844, 2005/0221384, 2005/0164301, 2005/0089932, 2005/0053973, 2005/0048512, 2004/0175756, all of which are hereby incorporated by reference in their entirety.

5 Versabodies are another antibody mimetic technology that could be used in the context of the instant invention. Versabodies are small proteins of 3-5 kDa with >15% cysteines, which form a high disulfide density scaffold, replacing the hydrophobic core that typical proteins have. The replacement of a large number of hydrophobic amino acids, comprising the hydrophobic core, with a small number of disulfides results in a
10 protein that is smaller, more hydrophilic (less aggregation and non-specific binding), more resistant to proteases and heat, and has a lower density of T-cell epitopes, because the residues that contribute most to MHC presentation are hydrophobic. All four of these properties are well-known to affect immunogenicity, and together they are expected to cause a large decrease in immunogenicity.

15 The inspiration for Versabodies comes from the natural injectable biopharmaceuticals produced by leeches, snakes, spiders, scorpions, snails, and anemones, which are known to exhibit unexpectedly low immunogenicity. Starting with selected natural protein families, by design and by screening the size, hydrophobicity, proteolytic antigen processing, and epitope density are minimized to levels far below the
20 average for natural injectable proteins.

 Given the structure of Versabodies, these antibody mimetics offer a versatile format that includes multi-valency, multi-specificity, a diversity of half-life mechanisms, tissue targeting modules and the absence of the antibody Fc region. Furthermore, Versabodies are manufactured in E. coli at high yields, and because of
25 their hydrophilicity and small size, Versabodies are highly soluble and can be formulated to high concentrations. Versabodies are exceptionally heat stable (they can be boiled) and offer extended shelf-life.

 Additional information regarding Versabodies can be found in US Patent Application Publication No. 2007/0191272 which is hereby incorporated by reference in
30 its entirety.

 The detailed description of antibody fragment and antibody mimetic technologies provided above is not intended to be a comprehensive list of all technologies that could be used in the context of the instant specification. For example,

and also not by way of limitation, a variety of additional technologies including alternative polypeptide-based technologies, such as fusions of complimentary determining regions as outlined in Qui et al., *Nature Biotechnology*, 25(8) 921-929 (2007), which is hereby incorporated by reference in its entirety, as well as nucleic acid-based technologies, such as the RNA aptamer technologies described in US Patent Nos. 5,789,157, 5,864,026, 5,712,375, 5,763,566, 6,013,443, 6,376,474, 6,613,526, 6,114,120, 6,261,774, and 6,387,620, all of which are hereby incorporated by reference, could be used in the context of the instant invention.

B. Antibodies

Embodiments of the invention include conjugates formed from monoclonal and polyclonal antibodies raised to antigens and epitopes of HCV. A variety of techniques known in the art may be used to generate antibodies for use with embodiments of the invention.

i. Generation of Human Monoclonal Antibodies

Monoclonal antibodies can be produced in a manner not possible with polyclonal antibodies. Polyclonal antisera vary from animal to animal, whereas monoclonal preparations exhibit a uniform antigenic specificity. Murine animal systems are useful to generate monoclonal antibodies, and immunization protocols, techniques for isolating and fusing splenocytes, and methods and reagents for producing hybridomas are well known. Monoclonal antibodies can be produced by a variety of techniques, including conventional monoclonal antibody methodology, *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, *Nature*, 256: 495, 1975. See generally, Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.

Although these standard techniques are known, it is desirable to use humanized or human antibodies rather than murine antibodies to treat human subjects, because humans mount an immune response to antibodies from mice and other species. The immune response to murine antibodies is called a human anti-mouse antibody or HAMA response (Schroff, R. *et al.*, *Cancer Res.*, 45, 879-885, 1985) and is a condition that causes serum sickness in humans and results in rapid clearance of the murine antibodies from an individual's circulation. The immune response in humans has been

shown to be against both the variable and the constant regions of murine immunoglobulins. Human monoclonal antibodies are safer for administration to humans than antibodies derived from other animals and human polyclonal antibodies. One useful type of animal in which to generate human monoclonal antibodies is a transgenic mouse that expresses human immunoglobulin genes rather than its own mouse immunoglobulin genes. Such transgenic mice, *e.g.*, "HuMAb™" mice, contain human immunoglobulin gene miniloci that encode unrearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (see *e.g.*, Lonberg, N. *et al.*, *Nature* 368(6474): 856-859, 1994, and U.S. Pat. 5,770,429). Accordingly, the mice exhibit reduced expression of mouse IgM or κ , and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgG κ monoclonal antibodies (Lonberg, N. *et al.*, *supra*; reviewed in Lonberg, N. *Handbook of Experimental Pharmacology* 113:49-101, 1994; Lonberg, N. and Huszar, D., *Intern. Rev. Immunol.*, 13: 65-93, 1995, and Harding, F. and Lonberg, N., *Ann. N.Y. Acad. Sci.*, 764:536-546, 1995).

The preparation of such transgenic mice is described in further detail in Taylor, L. *et al.*, *Nucleic Acids Research*, 20:6287-6295, 1992; Chen, J. *et al.*, *International Immunology* 5: 647-656, 1993; Tuaillon *et al.*, *Proc. Natl. Acad. Sci., USA* 90:3720-3724, 1993; Choi *et al.*, *Nature Genetics*, 4:117-123, 1993; Chen, J. *et al.*, *EMBO J.*, 12: 821-830, 1993; Tuaillon *et al.*, *J. Immunol.*, 152:2912-2920, 1994; Taylor, L. *et al.*, *International Immunology*, 6: 579-591, 1994; and Fishwild, D. *et al.*, *Nature Biotechnology*, 14: 845-851, 1996. See further, U.S. Pat. 5,545,806; U.S. Pat. 5,569,825, U.S. Pat. 5,625,126, U.S. Pat. 5,633,425, U.S. Pat. 5,661,016, U.S. Pat. 5,770,429, U.S. Pat. 5,789,650, U.S. Pat. 5,814,318, U.S. Pat. 5,874,299 and U.S. Pat. 5,877,397, all by Lonberg and Kay, and PCT Publication Nos. WO 01/14424, WO 98/24884, WO 94/25585, WO 93/1227, and WO 92/03918.

To generate fully human monoclonal antibodies to an antigen, HuMAb mice can be immunized with an immunogen, as described by Lonberg, N. *et al.* *Nature*, 368(6474): 856-859, 1994; Fishwild, D. *et al.*, *Nature Biotechnology*, 14: 845-851, 1996 and WO 98/24884. Preferably, the mice are 6-16 weeks of age upon the first immunization. For example, a purified preparation of inactivated HCV can be used to immunize the HuMAb mice intraperitoneally. To generate antibodies against HCV

proteins, lipids, and/or carbohydrate molecules, mice can be immunized with live, killed or nonviable inactivated and/or lyophilized HCV. In another embodiment, HCV envelope glycoproteins E1 and/or E2, or one or more fragments thereof, can be used as an immunogen.

5 HuMAb transgenic mice respond best when initially immunized intraperitoneally (IP) with antigen in complete Freund's adjuvant, followed by IP immunizations every other week (up to a total of 6) with antigen in incomplete Freund's adjuvant. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be
10 screened, for example by ELISA or flow cytometry, and mice with sufficient titers of anti-HCV human immunoglobulin can be used for fusions. Mice can be boosted intravenously with antigen 3 days before sacrifice and removal of the spleen. It is expected that multiple fusions for each antigen may need to be performed. Several mice are typically immunized for each antigen.

15 The mouse splenocytes can be isolated and fused with PEG to a mouse myeloma cell line based upon standard protocols. The resulting hybridomas are then screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from immunized mice are fused to one-sixth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (ATCC, CRL 1580) with 50%
20 PEG. Cells are plated at approximately 2×10^5 in flat bottom microtiter plate, followed by a two week incubation in selective medium containing 20% fetal Clone Serum, 18% "653" conditioned media, 5% origen (IGEN), 4 mM L-glutamine, 1 mM L-glutamine, 1 mM sodium pyruvate, 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml gentamycin and 1x HAT (Sigma; the HAT
25 is added 24 hours after the fusion). After two weeks, cells are cultured in medium in which the HAT is replaced with HT. Supernatants from individual wells are then screened by ELISA for human anti-HCV monoclonal IgM and IgG antibodies. The antibody secreting hybridomas are replated, screened again, and if still positive for human IgG, anti-HCV monoclonal antibodies, can be subcloned at least twice by
30 limiting dilution. The stable subclones are then cultured *in vitro* to generate small amounts of antibody in tissue culture medium for characterization.

In one embodiment, the transgenic animal used to generate human antibodies to HCV contains at least one, typically 2-10, and sometimes 25-50 or more copies of the

transgene described in Example 12 of WO 98/24884 (*e.g.*, pHCl or pHCl2) bred with an animal containing a single copy of a light chain transgene described in Examples 5, 6, 8, or 14 of WO 98/24884, and the offspring bred with the J_H deleted animal described in Example 10 of WO 98/24884, the contents of which are hereby expressly incorporated
5 by reference. Animals are bred to homozygosity for each of these three traits. Such animals have the following genotype: a single copy (per haploid set of chromosomes) of a human heavy chain unrearranged mini-locus (described in Example 12 of WO 98/24884), a single copy (per haploid set of chromosomes) of a rearranged human K light chain construct (described in Example 14 of WO 98/24884), and a deletion at each
10 endogenous mouse heavy chain locus that removes all of the functional J_H segments (described in Example 10 of WO 98/24884). Such animals are bred with mice that are homozygous for the deletion of the J_H segments (Examples 10 of WO 98/24884) to produce offspring that are homozygous for the J_H deletion and hemizygous for the human heavy and light chain constructs. The resultant animals are injected with
15 antigens and used for production of human monoclonal antibodies against these antigens.

The B cells isolated from such an animal are monospecific with regard to the human heavy and light chains because they contain only a single copy of each gene. Furthermore, they will be monospecific with regard to human or mouse heavy chains
20 because both endogenous mouse heavy chain gene copies are nonfunctional by virtue of the deletion spanning the J_H region introduced as described in Examples 9 and 12 of WO 98/24884. Furthermore, a substantial fraction of the B cells will be monospecific with regards to the human or mouse light chains, because expression of the single copy of the rearranged human kappa light chain gene will allelically and isotypically exclude
25 the rearrangement of the endogenous mouse kappa and lambda chain genes in a significant fraction of B-cells.

In one embodiment, the transgenic mouse will exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a native mouse. Thus, for example, in embodiments where the endogenous Ig genes have been
30 inactivated, the total immunoglobulin levels will range from about 0.1 to 10 mg/ml of serum, *e.g.*, 0.5 to 5 mg/ml, or at least about 1.0 mg/ml. When a transgene capable of affecting a switch to IgG from IgM has been introduced into the transgenic mouse, the adult mouse ratio of serum IgG to IgM is preferably about 10:1. The IgG to IgM ratio

will be much lower in the immature mouse. In general, greater than about 10%, *e.g.*, about 40 to 80% of the spleen and lymph node B cells will express exclusively human IgG protein.

The repertoire in the transgenic mouse will ideally approximate that shown in a non-transgenic mouse, usually at least about 10% as high, preferably 25 to 50% or more as high. Generally, at least about a thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending primarily on the number of different V, J, and D regions introduced into the mouse genome. Typically, the immunoglobulins will exhibit an affinity for preselected antigens of at least about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M, 10^{-11} M, 10^{-12} M, 10^{-13} M, 10^{-14} M, or greater, *e.g.*, up to 10^{-15} M or more.

HuMAb mice can produce B cells that undergo class-switching *via* intratransgene switch recombination (cis-switching) and express immunoglobulins reactive with HCV. The immunoglobulins can be human sequence antibodies, wherein the heavy and light chain polypeptides are encoded by human transgene sequences, which may include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences. These human sequence immunoglobulins can be referred to as being substantially identical to a polypeptide sequence encoded by a human VL or VH gene segment and a human JL or JH segment, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. With respect to such human sequence antibodies, the variable regions of each chain are typically at least 80 percent encoded by human germline V, J, and, in the case of heavy chains, D, gene segments. Frequently at least 85 percent of the variable regions are encoded by human germline sequences present on the transgene. Often 90 or 95 percent or more of the variable region sequences are encoded by human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic mutation and VJ and VDJ joining, the human sequence antibodies will frequently have some variable region sequences (and less frequently constant region sequences) that are not encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide positions) will cluster in or near CDRs, or in regions where somatic mutations are known to cluster.

The human sequence antibodies that bind to HCV can result from isotype switching, such that human antibodies comprising a human sequence gamma chain (such as gamma 1, gamma 2, or gamma 3) and a human sequence light chain (such as K) are produced. Such isotype-switched human sequence antibodies often contain one or more somatic mutation(s), typically in the variable region and often in or within about 10 residues of a CDR) as a result of affinity maturation and selection of B cells by antigen, particularly subsequent to secondary (or subsequent) antigen challenge. These high affinity human sequence antibodies have binding affinities of at least about 1×10^{-9} M, typically at least 5×10^{-9} M, frequently more than 1×10^{-10} M, and sometimes 5×10^{-10} M to 1×10^{-11} M or greater.

Anti-HCV antibodies can also be raised in other mammals, including non-transgenic mice, humans, rabbits, and goats.

ii. Anti-HCV Antibodies

Human monoclonal antibodies that specifically bind to HCV include antibodies produced by the clones 95-2, 95-14, 95-38, 83-128 and 073-1 described herein (referred to as, respectively, antibody clones 95-2, 95-14, 95-38, 83-128 and 073-1). Antibodies with variable heavy chain and variable light chain regions that are at least 80% identical to the variable heavy and light chain regions of 95-2, 95-14, 95-38, 83-128 and 073-1 can also bind to HCV. In related embodiments, anti-HCV antibodies include, for example, complementarity determining regions (CDRs) that are at least 80% identical to the CDRs of the variable heavy chains and/or variable light chains of 95-2, 95-14, 95-38, 83-128 and 073-1. The SEQ ID NOs. for the amino acid and nucleotide sequences of the variable light and heavy chains of these antibodies, as well as their CDRs, are given in Table 1 below.

Table 1: Anti-HCV antibody sequences

SEQ NO.	ID	Type	Description of Sequence
1		AA	VH of clone 83-128
2		AA	VL of clone 83-128
3		AA	VH of clone 95-2

SEQ NO.	ID	Type	Description of Sequence
4	AA		VL of clone 95-2
5	AA		VH of clone 073-1
6	AA		VL of clone 073-1
7	AA		CDR1 of VH of clone 83-128
8	AA		CDR2 of VH of clone 83-128
9	AA		CDR3 of VH of clone 83-128
10	AA		CDR1 of VH of clone 95-2
11	AA		CDR2 of VH of clone 95-2
12	AA		CDR3 of VH of clone 95-2
13	AA		CDR1 of VH of clone 073-1
14	AA		CDR2 of VH of clone 073-1
15	AA		CDR3 of VH of clone 073-1
16	AA		CDR1 of VL of clone 83-128
17	AA		CDR2 of VL of clone 83-128
18	AA		CDR3 of VL of clone 83-128
19	AA		CDR1 of VL of clone 95-2
20	AA		CDR2 of VL of clone 95-2
21	AA		CDR3 of VL of clone 95-2
22	AA		CDR1 of VL of clone 073-1
23	AA		CDR2 of VL of clone 073-1
24	AA		CDR3 of VL of clone 073-1
25	NA		VH of clone 83-128
26	NA		VL of clone 83-128
27	NA		VH of clone 95-2
28	NA		VL of clone 95-2
29	NA		VH of clone 073-1

SEQ NO.	ID	Type	Description of Sequence
30	NA		VL of clone 073-1
31	NA		E1/E2 1a H77
32	AA		VH of clone 95-14
33	AA		VH of clone 95-38
34	AA		VH of clone 95-25
35	AA		VH of clone 95-42
36	AA		VH of clone 95-43
37	AA		VH of clone 95-49
38	AA		VH of clone 95-54
39	AA		VH of clone 95-58
40	AA		VH of clone 95-62
41	AA		CDR1 of VH of clone 95-14
42	AA		CDR2 of VH of clone 95-14
43	AA		CDR3 of VH of clone 95-14
44	AA		VL of clone 95-14
45	AA		CDR1 of VL of clone 95-14
46	AA		CDR2 of VL of clone 95-14
47	AA		CDR3 of VL of clone 95-14
48	NA		VH of clone 95-14
49	NA		VL of clone 95-14
50	AA		CDR1 of VH of clone 95-38
51	AA		CDR2 of VH of clone 95-38
52	AA		CDR3 of VH of clone 95-38
53	AA		VL of clone 95-38
54	AA		CDR1 of VL of clone 95-38
55	AA		CDR2 of VL of clone 95-38

SEQ NO.	ID	Type	Description of Sequence
56	AA		CDR3 of VL of clone 95-38
57	NA		VH of clone 95-38
58	NA		VL of clone 95-38
59	AA		E2 412-423 (1a) QLINTNGSWHIN
60	AA		(1b) QLVNTNGSWHIN
61	AA		QLVNSNGSWHIN
62	AA		QLINSNGSWHIN
63	AA		HLINTNGSWHIN
64	AA		QLIKTNGSWHIN
65	AA		QLVNTNGSWHVN
66	AA		QFVNTNGSWHIN
67	AA		QLIKNGSSWHIN
68	AA		QLVKTNGSWHIN
69	AA		HLVNTNGSWHIN
70	AA		HLVNSNGSWHIN
71	AA		QLIHTNGSWHIN
72	AA		QLVKTEGNWHIN
73	AA		NLIKTNNGSWHIN
74	AA		QLIYTNGSWHIN
75	AA		QLINTNGSWHLN
76	AA		YLINTNGSWHIN
77	AA		SLINTNGSWHIN
78	AA		NLINTNGSWHIN
79	AA		HLVNSNGSWHIN genotype 2b
80	AA		QLVNSSGSWHIN genotype 3a
81	AA		QLINSNGSWHIN genotype 4a

SEQ NO.	ID	Type	Description of Sequence
82	AA		QLIQNGSSWHIN genotype 5
83	AA		QFVNTNGSWHIN genotype 5a
84	AA		QLIKNGSSWHIN genotype 6a
85	AA		QLIKTNGSWHIN genotype 6g
86	AA		QLINSNGSWHVN genotype 6k

87	AA		VH CDR1 consensus sequence
88	AA		VH CDR2 consensus sequence
89	AA		VH CDR3 consensus sequence
90	AA		VL CDR1 consensus sequence
91	AA		VL CDR2 consensus sequence
92	AA		VL CDR3 consensus sequence

CDRs are the portions of immunoglobulins that determine specificity for a particular antigen. In certain embodiments, CDRs corresponding to the CDRs in Table 1 having sequence variations (*e.g.*, conservative substitutions) may bind to HCV. For example, CDRs, in which 1, 2, 3, 4, or 5 residues, or less than 20% of total residues in the CDR, are substituted or deleted can be present in an antibody (or antigen binding portion thereof) that binds HCV.

Similarly, anti-HCV antibodies can have CDRs containing a consensus sequence, as sequence motifs conserved amongst multiple antibodies can be important for binding activity.

For example, the invention provides for the use of one or more CDR regions or derivatives of the disclosed CDRs. Such derivative CDRs are derived from a disclosed CDR or portion thereof and, optionally, altered at one more amino acid positions. Alterations include one or more amino acid additions, deletions, or substitutions as described herein. Exemplary residue positions for altering include those amino acid positions identified as subject to more variance than other amino acid positions, for example, positions subject to somatic mutations as known in the art. Alternatively, such positions can be identified by comparing two or more sequences known to have a desired binding activity and identifying CDR residues that vary and CDR residues that are constant. For example, a comparison of the variable regions of heavy and light chains are presented in Figures 37 and 38 and the CDR derivative or consensus sequences that can be determined therefrom are found in SEQ ID NO: 87, 88, 89 (VH consensus sequences for CDR1, CDR2, and CDR3, respectively), 90, 91 and 92 (VL consensus sequences for CDR1, CDR2, and CDR3, respectively).

It is also understood that one more of the CDRs disclosed herein (including CDR derivative or consensus sequences) can be used for identifying naturally occurring CDRs that are suitable for binding to a HCV epitope. The CDRs can also be combined or cross-cloned between variable regions, for example, light chain CDRs can be introduced into heavy chain variable regions and heavy chain CDRs can be introduced into light chain variable regions and screened to ensure that specific binding is retained.

Human anti-HCV antibodies can include variable regions that are the product of, or derived from, specific human immunoglobulin genes. For example, the antibodies can include a variable heavy chain region that is the product of, or derived from, a

human VH 3-30-3 or VH3-33 gene (see, *e.g.*, Acc. No.: AJ555951, GI No.: 29836865; Acc. No.: AJ556080, GI No.: 29837087; Acc. No.: AJ556038, GI No.: 29837012, and other human VH3-33 rearranged gene segments provided in GenBank®). The antibodies can also, or alternatively, include a light chain variable region that is the
5 product of, or derived from a human Vκ L6, Vκ L11, Vκ L13, Vκ L15, or Vκ L19 gene (see, *e.g.*, GenBank® Acc. No.: AJ556049, GI No.: 29837033 for a partial sequence of a rearranged human Vκ L19 gene segment). As known in the art, and described in this section, above, variable immunoglobulin regions of recombined antibodies are derived by a process of recombination *in vivo* in which variability is introduced to genomic
10 segments encoding the regions. Accordingly, variable regions derived from a human VH or VL gene can include nucleotides that are different than those in the gene found in non-lymphoid tissues. These nucleotide differences are typically concentrated in the CDRs.

Moreover, the above antibodies exhibit binding activity to HCV and, in
15 particular, to one or more HCV glycoprotein epitopes (*e.g.*, E1 or E2 envelope glycoproteins). Such antibodies further exhibit HCV neutralization activity *in vitro* and protective efficacy against hepatitis sequelae *in vivo*.

Additional anti-HCV antibodies for use in embodiments of the invention include GNI-104 (monoclonal antibodies to E1, from GENImmune) and HuMax-HepC
20 (monoclonal antibody to E2, broadly cross-reactive across multiple HC genotypes, from Genmab A/S) (*see* <http://www.hcvdrugs.com>).

Additional embodiments of the invention feature a variety of antibodies. Table 2 lists additional human anti-HCV antibodies known in the art that can be used in embodiments of the invention. Further additional antibodies for use with embodiments
25 of the invention can be found in U.S. Patent No. 6,171,782, issued January 9, 2001; European Patent No. 0 569 537 B1, published June 17, 1998; U.S. Patent No. 5,753,430, issued May 19, 1998; U.S. Patent No. 5,308,750, issued May 3, 1994; PCT publication WO 02/100900 A2, published December 19, 2002; PCT publication WO 02/060954 A1, published August 8, 2002; PCT publication WO 02/055560 A2, July 18,
30 2002; PCT publication WO 99/24466, published May 20, 1999; PCT publication WO 98/21338, published May 22, 1998; European Patent No. 0 569 537 B1, issued June 17, 1998; PCT publication no. WO 92/07001, published April 30, 1992; PCT publication no. WO 02/08292, January 31, 2002; PCT publication WO 00/26418, published May

11, 2000; U.S. Patent No. 6,692,908, issued February 17, 2004; U.S. Patent No. 5,645,983, issued July 8, 1997; U.S. Patent No. 5,595,868, issued January 21, 1997; European Patent Application No. 0 947 525 A1, published June 10, 1999; European Patent No. 0 599 913 B1, published December 2, 1998; PCT publication WO 00/05266, published February 3, 2000; U.S. Patent No. 6,747,136, issued June 8, 2004; U.S. Patent No. 6,538,114, issued March 24, 2003; and U.S. Patent No. 5,919,454, issued July 6, 1999; each of which is incorporated by reference herein in its entirety.

Table 2: Human Anti-HCV Antibodies

10

Name	Epitope	Source	Activity
H-111	E1,192-211	EBV infection of human B, Stanford	Blocks attachment of baculovirus VLPs, mild block of infection of wt HCV from SB cells
CBH series (10 abs)	E2	EBV infection of human B, Stanford	Many block sE2/CD81
503 and 108	E2	EBV infection of human B, Institute Pasteur	Blocks E2 interaction with CD81+ cells
e137 and 301	E2	Fab from phage library	Blocks VSV/HCV binding to cells
HCV-AB ^{XITL} 68	E2	XLT Pharma	Moderate protection of Trimer mice

b. Production and Modification of Antibodies

Many different forms of anti-HCV antibodies can be useful in embodiments of the present invention. The antibodies can be of the various isotypes, including: IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgM, IgA1, IgA2, IgD, or IgE. Preferably, the antibody is an IgG isotype, e.g., IgG1. The antibody molecules can be full-length (e.g., an IgG1, IgG2, IgG3, or IgG4 antibody) or can include only an antigen-binding fragment (e.g., a Fab, F(ab')₂, Fv or a single chain Fv fragment). These include monoclonal antibodies (e.g., human monoclonal antibodies), recombinant antibodies, chimeric antibodies, and humanized antibodies, as well as antigen-binding portions of the foregoing.

Anti-HCV antibodies or portions thereof useful in the present invention can also be recombinant antibodies produced by host cells transformed with DNA encoding

immunoglobulin light and heavy chains of a desired antibody. Recombinant antibodies may be produced by known genetic engineering techniques. For example, recombinant antibodies can be produced by cloning a nucleotide sequence, *e.g.*, a cDNA or genomic DNA, encoding the immunoglobulin light and heavy chains of the desired antibody.

5 The nucleotide sequence encoding those polypeptides is then inserted into an expression vector so that both genes are operatively linked to their own transcriptional and translational expression control sequences. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. Typically, both genes are inserted into the same expression vector. Prokaryotic or
10 eukaryotic host cells may be used.

Expression in eukaryotic host cells is preferred because such cells are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. However, any antibody produced that is inactive due to improper folding can be renatured according to well known methods (Kim and
15 Baldwin, *Ann. Rev. Biochem.*, 51:459-89, 1982). It is possible that the host cells will produce portions of intact antibodies, such as light chain dimers or heavy chain dimers, which also are antibody homologs according to the present invention.

The antibodies described herein also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene
20 transfection methods as is well known in the art (Morrison, S., *Science*, 229:1202, 1985). For example, in one embodiment, the gene(s) of interest, *e.g.*, human antibody genes, can be ligated into an expression vector such as a eukaryotic expression plasmid such as used in a GS gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338 841, or in other expression systems well known in the art. The purified
25 plasmid with the cloned antibody genes can be introduced in eukaryotic host cells such as CHO-cells or NS0-cells or alternatively other eukaryotic cells like a plant derived cells, fungi, or yeast cells. The method used to introduce these genes can be any method described in the art, such as electroporation, lipofectine, Lipofectamine, transfection (*e.g.*, calcium chloride-mediated), or ballistic transfection, in which cells are bombarded
30 with microparticles carrying the DNA of interest (Rodin, *et al. Immunol. Lett.*, 74(3):197-200, 2000). After introducing these antibody genes in the host cells, cells expressing the antibody can be identified and selected. These cells represent the transfectomas which can then be amplified for their expression level and upscaled to

produce antibodies. Recombinant antibodies can be isolated and purified from these culture supernatants and/or cells using standard techniques.

It will be understood that variations on the above procedures are useful in the present invention. For example, it may be desired to transform a host cell with DNA
5 encoding either the light chain or the heavy chain (but not both) of an antibody. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding, *e.g.*, the constant region may be modified by, for example, deleting specific amino acids. The molecules expressed from such truncated DNA molecules are useful in the methods
10 described herein. In addition, bifunctional antibodies can be produced in which one heavy and one light chain bind to HCV, and the other heavy and light chain are specific for an antigen other than HCV, or another epitope of HCV.

Also within the scope of the invention are antibodies in which specific amino acids have been substituted, deleted, or added. In particular, preferred antibodies have
15 amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, a selected, small number of acceptor framework residues of the immunoglobulin chain can be replaced by the corresponding donor amino acids. Preferred locations of the substitutions include amino acid residues adjacent to the CDR, or which are capable of interacting with a CDR (see *e.g.*, U.S. Pat. 5,585,089). Criteria
20 for selecting amino acids from the donor are described in U.S. Pat. 5,585,089 (*e.g.*, columns 12-16), the contents of which are hereby incorporated by reference. The acceptor framework can be a mature human antibody framework sequence or a consensus sequence. As desired, the Fc region of antibodies of the invention can be altered to modulate effector function(s) such as, for example, complement binding
25 and/or Fc receptor binding. Criteria and subsets of framework alterations and/or constant regions suitable for alteration (by, *e.g.*, substitution, deletion, or insertion) are described in U.S. Patent Nos. 6,548,640; 5,859,205; 6,632,927; 6,407,213; 6,054,297; 6,639,055; 6,737,056; and 6,673,580.

A "consensus sequence" is a sequence formed from the most frequently
30 occurring amino acids (or nucleotides) in a family of related sequences (See *e.g.*, Winnaker, From Genes to Clones (Verlagsgesellschaft, Weinheim, Germany 1987). In a family of proteins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur

equally frequently, either can be included in the consensus sequence. A "consensus framework" of an immunoglobulin refers to a framework region in the consensus immunoglobulin sequence.

An anti-HCV antibody, or antigen-binding portion thereof, can be derivatized or
5 linked to another functional molecule (*e.g.*, another peptide or protein). For example, an antibody can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody, a detectable agent, a cytotoxic agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association with another molecule (such as a streptavidin
10 core region or a polyhistidine tag).

One type of derivatized antibody (or fragment thereof) is produced by crosslinking two or more of such proteins (of the same type or of different types). Suitable crosslinkers include those that are heterobifunctional, having two distinct reactive groups separated by an appropriate spacer (*e.g.*, *m*-maleimidobenzoyl-*N*-
15 hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody (or fragment thereof) can be derivatized (or labeled) include fluorescent compounds, various enzymes, prosthetic groups, luminescent materials, bioluminescent materials, and radioactive materials.
20 Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, and phycoerythrin. A protein or antibody can also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, β -galactosidase, acetylcholinesterase, glucose oxidase and the like. When a protein is derivatized with a detectable enzyme, it is detected by adding additional reagents that
25 the enzyme uses to produce a detectable reaction product. For example, when the detectable agent horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. A protein can also be derivatized with a prosthetic group (*e.g.*, streptavidin/biotin and avidin/biotin). For example, an antibody can be derivatized with biotin, and detected
30 through indirect measurement of avidin or streptavidin binding.

Labeled proteins and antibodies can be used, for example, diagnostically and/or experimentally in a number of contexts, including (i) to isolate a predetermined antigen by standard techniques, such as affinity chromatography or immunoprecipitation; and

(ii) to detect a predetermined antigen (*e.g.*, HCV, or a HCV protein, carbohydrate, or lipid, or combination thereof, *e.g.*, in a cellular lysate or a patient sample) in order to monitor virus and/or protein levels in tissue as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen.

5 Any of the above protein derivatizing/labeling techniques can also be employed on a viral target, for example, one or more HCV proteins, such as an E1 or E2 glycoprotein or fragment(s) thereof.

 Anti-HCV antibodies can be characterized for binding to HCV by a variety of known techniques. Antibodies are typically characterized by ELISA first. Briefly,
10 microtiter plates can be coated with the target antigen in PBS, for example, HCV or the E1 and/or E2 glycoprotein or portion thereof, and then blocked with irrelevant proteins such as bovine serum albumin (BSA) diluted in PBS. Dilutions of plasma from mice immunized with the target antigen, for example, a HCV vaccine, are added to each well and incubated for 1-2 hours at 37°C. The plates are washed with PBS/Tween 20 and
15 then incubated with a goat-anti-human IgG Fc-specific polyclonal reagent conjugated to alkaline phosphatase for 1 hour at 37°C. After washing, the plates are developed with ABTS substrate, and analyzed at OD of 405. Preferably, mice which develop the highest titers will be used for fusions.

 An ELISA assay as described above can be used to screen for antibodies and,
20 thus, hybridomas that produce antibodies that show positive reactivity with HCV. Hybridomas that produce antibodies that bind, preferably with high affinity, to HCV can then be subcloned and further characterized. One clone from each hybridoma, which retains the reactivity of the parent cells (by ELISA), can then be chosen for making a cell bank, and for antibody purification.

25 To purify the anti-HCV antibodies, selected hybridomas can be grown in roller bottles, two-liter spinner-flasks or other culture systems. Supernatants can be filtered and concentrated before affinity chromatography with protein A-Sepharose (Pharmacia, Piscataway, NJ) to purify the protein. After buffer exchange to PBS, the concentration can be determined by spectrophotometric methods.

30 To determine if the selected monoclonal antibodies bind to unique epitopes, each antibody can be biotinylated using commercially available reagents (Pierce, Rockford, Ill.). Biotinylated MAb binding can be detected with a streptavidin labeled probe. Anti-

HCV antibodies can be further tested for reactivity with HCV or one or more HCV proteins by immunoprecipitation or immunoblot.

Particular antibodies of the invention are characterized as binding to one or more epitopes of a HCV glycoprotein. For example, the HCV E1 or E2 glycoprotein epitopes
5 can be linear epitopes, conformational epitopes, discontinuous epitopes, or combinations of such epitopes.

Without wishing to be bound by any particular theory, in some embodiments, the epitope of HCV E1 glycoprotein is found within amino acid residues 190-330. In some embodiments, the HCV E1 glycoprotein is found within amino acid residues 315-328.

10 Without wishing to be bound by any particular theory, in some embodiments, the epitope of HCV E2 glycoprotein is found within amino acid residues 412-464. In some embodiments, the HCV E2 glycoprotein is found within amino acid residues 412-423. In some embodiments, the HCV E2 glycoprotein is found within residues 413, 418, 420. In some embodiments, an epitope of a HCV glycoprotein comprises alterations to the
15 canonical or published sequences of HCV, such as substitutions or deletions.

Other assays to measure activity of the anti- HCV antibodies include neutralization assays. *In vitro* neutralization assays can measure the ability of an antibody to inhibit a cytopathic effect, infectivity, or presence of a virus on or in cells in culture (see Example 1, below). *In vivo* neutralization or survival assays can be used to
20 measure HCV neutralization as a function of reduced morbidity and/or mortality in an appropriate animal model (see Example 5 of PCT/US08/01922, filed February 13, 2008, hereby incorporated by reference).

iii. Engineered Antibodies

25 An antibody of the invention can be prepared using an antibody having one or more V_H and/or V_L sequences as starting material to engineer a modified antibody, which modified antibody may have altered properties from the starting antibody. An antibody can be engineered by modifying one or more residues within one or both variable regions (*i.e.*, V_H and/or V_L), for example within one or more CDR regions
30 and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

One type of variable region engineering that can be performed is CDR grafting. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain CDRs. For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences
5 outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, e.g., Riechmann *et*
10 *al.*, 1998 *Nature* 332:323-327; Jones *et al.*, 1986 *Nature* 321:522-525; Queen *et al.*, 1989 *Proc. Natl. Acad. Sci. U.S.A.* 86:10029-10033; U.S. Pat. No. 5,225,539, and U.S. Pat. Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370).

Framework sequences can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example, germline DNA
15 sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the Internet at www.mrc-cpe.cam.ac.uk/vbase), as well as in Kabat *et al.*, 1991 *Sequences of Proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson *et al.*, 1992 *J. Mol. Biol.* 227:776-798; and
20 Cox *et al.*, 1994 *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference.

The V_H CDR1, 2 and 3 sequences and the V_L CDR1, 2 and 3 sequences can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence is derived, or the
25 CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (see e.g., U.S. Pat. Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370).

30 CDRs can also be grafted into framework regions of polypeptides other than immunoglobulin domains. Appropriate scaffolds form a conformationally stable framework that displays the grafted residues such that they form a localized surface and bind the target of interest (*e.g.*, an epitope of a HCV protein). For example, CDRs can

be grafted onto a scaffold in which the framework regions are based on fibronectin, ankyrin, lipocalin, neocarzinostatin, cytochrome b, CP1 zinc finger, PST1, coiled coil, LACI-D1, Z domain or tendramisat (See e.g., Nygren and Uhlen, 1997 Current Opinion in Structural Biology, 7, 463-469).

5 Another type of variable region modification is mutation of amino acid residues within the V_H and/or V_L CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (e.g., affinity) of the antibody of interest, known as "affinity maturation." Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s), and the effect on antibody binding, or other functional
10 property of interest, can be evaluated in *in vitro* or *in vivo* assays as described herein. Conservative modifications can be introduced. The mutations may be amino acid substitutions, additions or deletions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

 Engineered antibodies of the invention include those in which modifications
15 have been made to framework residues within V_H and/or V_L, e.g., to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody that has undergone somatic mutation may contain
20 framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived. To return the framework region sequences to their germline configuration, the somatic mutations can be "backmutated" to the germline sequence by, for example, site-directed mutagenesis
25 or PCR-mediated mutagenesis. Such "backmutated" antibodies are also intended to be encompassed by the invention.

 Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell
-epitopes to thereby reduce the potential immunogenicity of the antibody. This
30 approach is also referred to as "deimmunization" and is described in further detail in U.S. Pat. Pub. No. 20030153043 by Carr *et al.*

 In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within

the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (e.g., one or more chemical moieties can be attached to the antibody) or be
5 modified to alter its glycosylation, again to alter one or more functional properties of the antibody.

In one embodiment, the hinge region of CH1 is modified such that the number of cysteine residues in the hinge region is altered, e.g., increased or decreased. This approach is described further in U.S. Pat. No. 5,677,425 by Bodmer *et al.* The number
10 of cysteine residues in the hinge region of CH1 is altered to, for example, facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half-life of the antibody. More specifically, one or more amino
15 acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcal protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Pat. No. 6,165,745 by Ward *et al.*

In another embodiment, the antibody is modified to increase its biological half-
20 life. Various approaches are possible. For example, U.S. Pat. No. 6,277,375 describes the following mutations in an IgG that increase its half-life *in vivo*: T252L, T254S, T256F. Alternatively, to increase the biological half life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Pat. Nos.
25 5,869,046 and 6,121,022 by Presta *et al.*

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector functions of the antibody. For example, one or more amino acids can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand
30 but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Pat. Nos. 5,624,821 and 5,648,260, both by Winter *et al.*

In another embodiment, one or more amino acids selected from amino acid residues can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Pat. Nos. 6,194,551 by

5 Idusogie *et al.*

In another embodiment, one or more amino acid residues are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in WO 94/29351 by Bodmer *et al.*

In yet another embodiment, the Fc region is modified to increase the ability of
10 the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase the affinity of the antibody for an Fc γ receptor by modifying one or more amino acids. This approach is described further in WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for Fc γ RI, Fc γ RII, Fc γ RIII and FcRn have been mapped and variants with improved binding have been described (see Shields, R.L. et
15 al., 2001 J. Biol. Chem. 276:6591-6604).

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycosylated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered, for example, to increase the affinity of the antibody for an antigen. Such carbohydrate modifications can be accomplished by, for example,
20 altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Pat. Nos. 5,714,350 and
25 6,350,861 by Co *et al.*

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of
30 antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an

antibody with altered glycosylation. For example, EP 1,176,195 by Hang *et al.* describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation. PCT Pub. WO 03/035835 by Presta describes a variant CHO cell line, Lec13 cells, with
5 reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, R.L. *et al.*, 2002 J. Biol. Chem. 277:26733-26740). WO 99/54342 by Umana *et al.* describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed
10 in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana *et al.*, 1999 Nat. Biotech. 17:176-180).

Another modification of the antibodies herein that is contemplated by the invention is pegylation. An antibody can be pegylated, for example, to increase the
15 biological (*e.g.*, serum) half-life of the antibody. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG moieties become attached to the antibody or antibody fragment. The pegylation can be carried out by an acylation reaction or an alkylation reaction with a reactive PEG
20 molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody to be pegylated is an aglycosylated antibody. Methods for pegylating proteins
25 are known in the art and can be applied to the antibodies of the invention. See for example, EP 0 154 316 by Nishimura *et al.* and EP 0 401 384 by Ishikawa *et al.*

In addition, pegylation can be achieved in any part of an anti- HCV binding polypeptide of the invention by the introduction of a nonnatural amino acid. Certain nonnatural amino acids can be introduced by the technology described in Deiters *et al.*, J
30 Am Chem Soc 125:11782-11783, 2003; Wang and Schultz, Science 301:964-967, 2003; Wang *et al.*, Science 292:498-500, 2001; Zhang *et al.*, Science 303:371-373, 2004 or in US Patent No. 7,083,970. Briefly, some of these expression systems involve site-directed mutagenesis to introduce a nonsense codon, such as an amber TAG, into the

open reading frame encoding a polypeptide of the invention. Such expression vectors are then introduced into a host that can utilize a tRNA specific for the introduced nonsense codon and charged with the nonnatural amino acid of choice. Particular nonnatural amino acids that are beneficial for purpose of conjugating moieties to the polypeptides of the invention include those with acetylene and azido side chains. The polypeptides containing these novel amino acids can then be pegylated at these chosen sites in the protein.

VII. Pharmaceutical Compositions and Kits

In another aspect, the present invention provides compositions, *e.g.*, pharmaceutically acceptable compositions, which include conjugates of the present invention, formulated together with a pharmaceutically acceptable carrier, as well as methods of treating subjects with said compositions. In exemplary embodiments, the conjugates feature targeting antibodies or antibody variants. In particular embodiments, the antibodies are therapeutic antibodies, for example neutralizing antibodies. As such, exemplary conjugates of the invention have a “dual modality”. For example, exemplary conjugate are capable of neutralizing virus outside the cell (*e.g.*, free virus) to inhibit viral infection and accessing infected cells to prevent viral replication.

“Pharmaceutically acceptable carriers” include any and all solvents, dispersion media, isotonic and absorption delaying agents, and the like that are physiologically compatible. The carriers can be suitable for intravenous, intramuscular, subcutaneous, parenteral, rectal, spinal, or epidermal administration (*e.g.*, by injection or infusion).

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Useful compositions are in the form of injectable or infusible solutions. A useful mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). For example, conjugates can be administered by intravenous infusion or injection. In another embodiment, conjugates can be administered by intramuscular or subcutaneous injection.

The composition of the invention may be co-administered with a) one or more other antibodies, *e.g.*, anti- HCV antibodies, b) HCV protein(s), *e.g.*, a HCV vaccine, c) toxin(s) d) other therapeutic agent(s) (*e.g.*, antivirals), and/or e) label(s). In some

embodiments, administration of one or more anti-HCV antibodies can precede administration of one or more anti-HCV antibody-silencing agent conjugates.

The phrases "parenteral administration" and "administered parenterally" as used herein mean modes of administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intracranial, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural, and intrasternal injection and infusion.

Therapeutic compositions typically should be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high antibody concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody or antibody portion) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the useful methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The conjugates described herein can be administered by a variety of methods known in the art, and for many therapeutic applications. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

In certain embodiments, a conjugate may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule,

compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention
5 by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. Therapeutic compositions can be administered with medical devices known in the art.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided
10 doses may be administered over time, or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each
15 unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of
20 compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of a conjugate of the invention is 0.1-60 mg/kg, *e.g.*, 0.5-25 mg/kg, 1-2 mg/kg, or 0.75-10 mg/kg. In one embodiment, the amount of conjugate administered, is at or about 0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg, or at an interval or range thereof. It is
25 to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Also within the scope of the invention are kits including conjugates of the
30 invention, for example, anti-HCV conjugates. The kits can include one or more other elements including: instructions for use; other reagents, *e.g.*, a label, a therapeutic agent, or an agent useful for chelating, or otherwise coupling, an antibody to a label or

therapeutic agent, or other materials for preparing the antibody for administration; pharmaceutically acceptable carriers; and devices or other materials for administration to a subject.

Various combinations of conjugates, with or without unconjugated antibodies, can be packaged together. For example, a kit can include anti-HCV conjugates, antibodies that bind to HCV (*e.g.*, antibodies that include the variable heavy and/or light chain regions of 95-2, 95-14, 95-38, 83-128 and 073-1), or a combination thereof. In certain embodiments, the anti-HCV antibodies (either within or administered with the conjugates) are blocking or neutralizing antibodies. In instances where HCV resistance is an issue, kits of the invention may include more than one conjugate, for example, conjugates delivering different silencing agents targeting distinct genes. The compounds can be mixed together, or packaged separately within the kit.

Instructions for use can include instructions for therapeutic application including suggested dosages and/or modes of administration, *e.g.*, in a patient with a symptom or indication of a hepatitis virus/HCV infection, or exposure or suspected exposure to a hepatitis virus/HCV. Other instructions can include instructions on coupling of the antibody to a chelator, a label or a therapeutic agent, or for purification of a conjugated antibody, *e.g.*, from unreacted conjugation components.

The kit can include a detectable label, a therapeutic agent, and/or a reagent useful for chelating or otherwise coupling a label or therapeutic agent to a conjugate or antibody. Coupling agents include agents such as N-hydroxysuccinimide (NHS). In such cases the kit can include one or more of a reaction vessel to carry out the reaction or a separation device, *e.g.*, a chromatographic column, for use in separating the finished product from starting materials or reaction intermediates.

The kit can further contain at least one additional reagent, such as a diagnostic or therapeutic agent, *e.g.*, a diagnostic or therapeutic agent as described herein, and/or one or more additional anti-HCV antibodies or conjugates (or portions thereof), formulated as appropriate, in one or more separate pharmaceutical preparations.

Other kits can include optimized nucleic acids encoding anti-HCV antibodies, for use as passive immunotherapy, and/or HCV protein(s), or fragments thereof, for use as, *e.g.*, vaccines (active immunotherapy), and instructions for expression of the nucleic acids.

VII. Therapeutic Methods and Compositions

In particular embodiments, conjugates of the present invention can have *in vitro* and *in vivo* therapeutic, prophylactic, and diagnostic utilities. For example, conjugates can be administered to cells in culture, *e.g.*, *in vitro* or *ex vivo*, or *in vivo*, to an animal, preferably a human subject, to treat, inhibit, prevent relapse, and/or diagnose HCV and disease associated with HCV.

As used herein, the term "subject" is intended to include human and non-human animals. The term "non-human animals" includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, mice, dogs, cats, pigs, cows, and horses.

Conjugates of the invention can be used on cells in culture, *e.g.*, *in vitro* or *ex vivo* in some embodiments. For example, cells can be cultured *in vitro* in culture medium and the contacting step can be affected by adding a conjugate to the culture medium. The methods can be performed on virions or cells present in a subject, as part of an *in vivo* (*e.g.*, therapeutic or prophylactic) protocol. For *in vivo* embodiments, the contacting step is effected in a subject and includes administering conjugate to the subject under conditions effective to permit binding of the binding protein, antibody, or portion thereof, to HCV or any portion thereof present in the subject, *e.g.*, in or around a wound or on or near cells of neuronal origin. In some embodiments, the subjects are subjects having a liver disorder, liver dysfunction, or liver transplant patients. In other embodiments, the subjects suffer from chronic HCV infection.

Methods of administering compounds of the invention are described herein. Suitable dosages of the molecules used will depend on the age and weight of the subject and the particular drug used. In some embodiments, the compounds can be used as competitive agents for ligand binding to inhibit or reduce an undesirable interaction, *e.g.*, to inhibit binding and/or infection of HCV of cells, *e.g.*, neuronal cells.

In some embodiments, an anti-HCV conjugate can be administered in combination with other anti-HCV therapies (*e.g.*, other monoclonal antibodies, polyclonal gamma-globulin, *e.g.*, human serum comprising anti-HCV immunoglobulins). Combinations of conjugates and therapies that can be used in some embodiments include an anti-HCV antibody or antigen binding portion thereof and/or an anti-HCV E1 or E2 protein antibody or antigen binding portion thereof. The anti-HCV or HCV E1 or E2 protein antibody for use in the formation of a conjugate of the invention can be antibody clone 95-2, 95-14, 95-38, 83-128 and/or 073-1 that includes the variable regions of such an antibody or antibodies, or an antibody with variable

regions at least 90% identical to the variable regions of such an antibody or antibodies. In one embodiment, conjugates comprising the anti-HCV virus antibody can be 83-128, 95-2, 073-1 or portion thereof or an antibody with variable regions at least 90% identical to the variable regions of the foregoing, *e.g.*, 95-2, 95-14, 95-38, 83-128 and/or 073-1.

5 Combinations of anti-HCV virus conjugates based on anti-HCV antibodies (*e.g.*, 95-2, 95-14, 95-38, 83-128 and/or 073-1) can provide potent inhibition of HCV, especially, *e.g.*, particular HCV genotypes and subgenotypes. Characteristic HCV genotypes and subgenotypes for which the conjugates of the invention are suitable for treating, detecting, diagnosing and the like include genotypes 1, 2, 3, 4, 5, and 6, subgenotypes
10 1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a, 4b, 4c, 4d, 4e, 5a, 6a and combinations thereof.

It is understood that any of the agents of the invention, for example, conjugates comprising anti-HCV antibodies, or fragments thereof, can be combined, for example in different ratios or amounts, for improved therapeutic effect. Indeed, the agents of the invention can be formulated as a mixture, or chemically or genetically linked using art
15 recognized techniques thereby resulting in covalently linked conjugates having anti-HCV binding properties, for example, multi-epitope binding properties to, for example, HCV E1 and/or E2 glycoprotein. The combined formulation may be guided by a determination of one or more parameters such as the affinity, avidity, or biological efficacy of the agent alone or in combination with another agent. The agents of the
20 invention can also be administered in combination with other agents that enhance access, half-life, or stability of the therapeutic agent in targeting, clearing, and/or sequestering HCV or an antigen thereof.

Such combination therapies are preferably additive and even synergistic in their therapeutic activity, *e.g.*, in the inhibition, prevention, infection, and/or treatment of
25 HCV -related disease or disorders. Administering such combination therapies can decrease the dosage of the therapeutic agent (*e.g.*, a conjugate mixture, with or without an antibody or antibody fragment mixture, or cross-linked or genetically fused bispecific antibody or antibody fragment) needed to achieve the desired effect.

Immunogenic compositions can be used with the conjugates of the invention in
30 certain embodiments. Immunogenic compositions typically contain an immunogenically effective amount of a HCV component, for example, HCV E1 and/or E2 glycoprotein, or fragments thereof, and can be used in generating anti-HCV antibodies. Immunogenic epitopes in a HCV protein sequence can be identified as

described herein (see *e.g.* Example 4 of PCT/US08/01922, filed February 13, 2008, hereby incorporated by reference) or according to methods known in the art, and proteins, or fragments containing those epitopes can be delivered by various means, in a vaccine composition. Suitable compositions can include, for example, lipopeptides
5 (e.g., Vitiello *et al.*, *J. Clin. Invest.* 95:341 (1995)), peptide compositions encapsulated in poly (DL-lactide-co-glycolide) ("PLG") microspheres (see, *e.g.*, Eldridge *et al.*, *Molec. Immunol.* 28:287-94 (1991); Alonso *et al.*, *Vaccine* 12:299-306 (1994); Jones *et al.*, *Vaccine* 13:675-81 (1995)), peptide compositions contained in immune stimulating complexes (ISCOMS) (see, *e.g.*, Takahashi *et al.*, *Nature* 344:873-75 (1990); Hu *et al.*,
10 *Clin. Exp. Immunol.* 113:235-43 (1998)), and multiple antigen peptide systems (MAPs) (see, *e.g.*, Tam, *Proc. Natl. Acad. Sci. U.S.A.* 85:5409-13 (1988); Tam, *J. Immunol. Methods* 196:17-32 (1996)).

Useful carriers that can be used with immunogenic compositions of the invention are well known, and include, for example, thyroglobulin, albumins such as human
15 serum albumin, tetanus toxoid, polyamino acids such as poly L-lysine, poly L-glutamic acid, influenza, hepatitis B virus core protein, and the like. The compositions can contain a physiologically tolerable (*i.e.*, acceptable) diluent such as water, or saline, typically phosphate buffered saline. The compositions and vaccines also typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum
20 phosphate, aluminum hydroxide, or alum are examples of materials well known in the art. Additionally, CTL responses can be primed by conjugating target antigens, for example a HCV protein(s) (or fragments, inactive derivatives or analogs thereof) to lipids, such as tripalmitoyl-S-glycerylcysteinyl-serine (P₃CSS).

The anti-HCV conjugates can be administered in combination with other agents,
25 such as compositions to treat HCV -mediated disease. For example, therapeutics, including antiviral agents, serum immunoglobulin, and/or vaccines, can be administered in combination with anti-HCV conjugates for treating, preventing, or inhibiting HCV. The conjugate can be administered before, after, or contemporaneously with a HCV vaccine.

30

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.

Incorporation by Reference

5 This application is also related to International Patent Application No. PCT/US08/01922, filed February 13, 2008, and entitled: "Human Antibodies Against Hepatitis C Virus (HCV) an Uses Thereof". The content of this application and the entire contents of all patents, published patent applications and other references cited
10 herein are all hereby expressly incorporated herein in their entirety by reference.

EXAMPLES

The invention is further described in the following examples, which do not limit
5 the scope of the invention described in the claims.

Recent studies have demonstrated a promising therapeutic potential of duplex
small interfering RNAs (siRNAs) in prevention and treatment of infectious disease,
cancer and neurodegenerative disease *in vitro*. However, the major hurdle in therapeutic
10 application of siRNA is how to direct the molecule into a desired cell population to
achieve maximal therapeutic effect and avoid non-specific silencing or other toxicity in
cells other than intended targets. Since duplex siRNAs cannot choose their cellular
targets, it is necessary to design a “targeting agent” to deliver these siRNAs. Cell
surface receptors have been explored as potential targets for gene delivery. Antibodies
15 specifically binding to these surface receptors can serve as a carrying vector to delivery
siRNAs into target cells expressing the corresponding cell surface protein recognized by
the antibody. As one way to realize the antibody-siRNA interaction, full length or
truncated human protamine polypeptides, which is positive-charged and interacts with
negative-charged RNA non-covalently, has been suggested to fuse to the C-terminus of
20 a human single-chain antibody fragment (ScFv) or of a human heavy chain. However,
even with the usage of short protamine fragments, there are still concerns that fusion to
polypeptides may destroy the activity of antibodies. Moreover, the non-covalent binding
between the full-length protamine or its fragment and the siRNA can be disrupted by
other charged molecules *in vitro* or *in vivo*, in which case it cannot promise the safe
25 delivery of the siRNA into target cells. Therefore, a better way to realize the antibody-
siRNA interaction is needed.

Materials and Methods

In general, the practice of the present invention employs, unless otherwise
30 indicated, conventional techniques of chemistry, molecular biology, recombinant DNA
technology, immunology (especially, *e.g.*, antibody technology), and standard
techniques in polypeptide preparation. See, *e.g.*, Sambrook, Fritsch and Maniatis,
Molecular Cloning: Cold Spring Harbor Laboratory Press (1989); *Antibody*

Engineering Protocols (Methods in Molecular Biology), 510, Paul, S., Humana Pr
(1996); *Antibody Engineering: A Practical Approach* (Practical Approach Series, 169),
McCafferty, Ed., Irl Pr (1996); *Antibodies: A Laboratory Manual*, Harlow *et al.*,
C.S.H.L. Press, Pub. (1999); and *Current Protocols in Molecular Biology*, eds. Ausubel
5 *et al.*, John Wiley & Sons (1992).

Example 1: Antibody Characterization

HCV genotype 1a and 1b – soluble E2 ELISA

10 The reactivity of human antibody 95-2 (Figure 1A) and human antibody 83-128 (Figure
1B) to soluble HCV genotype 1a E2-660 (triangles) and soluble HCV genotype 1b E2-
661 (squares) was compared. ELISA plates were coated with 2 µg/ml of antigen and
probed with antibody in two-fold dilutions. Bound antibody was detected with goat-
anti-human secondary antibody conjugated to alkaline phosphatase and PNPP substrate.
15 Both human antibodies, 95-2 and 83-128 were found to react equivalently to both
genotypes (see Figure 1). These experiments were also performed with the 95-14, 95-38
and 073-1 antibodies and all three recognized genotype 1a and 1b equivalently (data not
shown).

Neutralization of HCV pseudovirus from multiple genotypes

20 The capacity of human antibodies 95-2 and 83-128 to neutralize both multiple
genotypes of HCV pseudovirus was determined using Hep3B cells. Five-fold dilutions
of antibody were incubated with HCV pseudovirus for one hour at room temperature.
The virus-antibody mixture was added to Hep3B cells followed by incubation at 37°C
25 for 72 hours. Infection was quantitated with Brightglo luciferase assay and read in a
Victor3 plate reader for light output. An isotype-matched irrelevant human antibody
was used as a negative control. Both human antibodies, 95-2 and 83-128, were found to
neutralize HCV genotypes 1a, 1b, 2b, 3a and 4a pseudovirus (see Figures 2A, 2B, 2C,
2D and 2E, respectively). Antibody 073-1 was also shown to neutralize all of the above
30 listed pseudovirus (data not shown).

Reducing and non-reducing Western blots of genotype 1a and 1b E2 soluble protein

The reactivity of human antibodies 95-2 and 83-128 to soluble E2 protein (E2-661) from HCV genotype 1a and 1b subjected to reducing or non-reducing SDS-PAGE was analyzed. Westerns blots were performed with anti-his tag monoclonal (his), 83-128 and 95-2 using anti-mouse IgG (his) or anti-human IgG (95-2 and 83-128) conjugated to HRP with enhanced chemiluminescent detection reagent. Both human antibodies 95-2 and 83-128 were found to recognize E2-661 subjected to denaturing, reducing gel followed by transfer to PVDF membrane (see Figure 3).

Binding affinity of human antibodies 95-2 and 83-128 for E2 412-423 epitope expressed as a bacterial fusion protein

Binding affinity of human antibodies 95-2 and 83-128 for E2 412-423 epitope expressed as a bacterial fusion protein was determined and summarized in Figure 4. Goat anti-human IgG Fc was amide coupled to the Biacore chip. Human antibodies 95-2 and 83-128 were separately captured on the chip and E2-G bacterially expressed protein containing E2 412-423 amino acids was flowed over at varying concentrations. The BIAevaluationTM software was used to fit the curves and calculate the affinity constants.

Example 2: Oligo modification with SFB (succinimidyl 4-formylbenzoate)

The present application discloses methods to form bio-functional molecules between DNA or RNA oligos and proteins using cross-linking reagents. These reagents can be, for example, heterobifunctional, containing an N-hydroxysuccinimide ester (NHS-ester) and either a hydrazine/hydrazide or an aldehyde group. In this Example, the RNA/DNA oligos containing a 5' amino group (introduced during synthesis) were modified with SFB (succinimidyl 4-formylbenzoate, Pierce Cat. #22419) to form an active biomolecule for further conjugation with a protein or polypeptide. The exemplary experimental procedures are given below.

Step 1 Anneal Duplex

The RNA/DNA oligos used in this Example had an amino modification of an N6 linker (Figure 5) for SFB modification. For example, a DNA duplex formed by annealing may have an N6 linker on the 5' terminus of the Sense Strand as

5 Sense 5'-N6-CAAGCTGACCCTGAAGTTCTT-3'

Antisense 3'-TTGTTCTGACTGGGACTTCAAG-5'.

For the annealing procedure, two complimentary strands of RNA/DNA (*e.g.*, strand A and strand B) were resuspended in RNase-free water with a final concentration of 250 pmol/ul, as confirmed by common spectrophotometry. Solutions of each strand
10 was mixed together in an equal molar ratio. To create 100 nmol of annealed RNA/DNA duplex, 400 ul strand A (250 pmol/ul) was mixed with 400 ul strand B (250 pmol/ul). 200 ul 5X lysis buffer (Dharmacon's 5X buffer) was then added to this nucleic acid solution mixture. The mixture was incubated at 95°C for 5 minutes and then at 37°C for 1.5 hour. To confirm duplex formation, 1 pmol duplex was analyzed via electrophoresis
15 (mini TBE gel). The duplex solution was then dialyzed into modification buffer (100 mM Naphosphate, Pierce, Cat#28372, 150 mM NaCl, PH7.0) for 3 hours before proceeding with the SFB modification step.

Step 2: Modify Duplex with SFB

20 In this step, the N6 linker at the 5' terminus of the sense strand of the RNA/DNA duplex is modified by SFB to form an aldehyde group, as illustrated in Figure 5. (Modification may also be performed on non-duplexed, N6-linked oligonucleotides.)

Before the modification reaction, a fresh solution of SFB was prepared by dissolving 12 mg SFB in 500 ul DMF (Dimethylformamide, Sigma Cat. #227056), to a
25 final concentration of 97 nmol/ul.

To perform the modification reaction, 800 ul (0.125 nmol/uL, thus 100 nmol) of RNA/DNA duplex, 400 ul DMF (half volume of the duplex volume, added to increase solubility of SFB in the reaction mixture) and 25.7 ul SFB solution (volume of SFB = nmol of oligo * 25 molar excess/ SFB concentration). The reaction mixture was
30 incubated at room temperature for 3 hours. Excess SFB was then removed from the mixture by precipitating the modified duplex molecules with ethanol and sodium acetate (the sample was mixed with 2.5 volume EtOH and 0.1 volume sodium acetate, followed by incubation at -80°C for 2 hours). The modified duplex molecules were collected by

centrifugation, the pellet was further washed with 70% EtOH, followed by resuspension in conjugation buffer (100 mM Naphosphate, 150mM NaCl, PH5.0) at a concentration between 1-2 nmol/ul. The final concentration of modified RNA/DNA duplex was confirmed by Quan-it RNA kit (Invitrogen). A small sample of the modified duplex (1
5 pmol) was analyzed using a 20 cm 15% TBE urea gel, to assess modification efficiency.

Figure 6 shows the results of modification of the sense strand of the duplex. In Figure 6, lane 1 shows unmodified sense strand RNA, whereas lanes 2, 3, 4 and 5 show sense strand RNA oligonucleotides that had been modified via the procedure above, with different amounts of SFB in each reaction (25:1, 35:1, 45:1 and 55:1 molar ratios
10 of SFB:N6 GTP duplex in the reaction mix, respectively). Due to a greater molecular weight, the sense strand RNA oligonucleotides with SFB modification (Figure 6, lanes 2-5) moved more slowly than those without modification (Figure 6, lane 1). The modification efficiency was at least 90% and these modifications were seen at the lowest molar ratio of SFB:N6 GTP duplex (25:1) (Figure 6, lane 2). No significant
15 increase in modification was seen with greater SFB:N6 GTP duplex molar ratios (*i.e.*, when the SFB: N6 GTP duplex molar ratio was 35:1, 45:1 or 55:1).

To compare the efficiency of the modification reaction when using a oligo duplex as a substrate as opposed to using a single strand oligo, parallel modification reactions were carried out using N6-tagged RNA/DNA duplex molecules or
20 unduplexed, single sense RNA or DNA strands (with identical sequences to the corresponding oligonucleotides of the duplex molecules). Using a 25 fold molar ratio excess of SFB in the reaction, both duplex (Figure 7, lane 1) and single sense strand oligo (Figure 7, lane 2) had modification efficiency higher than 90%. Two bands are seen for the modified duplex sample (lane 1). The upper band in the duplex oligo lane
25 had the same mobility during gel electrophoresis as the modified sense strand oligo in lane 2; the lower band in the duplex oligo lane had the same mobility during gel electrophoresis as the *unmodified* anti-sense strand oligo in the last lane on the right hand side of the figure. This confirms that only the RNA sense strand is modified when the RNA/DNA duplex is used as the substrate in the modification reaction.

30

Example 3: SFB modification of siRNA (N6-GFPz stable 2) and result detection using 2-HP method

In this Example, a specific siRNA (N6-linked GFPz stable 2) was used for SFB modification as part of a RNA/DNA duplex, as demonstrated in Example 2. After SFB modification to the siRNA duplex, the sample was diluted 20 to 50 fold into 2-HP (2-hydrazino pyridine.2HCl) solution (0.5 mM 2-HP in 100mM MES, pH 5.0 and incubated at 37°C for 1 hour. The aldehyde-modified siRNA duplex and 2-HP will form a hydrazone structure, which has a spectroscopic UV signature and can be read at 260 nm and 350 nm (Figure 8). Spectroscopy results can be converted via a Solulink calculator to quantitatively determine the percentage of oligo being modified. As shown in Figure 9, using the 2-HP detection method, 94.3% and 94.5% of the siRNA duplex was modified with SFB in two separate trials (right panel), which suggests that this detection methods is comparable to results visualized on gel (middle panel). On the contrary, in this example the efficiency for SFB modification of the passenger strand (DNA) of the siRNA duplex was quite low, at only 28.8% efficiency (left panel).

Example 4: Confirmation of siRNA activity after SFB modification and IC 50 measurement

The activities of SFB-modified RNA/DNA duplexes were assessed before further conjugation process with any proteins or polypeptides, by measuring the activity of a specific siRNA (GFPz) before and after SFB modification. The GFPz siRNA duplexes were previously modified with various 2' O-Met nucleotides (named Stable 6) and the N6 linker to become N6 GFPz Stable 6 siRNA oligo duplex. (This was done using psiCheck with GFP cloned into the psiCheck vector. 293T cells were transfected with siRNAs and psiCheck vector containing GFP DNA. Then luciferase reduction was measured to assess delivery of the anti-GFP siRNA). The ability of the siRNA oligos, both with and without SFB modification on the N6 linker at the 5' terminus, to knock down targeted genes was measured. As shown in Figure 10, the knockdown efficiency of SFB-N6-GFPz Stable 6 oligos was similar to that of unmodified GFPz oligos.

Example 5: Antibody modification with SANH (succinimidyl 4-hydrazinonicotinate acetone hydrazone)

The present application includes methods of cross-linking DNA or RNA oligos and proteins or polypeptides to form bio-functional molecules. The proteins include, but are not limited to, antibodies or fragments thereof. In this Example, an antibody was modified with SANH (succinimidyl 4-hydrazinonicotinate acetone hydrazone) to form an active biomolecule for further conjugation with a RNA or DNA oligo. Exemplary experimental procedures are given below.

Step 1 Antibody preparation

5-10 ml (5mg/ml) of antibody solution was dialyzed into modification buffer (100 mM Naphosphate, Pierce, Cat. # 28372, 150 mM NaCl, pH 7.0) using slide-A-lyzer dialysis cassette (Pierce, Cat. #66710). The concentration of the antibody was measured by Octet QK (Fortebio). (The octet measures surface plasmon resonance. Protein A sensors are placed within a well of a microtiter plate containing antibody. The Ab binds to the protein A tip and the increase in mass can be detected by SPR. Using a standard curve, antibody can be quantitated using this method). The dialyzed antibody solution with a final concentration of 4-5 mg/ml was stored at 4 °C until required for the modification process.

Step 2: Preparation of SANH solution

9 mg of SANH (Pierce, Cat. # 22400) was dissolved in 1 ml DMF (Dimethylformamide, Sigma Cat. #227056-100 mL) to make final concentration of 30 nmol/ul).

Step 3: Antibody modification with SANH

The SANH solution was added 25-50 fold in molar excess to 2 mg of the antibody in a volume of 400 ul of modification buffer (*e.g.*, 100 mM Naphosphate, Pierce, Cat. #28372, 150 mM NaCl, pH 7.0) to make final concentration of 5mg/ml. For example, 2 mg of the antibody (13.33 nmol) requires with 333 nmol SANH ($13.33 \times 25 = 333$) for the modification reaction, thus 500ul of the antibody solution (4 mg/ml) is combined with 11 ul of the SANH solution (30 nmol/ul). The reaction

mixture was incubated at room temperature for 3 hours. SANH will react with side chains of lysine residues on the antibody to form a hydrazine group.

Step 4: Removal of excess SANH using Zeba Desalting columns

5 After the SANH-antibody modification step, excess SANH in the reaction mixture was removed using Zeba Desalting columns (Pierce Cat. #89889, 2ml columns). After loosening the cap and snapping off the bottom tip, the Zeba column was placed in a 15 ml tube and centrifuged for 1 minute to remove the storage solution. A mark was placed on the side of the column, indicating where the highest point of the upwardly slanted compacted resin was located. The column was placed in centrifuge
10 with the mark facing outward in all subsequent steps. 1 ml (pH 5) conjugation buffer (e.g., 100 mM Naphosphate, 150mM NaCl, pH 5.0) was used to equilibrate the column. After centrifuging at 1,000 x g for 2 minutes, the flow-through was discarded. This step was repeated for 3-4 times, until the pH of final flow-through was between 5.0 and 6.0.
15 The pH balanced column was placed in a new collection tube. The antibody-SANH reaction mixture was applied slowly to the center of the resin bed (200-700 ul volume capacity). To ensure maximal protein recovery, a stacker of pH 5 conjugation buffer (40 ul) was applied to resin bed after sample has been fully absorbed. The sample was then collected using a Mini quick spin oligo column (Roche Cat#11814397001)
20 after centrifuging at 1,000 x g for 2 minutes.

Step 5: Estimation of the number of SANH linkers per antibody

Before measurement, a p-NB (p-nitrobenzaldehyde, Solulink) solution was prepared by dissolving 5 mg p-NB in 100 ul DMF and adding a 75 ul aliquot of this
25 solution to a 50 ml volume of 100 mM MES buffer (Sigma C/N M2933, pH 5.0). The solution was protected from light and kept refrigerated. This solution will remain stable for 30 days at 4° C.

40 ul of the antibody-SANH reaction mixture was mixed with 460 ul of p-NB solution and incubated at 37°C for 30 minutes. The UV absorption at 390 nm was
30 measured to quantitatively determine the number of SANH linker per antibody. For example, if SANH is added 25 fold molar excess to the Ab, the average SANH linker per antibody should be around 4. After confirming the number of SANH linkers per antibody, the antibody-SANH solution was stored in aliquots at -80°C.

The following provides an example of the calculations and results of the procedure of Step 5:

1. Calculate protein concentration in mM

5 Protein concentration= 5mg/ml Ab MW=150,000
mM protein concentration=(5/150000)/1*1000=0.0333 mM

2. Calculate final assay protein concentration

(40/500 dilution)*0.0333=0.00264 mM

10

3. Calculate # of hydrazone bond

Ext.coeff. of hydrazone bond is 24,000

Final assay protein concentration= 0.00264mM

A390=0.268

15

Number of bonds= [A390/ 24]/ final assay protein concentration= 4.2

Example of A390 reading

	Concentration	A390	#Hydrazine
Ab-SANH	5 mg/ml	0.268	4.2
Ab	5 mg/ml	0.006	

20 Example 6: Conjugation of Ab-SANH with Oligo-SFB

The amount of Oligo-SFB and Ab-SANH to be used in the final conjugation reaction was calculated. Modified oligo is added in molar excess to the modified antibody, taking into account the number of SANH linkers. Essentially, the formula is:
[(amount of oligo) = (nmol of Ab) * (fold molar excess oligo) * (# of SANH linkers per
25 Ab)]. The final Ab concentration should be around 2-3 mg/ml. Conjugation buffer was used to adjust final volume.

The following provides a sample of the calculations described above:

30

Conjugates								
Ab-SANH	Ab-SANH concentration (mg/ml)	Average # of SANH linker per Ab	Ab-SANH Oligo (SFB)	Ab-SANH amount (mg)	Ab-SANH nmol (mg/1500)	Ab-SANH volume (µl)	Final volume (µl)	Final Ab concentration (mg/ml)
17C7-siRNA1	4.45	5.39	2	1000	0.7	224.7	339.0	3.0
17C7-siRNA2	4.45	7.34	2	1000	0.7	224.7	468.0	2.1
Oligo-SFB	Oligo-SFB (nmol) (44 nmol/1000 ratio)	Oligo-SFB Concentration (nmol/µl)	Oligo-SFB Volume (µl)					
17C7-siRNA1	44.8	2	22.4					
17C7-siRNA2	97.9	2	48.9					

The conjugation mechanism and procedures are illustrated in Figure 11, 12 and 13. The free aldehyde formed by the SFB modification of the oligo reacts with SANH on the modified antibody. Using the proper amounts of Ab-SANH and oligo-SFB, the reaction mixture was incubated at room temperature overnight, or for at least 3 hours, and then stored at -80°C. 1-2 ug of the conjugates were then analyzed by electrophoresis on a Tris-glycine gel, while 20 ug was analyzed by IEF gel. Additionally, the antigen binding activity of the conjugates was analyzed by ELISA.

Conjugates were assessed for variation in conjugation efficiency. As illustrated in the isoelectric focusing gel (Figure 14), two previous independent experiments resulted in significantly different amount of Ab-oligo conjugates. For optimization of the experiment result, different numbers of SANH linkers on the antibody and different molar ratios of Oligo to Ab-SANH were used for the conjugation process. As seen in Figure 15, the conjugation efficiency improved with higher number of SANH linkers on the antibody as well as higher molar ratio of oligo to Ab-SANH. Further optimization confirmed that with 7 SANH linkers on every antibody molecule, the conjugation efficiency was nearly 100% with 6 fold excess molar ration of oligo to Ab-SANH (Figure 16). While keeping number of SANH linkers on antibody constant, adding more Oligos (increasing the ratio to 10 or 15) did not further increase the conjugation efficiency (Figure 16). Similarly, the comparatively highest conjugation efficiency was achieved under the condition of 7 SANH linkers on every antibody molecule and 6 fold excess oligo to Ab-SANH, confirmed by electrophoresis using a 14% Tris-Glycine gel (Figure 17 and 18, Sample 10). Results for the optimization of the antibody-GFPz stable 2 siRNA conjugation can be seen in Figure 19. As before, it was observed that, with an average of 7 SANH linkers per antibody, the conjugation efficiency was comparatively higher when increasing the ratio of Oligo to Ab-SANH.

Example 7: Oxidation/ β -elimination of siRNA

This Example illustrates one of other oligo modification methods for Oligo-protein conjugations disclosed in the present application. Specifically, sodium periodate

(NaIO_4) was used to treat the siRNA oligos, to oxidize hydroxyls (-OH) at the 2' and 3' positions of the ring to generate aldehydes for subsequent conjugations (Figure 20).

Step 1: Set up annealing reactions to make duplex siRNA

5 200 pmoles of passenger strand RNA was 5'-radiolabelled with ^{32}P using T4 polynucleotide kinase. 4 uL 5X lysis buffer (Dharmacon's 5X buffer) was mixed with 5 uL of 10 uM passenger strand solution (50 pmol), 5 uL of 10uM guide strand solution (50 pmol) and 6 uL dH_2O . The mixture was incubated at 95°C for 2 minutes and then at 37°C for 1 hour for annealing. The resulting siRNA duplex contained 50 pmol of siRNA
10 in a total volume of 20 uL. Therefore the concentration of siRNA in this reaction was 50 pmol/20uL or 2.5pmol/uL (2.5uM).

Step 2: Oxidation of siRNA

Before siRNA oxidation, fresh 200 mM NaIO_4 solution was prepared by
15 resolving 42.7 mg of NaIO_4 in 1 mL RNase free water. The NaIO_4 solution was stored in vials wrapped with foil and placed on ice until needed.

For the siRNA oxidation process, 1.25 uL of fresh NaIO_4 solution was mixed with 5 uL duplex siRNA (12.5 pmol siRNA), 1 uL PBS (100mM, pH 7.2, 150mM NaCl), and 2.75 uL water. For a control reaction (mock oxidation), the same amounts of
20 siRNA duplex, PBS and water were mixed without NaIO_4 . The reaction mixture (or the control) was incubated without light at room temperature for 30 min. Ethanol precipitation according to the protocol above was then performed to create a pellet of oxidized duplex, which was then resuspended in 20 uL RNase free water.

Samples were tested via gel electrophoresis. 10 uL of each sample was mixed
25 with 5 uL water and 15 uL formamide loading dye and stored in freezer. The other 10 uL of each sample was first tested via β -elimination, in order to ascertain the completeness of the previous oxidation reaction. To perform this test, 10 uL of each sample was mixed with 0.5 uL 1 M NaOH and 4.5 uL water and incubated at 42°C for 90 minutes. The resulting mixture was mixed with 15 uL formamide loading dye and
30 were analyzed along with the samples without β -elimination on a 15% TBE-Urea gel. The gel was analyzed using a phosphoimager to determine the oxidation efficiency. Since β -elimination removes the 3' terminal nucleotide, it reduced the 21-mer siRNA oligos into 20-mer oligos. As shown in Figure 21, the oxidation reaction was complete

since the band at the 21-mer position in the oxidized samples completely disappeared after β -elimination. Only oxidized siRNA was β -eliminated, while the control (no NaIO_4 , or mock oxidized sample) did not. The addition of NaOH also led to some degradation of siRNA (Figure 21). With the same β -elimination testing for oxidation efficiency in different pH conditions, it was found that oxidation was complete in every condition tested (Figure 22). When tested with various siRNA concentrations, 25 mM NaIO_4 was sufficient to oxidize at least 100 μM siRNA (Figure 23).

Step 3: Reaction of oxidized siRNA with the EMCH linker

3,3'-N-[e-Maleimidocaproic acid] hydrazide, trifluoroacetic acid salt (EMCH) (*e.g.*, Pierce Cat. #22106) is a water-soluble, heterobifunctional, membrane permeable cross-linker. It contains a sulfhydryl reactive maleimide group and carbonyl reactive hydrazide group at each end of a 6-carbon spacer arm. Maleimides react with sulfhydryls at pH 6.5-7.5 to form stable thiol ether bonds, along with release of the maleimide leaving group. Proteins with cysteine residues that are not involved in disulfide bond formation are targets for maleimide reactive groups. Carbonyl groups, while not found naturally on proteins, can be formed by ring sugar reduction to form aldehydes which react with hydrazides at pH 5.5-7.5. In this Example, the oxidized siRNA oligo was linked with EMCH to form an siRNA-EMCH conjugate for subsequent siRNA-antibody conjugation (Figure 24). The reaction was carried out for different lengths of time and the siRNA-EMCH conjugation was compared after electrophoresis. As shown in Figure 25, the conjugation with EMCH led to a band shift of oxidized siRNA (21-mer). This conjugation was complete, since the unconjugated oxidized siRNA band disappeared almost completely (Figure 25). Further optimization experiments with varying concentrations of NaIO_4 , along with varying durations of oxidation, indicated that: 1) the oxidation was not complete after 1min in NaIO_4 (25 mM or 2.5 mM); 2) the reaction continued during the overnight precipitation; 3) the addition of EMCH prevented the completion of the oxidation; 4) a 30 minute (or longer) oxidation was necessary; 5) 25 mM NaIO_4 eliminated the reactivity of EMCH while 2.5 mM NaIO_4 did not significantly do so (Figure 26).

Step 4: Removal of unconjugated siRNA from the siRNA-EMCH conjugate using HPLC

To prepare for subsequent siRNA-antibody conjugation, the siRNA-EMCH conjugate was separated from free, unconjugated siRNA oligos in the conjugation reaction. HPLC was used for the separation (0.1 M ammonium acetate solution for Buffer A and 0.1 M ammonium acetate in 50% acetonitrile for Buffer B) (Figure 27). The sample reaction mixture was loaded for HPLC and the different components in the mixture were eluted out individually after their characteristic retention time in the column (Figure 28). After removing the unmodified passenger and 2'OMe guide fractions (Figure 29), the oxidized passenger-EMCH conjugate fraction was concentrated and purified.

Example 8: Modification of Antibody with Traut's Reagent

In this example, Traut's Reagent was used to modify lysine residues on the antibody to generate sulfhydryls (-SH) (Figure 30), which were used in subsequent conjugations with the siRNA-EMCH conjugates.

Antibody solution was concentrated down to ~ 5mg/mL in 1 X PBS. At the same time, a 14 mM stock of Traut's Reagent was prepared by dissolving approximately 2 mg of Traut's Reagent (Pierce, Cat. #26101) in 1 mL of water. 100 uL of antibody solution was transferred to an eppendorf tube and diluted to a final concentration of 5 mM using EDTA solution. Then varying molar excesses of Traut's reagent was added to the antibody solution, while total volume of each sample was brought up to the same volume in water. The molar concentration of antibody can be calculated as follows (for 5 mg/ml antibody):

$(5 \text{ mg/ml})(1 \text{ mole}/150,000 \text{ g})(1 \text{ g}/1000 \text{ mg})(1000 \text{ mmole}/1 \text{ mole})(1000 \text{ ml}/1\text{L}) = 0.033 \text{ mmole/L or } 0.033 \text{ mM}.$

The volume of Traut's reagent to add to each molar excess reaction can be determined as follows (to modify 200µl of antibody with a concentration of 5mg/ml at a 25 Fold molar excess):

$(X)(14 \text{ mM Traut's}) = (200 \text{ µl})(0.033 \text{ mM})(25 \text{ fold excess})$
 $X = 11.8 \text{ µl of Traut's needed}.$

After mixing Traut's reagent with the antibody solution, the reaction mixture was incubated for 1 hour at room temperature. The resulting mixture was desalted using

Zeba desalting columns as illustrated above and then measured for the number of free sulfhydryls using Ellman's Reagent.

To prepare a stock of Ellman's reagent, 4 mg of Ellman's reagent (Pierce Cat. #22582) was dissolved in 1 mL of 100 mM PBS, pH 8, 1 mM EDTA. A cysteine
5 standard curve was made by serial dilutions of a 1.5 mM cysteine stock using a 1:2 dilution in 100 mM PBS, pH8, 1 mM EDTA.

For the measurement process, 5 uL of Ellman's Reagent (4 mg/mL stock) was added to each well of a 96-well plate and then mixed with 250 uL of 100 mM PBS, pH8, 1 mM EDTA and 25 uL of unknown or cysteine standard. The plates incubated for
10 10 minutes at room temperature before measuring free sulfhydryls via spectrometry at 405 nm.

The concentration of sulfhydryl groups in a reaction was determined using the cysteine standard curve. The number of free SH groups was determined by dividing the sulfhydryl concentration in each sample by the corresponding antibody concentration
15 (as determined by Octet).

As shown in two parallel experiments, the number of modified lysine residues on the antibody increased with more Traut's reagent in the reaction (Figure 31). For example, with 50-fold excess Traut's reagent in two side-by-side experiments, an average of 0.8 and 1.6 lysine residues per antibody were accessed and modified by
20 Traut's reagent. When the amount of Traut's reagent increased to 200-fold excess, the average number of modified lysine residues on the antibody increased to 6.1 and 6.7.

To confirm that the antibody retained its antigen-binding activity after Traut's reagent modification, spectrometry measurements at 405nm were carried out for Traut's-modified antibody versus unmodified antibody. As shown in Figure 32, the
25 antigen-recognition activity of the modified antibody was not reduced significantly, even with Traut's reagent in 200-fold excess, which suggests that the antibody retains its biological activity after modifications.

30 Example 9: Conjugation of siRNA to a human monoclonal antibody

The present invention further includes methods to conjugate the modified antibody with the modified siRNA with a linker. In this Example, an siRNA-EMCH conjugate was further linked with a human monoclonal antibody modified by Traut's

reagent to form an siRNA-mAb conjugate (Figure 33). The antibody modified by Traut's was reacted with a 5-fold molar excess of the siRNA-EMCH conjugate. The reaction mixture was incubated at room temperature for 2 hours and the resulting conjugates were resolved on a native 4-20% gradient TRIS-HCL gel. Nucleic acids were analyzed by SYBR-Gold staining. Protein was analyzed by SYPRO-Ruby staining. As the result, only the combination of modified antibody and siRNA-EMCH resulted in the conjugation of siRNA to antibody, as demonstrated by the band shift on the gel, while any other combination containing unmodified siRNA and/or antibody led to no conjugation or band shifts (Figure 34). In another experiment, the 3' oxidized siRNA reacted with the EMCH linker (siRNA-EMCH) conjugated with the Traut's modified monoclonal antibody (with 5 free sulfhydryls per mAb quantitated with Ellman's reagent) in ~ 2:1 molar ratio (siRNA:free sulfhydryls on the mAb), resulting in the band shift on the 12% native TRIS-Glycine gels (Figure 35). Other unmodified mAb or siRNA could not conjugate together. Similar results were seen with electrophoresis using the IEF (isoelectric focusing) gel: the siRNA-antibody conjugates were only formed from modified siRNA and antibody, leading to a band shift to a region representing pI = 4 (Figure 36).

Example 10: siRNAs, antibodies and linkers used in the conjugation

Recent studies have demonstrated a promising therapeutic potential of duplex small interfering RNAs (siRNAs) in prevention and treatment of infectious disease, cancer and neurodegenerative disease *in vitro*. However, the major hurdle in therapeutic application of siRNA has been finding a method to direct the molecule into a desired cell population to achieve maximal therapeutic effect and avoid non-specific silencing or toxicity in cells other than intended targets.

Since duplex siRNAs cannot choose their cellular targets, it is necessary to design a "targeting agent" to deliver these siRNAs. Cell surface receptors have been explored as potential targets for gene delivery. Antibodies specifically binding to these surface receptors can serve as a carrying vector to delivery siRNAs into target cells expressing the corresponding cell surface protein recognized by the antibody. In the case of HCV, HCV infected cells will express the viral protein E1 and E2 on the cell surface. E1 and/or E2 specific antibodies will then deliver the siRNA to only HCV infected cells.

In this present study, covalent linkages between antibodies and siRNAs are utilized to form siRNA-antibody bioconjugates. siRNA-antibody bioconjugates are shown to specifically and effectively deliver siRNAs into cells via cell surface receptors. Techniques employed can be modified to create conjugates with RNA linked to receptor
5 ligands in lieu of antibodies. A heterobifunctional linker is used to link the chosen antibody and the chosen RNA at each of the side of the linker. The list of possible heterobifunctional linkers that could be used includes, but is not limited to: MaleimidoCaproic acid Hydrazide, HCl (EMCH, with the formula as $C_{10}H_{16}N_3O_3Cl$); MaleimidoPropionic acid Hydrazide, HCl (MPH, with the formula as $C_8H_{12}N_3O_3Cl$); N-
10 (k-Maleimidoundecanoic acid)hydrazide (KMUH, with the formula as $C_{15}H_{25}N_3O_3$); and 4-(4-N-MaleimidoPhenyl)butyric acid Hydrazide, HCl (MPBH).

In this example, the heterobifunctional linker is comprised of a maleimide molecule and a hydrazide molecule linked together. The maleimide side of this linker is further linked to one molecule of thiolated antibodies, through a thioether bond with a
15 thiol group on a $-NH_2$ side chain of a lysine residue found in the sequence of a heavy chain of the antibody. The thiolation agent for use in this example is 2-Iminothiolane (Traut's Reagent). The hydrazide side of this heterobifunctional linker is further linked through a pH-labile hydrazone bond to the aldehyde on one molecule of alkylated
20 siRNAs. This hydrazone bond in the linker is stable in serum at pH 7.4, but can hydrolyze at pH 5.0 in the endosomes, leading to the release of the siRNA conjugated to the antibody. In some embodiments, the linker bond is stable in the endosomes. In some embodiments, a stable linker bond in the endosome leads to the guide strand remaining attached to the antibody for some time after the passenger strand separates from the guide strand.

25 Using this approach, bioconjugates are created with a human monoclonal antibody, which specifically bind to an epitope either on the surface of HCV or on the surface of virally infected cells (*e.g.*, the E1 and/or E2 protein (glycoprotein) of HCV). Specifically, bioconjugates are created by using one of the antibodies of the invention, including 95-2, 95-14, 95-38, 83-128 and 073-1 and an anti-HCV siRNA, such as those
30 described above in Section IV, for example.

Example 11: Testing of anti-HCV siRNAs

One obstacle in the study of HCV has been the absence of an efficient tissue culture system for HCV. Viral replication and the efficiency of anti-HCV conjugates and siRNAs can be studied, however, using the HCV replicon system (Blight *et al.* Science 290:1972-74 (2000); Lohmann *et al.*, J. Virol. 77:3007-19 (2003)). A replicon is a subgenomic RNA that contains all essential elements and genes required for replication in the absence of structural genes. The HCVcc system also provides a means to study HCV *in vitro* (Lindenbach *et al.*, Science 309:623-626 (2005)). The HCVcc system features a genetically engineered, chimeric full-length HCV genome (including structural genes) that replicates and produces virus particles *in vitro*.

The siRNAs and protein conjugates of the invention are tested using a replicon or chimeric version of HCV. Anti-HCV antibodies are conjugates to anti-HCV siRNA molecules and to control siRNA molecules, either with a randomized sequence or with a sequence containing point mutations in an anti-HCV siRNA sequence that render the siRNA ineffective at knocking down HCV RNA, gene expression and/or viral particle production.

Other Embodiments

It is evident that those skilled in the art, given the benefit of the foregoing disclosure may make numerous modifications thereof, and departures from the specific embodiments described herein, without departing from the inventive concepts. For example, the antibody in the bioconjugate can be substituted by any antibody or antigen binding portion of antibody recognizing other viral proteins. The siRNA in the bioconjugate can be substituted by any siRNA targeting the genome of the virus, different isolates of HCV or genetic elements of cells that are or may be infected with HCV. The heterobifunctional linker connecting the siRNA and the antibody can comprise of any spacer moiety between a maleimide moiety and a hydrazide moiety.

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.

WHAT IS CLAIMED IS:

1. An nucleic acid-protein conjugate, comprising
 - (i) a protein comprising a free sulfhydryl moiety; and
 - (ii) an nucleic acid modified to comprise a free carbonyl moiety at the 3' or 5' terminuswherein the protein and nucleic are linked by a heterobifunctional crosslinker linking the sulfhydryl and carbonyl moieties to form the nucleic acid-protein conjugate.
2. The conjugate of claim 1, wherein the protein is a binding protein.
3. The conjugate of claim 1 or 2, wherein the nucleic acid is a nucleic acid silencing agent
4. A nucleic acid silencing agent -binding protein conjugate, comprising
 - (i) a binding protein that specifically binds to an epitope of a Hepatitis C Virus (HCV); and
 - (ii) a nucleic acid silencing agent having a sequence that is substantially complementary to a target mRNA associated with the Hepatitis C Virus to silence the target mRNA in a cell infected with the virus;wherein the protein and nucleic acid are linked by a heterobifunctional crosslinker to form the conjugate.
5. The conjugate of claim 4, wherein the binding protein comprises a free sulfhydryl moiety, the nucleic acid silencing agent is modified to contain a free carbonyl moiety at the 3' or 5' terminus, and the heterobifunctional crosslinker links the sulfhydryl and carbonyl moieties to form the nucleic acid -protein.
6. The conjugate of claim 3 or 5, wherein the nucleic acid silencing agent is a RNA silencing agent.
7. The conjugate of any one of claims 2 to 5, wherein the binding protein is an antibody or antigen binding portion that specifically binds to one or more protein epitopes within Hepatitis C Virus (HCV).

8. The conjugate of claim 1 or 7 wherein the protein is an antibody or antigen binding portion that specifically binds to one or more epitopes within the E2 protein of Hepatitis C Virus (HCV).

5

9. The conjugate of claim 1, 7 or 8 wherein the protein, antibody or antigen binding portion thereof inhibits the ability of the virus to infect cells.

10. The conjugate of any one of the preceding claims, wherein the protein is a human, humanized or chimeric antibody, an antibody competing therewith or an antibody binding other HCV-associated antigens.

11. The conjugate of any one of the preceding claims, wherein the antibody or antigen binding portion binds an epitope within amino acid residues 412-464, 412-423, or 413-420 of the E2 protein.

12. The conjugate of any one of the preceding claims, wherein the conjugate binds to two or more HCV genotypes of an HCV E2 protein or fragment thereof.

13. The conjugate of any one of the preceding claims, wherein the conjugate binds an HCV E2 protein from a HCV virus having a genotype selected from the group consisting of 1a, 1b, 2b, 3a, 4a, 5, 5a, 6a, 6g, 6k, and combinations thereof.

14. The conjugate of any one of the preceding claims, wherein the binding protein is an antibody variant selected from the group consisting of a multispecific antibody, a multivalent antibody, a nanobody and a single chain antibody.

15. The conjugate of any one of claims 1 to 11, wherein the binding protein is a fusion protein.

30

16. The conjugate of any one of the preceding claims, wherein the protein, antibody or antigen binding portion thereof specifically binds to the E2 protein, or fragment thereof, of a HCV virus with a K_D of at least about 1×10^{-7} M, 1×10^{-8} M, 1×10^{-9} M, 1×10^{-10} M, 1×10^{-11} M, 1×10^{-12} M or better.

35

17. The conjugate of any one of the preceding claims, wherein the antibody is a human monoclonal antibody selected from the group consisting of 83-128, 95-2, 073-1, 95-14, 95-15, 95-18, 95-20, 95-21, 95-25, 95-26, 95-30, 95-38, 95-39, 95-42, 95-43, 95-48, 95-49, 95-52, 95-54, 95-58, and 95-62.

18. The conjugate of any one of the preceding claims, wherein the antibody is a human monoclonal antibody selected from the group consisting of 83-128, 95-2 and 073-1.

5

19. The conjugate of any one of the preceding claims, wherein the antibody comprises CDRs derived from a human monoclonal antibody selected from the group consisting of 83-128, 95-2, 073-1, 95-14, 95-15, 95-18, 95-20, 95-21, 95-25, 95-26, 95-30, 95-38, 95-39, 95-42, 95-43, 95-48, 95-49, 95-52, 95-54, 95-58, and 95-62.

10

20. The conjugate any one of the preceding claims, wherein the antibody comprises CDRs derived from antibody 83-128, 95-2 or 073-1.

21. The conjugate of any one of the preceding claims, wherein the antibody comprises a heavy chain variable region that is encoded from gene VH 3-33 and, optionally, a light chain variable region encoded from gene V κ L6.

15

22. The conjugate of any one of the preceding claims, wherein the RNA silencing agent is selected from the group consisting of a siRNA, a miRNA, a shRNA and an antagomir.

20

23. The conjugate of any one of the preceding claims, wherein the RNA inhibits replication of HCV.

24. The conjugate of any one of the preceding claims, wherein the target mRNA is encoded by the genome of the HCV.

25

25. The conjugate of any one of the preceding claims, wherein the crosslinker comprises a maleimide moiety and a hydrazide moiety, linked by a spacer moiety.

30

26. The conjugate of any one of the above claims, wherein a maleimide moiety of a crosslinker reacts with a free sulfhydryl group on the antibody, or antigen binding portion thereof, to form a thioester bond.

35

27. The conjugate of claim 18, wherein the free sulfhydryl group on the antibody, or antigen binding portion thereof, is added to lysines via a thiolation agent.

28. The conjugate of claim 27, wherein the thiolation agent is 2-Iminothiolane (Traut's Reagent).

5 29. The conjugate of any one of the preceding claims wherein the heterobifunctional crosslinker is selected from the group consisting of *N*-(ϵ -maleimidocaproic acid) hydrazide, *N*-(β -maleimidopropionic acid) hydrazide and *N*-(κ -maleimidoundecanoic acid) hydrazide, including EMCH, BPMH, KMH and other linkers containing a maleimide and a hydrazide.

10

30. The conjugate of claims 25-29, wherein the hydrazide moiety of the crosslinker reacts with a carbonyl group on the RNA to form a hydrazone bond.

15 31. The conjugate of claims 25-29, wherein the carbonyl group on the 3' or 5' end of the RNA, is added by chemical means.

32. The conjugate of claims 25-29, wherein the carbonyl group on the 3' end of the RNA is formed by reaction with sodium periodate.

20 33. The conjugate of claims 25-29, wherein the carbonyl group on the 5' is introduced by standard RNA synthesis techniques, e.g. chemically synthesized phosphoramidites.

25 34. The conjugate of claims 25-29, wherein the carbonyl group on the 5' end is introduced by a chemical cross linker containing a carbonyl at one end and an NHS-ester at the other in which the NHS ester is reactive with an amine group at the 5' end of RNA generated by standard RNA synthesis techniques.

30 35. The conjugate of any one of the preceding claims, wherein the RNA is double stranded.

36. The conjugate of any one of the preceding claims, wherein the RNA comprises sense and antisense strands of 18-30 nucleotides in length.

35 37. The conjugate of claim 36, wherein the RNA comprises sense and antisense strands of 21-23 nucleotides in length.

38. The conjugate of claim 36, wherein the RNA comprises sense and antisense strands of 25-30 nucleotides in length.

39. The conjugate of claim 38, wherein the RNA comprises sense and antisense strands of 27 nucleotides in length.

5 40. The conjugate of claims 35-38, wherein the RNA comprises 3' overhangs of 1-3 nucleotides.

41. The conjugate of claim 40, wherein the 3' overhangs are dTdT overhangs.

10 42. The conjugate of claims 35-39, wherein the RNA lacks 3' overhangs.

43. The conjugate of any one of the preceding claims, wherein the RNA comprises an antisense strand that is complementary to target sequences in the mRNA encoding E1 protein or E2 protein from a HCV isolate.

15

44. The conjugate of any one of the preceding claims, wherein the conjugate is internalized by receptor-mediated endocytosis.

20 45. The conjugate of any one of the preceding claims, wherein the RNA silencing agent is released from the conjugate by acid hydrolysis.

46. The conjugate of any one of the preceding claims wherein the RNA exhibits greater than 50% silencing efficiency of the target mRNA in a silencing assay relative to a control RNA.

25

47. The conjugate of any one of the preceding claims, wherein silencing is measured using a replicon system or a HCVcc system.

30 48. The conjugate of any one of the preceding claims, further comprising at least a second RNA linked to the protein, antibody or antigen binding portion by a heterobifunctional crosslinker, wherein the second RNA silencing agent has a sequence that is substantially complementary to a second HCV-associated target mRNA in the HCV-infected cell to mediate cleavage of the second target mRNA.

35 49. The conjugate of claim 48, wherein the crosslinker linking the first RNA silencing agent and the crosslinker linking the second siRNA silencing agent are the same.

50. The conjugate of claim 48, wherein the crosslinker linking the first RNA silencing agent and the crosslinker linking the second RNA silencing agent are different.

5 51. The conjugate of any of the preceding claims, wherein the RNA portion thereof inhibits HCV infection *in vitro*.

52. The conjugate of any of the preceding claims, wherein the RNA portion thereof inhibits HCV replication *in vitro*.

10

53. The conjugate of any of the preceding claims, wherein the RNA portion thereof treats HCV-mediated disease *in vivo* in a mammal.

15 54. The conjugate of any of the preceding claims, wherein the conjugate is used to treat HCV-mediated pathology *in vivo* in a mammal.

55. The conjugate of any of the preceding claims, wherein the RNA protects from or inhibits HCV-mediated hepatocyte pathology in a subject.

20 56. A method of treating or preventing a HCV-associated disease or disorder in a subject comprising, administering to the subject the RNA-protein conjugate of any of the preceding claims in an amount effective to treat or prevent a symptom or disorder associated with the HCV infection.

25 57. The method of claim 56, wherein said subject is resistant to antiviral therapy.

58. The method of claim 56, where said subject has received a liver transplant.

30

59. The method of any of claim 56, wherein the conjugate is administered in combination with a second therapeutic agent.

60. The method of claim 59, wherein the second agent is an antiviral agent.

35

61. The method of any of claim 59 or 60, wherein said second therapeutic agent is administered sequentially with the conjugate.

62. The method of claim 61, wherein said second therapeutic agent is administered prior to the administration of the conjugate.

5 63. A pharmaceutical composition suitable for treating a HCV-mediated disease or disorder in a subject comprising the conjugate of any of claims 1-55 in a pharmaceutically acceptable carrier.

64. The composition of claim 63, wherein the composition further comprises
10 a second therapeutic agent.

65. The composition of any of claim 63 or 64, wherein the conjugate is present in the composition in an amount that is less than 10% than the amount of the second therapeutic agent.

15

66. A method of making a nucleic acid-binding protein conjugate, comprising

(i) providing a binding protein comprising a free sulfhydryl group
(ii) providing a nucleic acid comprising a modified ribonucleotide at a 3' or 5'
20 terminus of the nucleic acid, wherein the nucleic acid comprises a nucleic acid strand that is sufficiently complementary to a target mRNA to silence expression of the target mRNA, and wherein the modified ribonucleotide comprises a modified carbonyl moiety;

(iii) combining the binding protein and the nucleic acid with a heterobifunctional
25 linker under condition such that the binding protein and the nucleic acid are linked by the heterobifunctional crosslinker to form the nucleic acid-binding protein conjugate.

67. The method of claim 66, wherein the crosslinker comprises a maleimide moiety and a hydrazide moiety, linked by a spacer moiety.

30

68. The method of claim 66 or 67, wherein a maleimide moiety of a crosslinker reacts with the free sulfhydryl group on the binding protein to form a thioester bond.

69. The method of any one of claim 66-68, wherein the free sulfhydryl group
35 is added to a lysine residue of the binding protein via a thiolation agent.

70. The method of claim 69, wherein the thiolation agent is 2-Iminothiolane (Traut's Reagent).

71. The method of any one of claims 67-70 wherein the heterobifunctional crosslinker is selected from the group consisting of *N*-(ϵ -maleimidocaproic acid) hydrazide, *N*-(β -maleimidopropionic acid) hydrazide and *N*-(κ -maleimidoundecanoic acid) hydrazide, including EMCH, BPMH, KMUH and other linkers containing a maleimide and a hydrazide.

72. The method of claim 67, wherein the hydrazide moiety of the crosslinker reacts with the free carbonyl group of the nucleic acid to form a hydrazone bond.

73. The method of claims 67-72, wherein the free carbonyl group is formed by oxidizing the modified ribonucleotide with sodium periodate.

74. The method of claim 73, wherein the modified ribonucleotide is at the 3' terminus of the nucleic acid.

75. The method of any one of claims 67-72, wherein the free carbonyl group is introduced by chemical synthesis with phosphoramidites.

76. The method of any one of claims 67-72, wherein the free carbonyl group is introduced by a chemical cross linker containing a carbonyl at one end and an NHS-ester at the other in which the NHS ester is reactive with an amine group at the 5' end of RNA generated by standard RNA synthesis techniques.

77. The method of claim 75 or 76, wherein the modified ribonucleotide is at the 5' terminus of the nucleic acid.

Figure 1A: 95-2

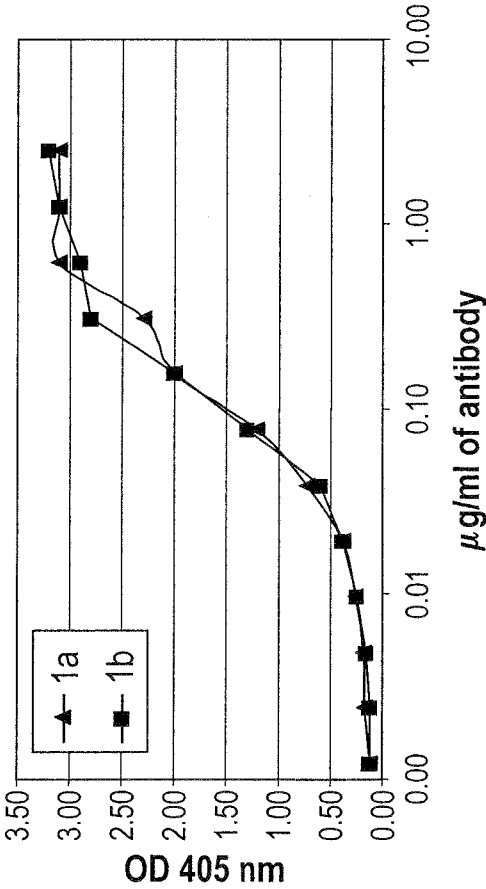
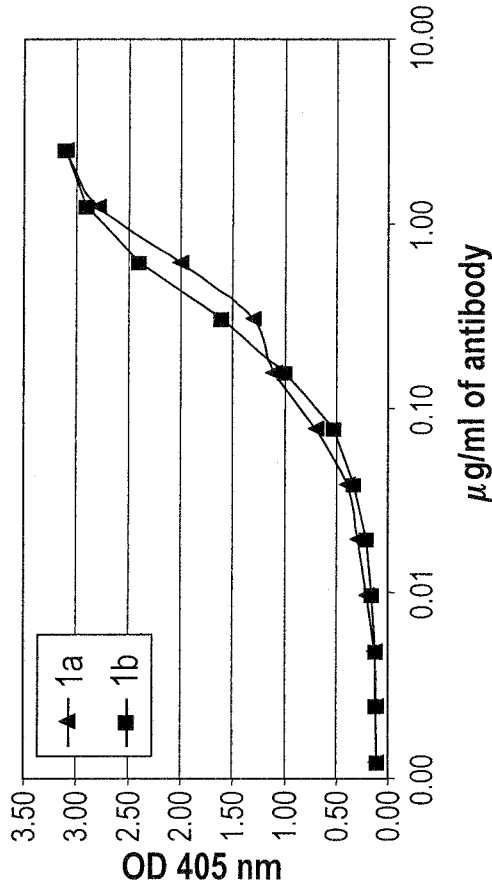
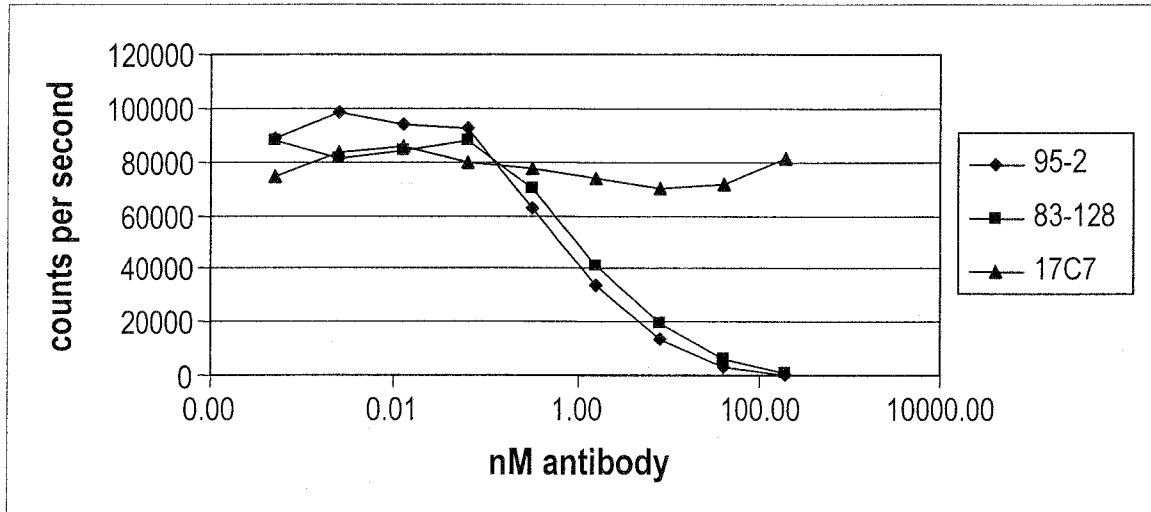
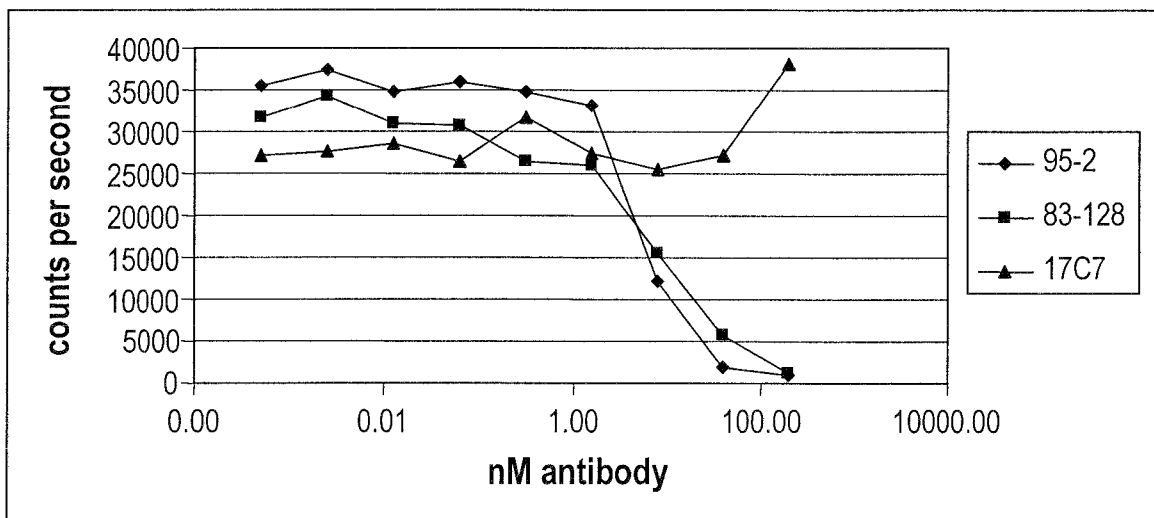


Figure 1B: 83-128



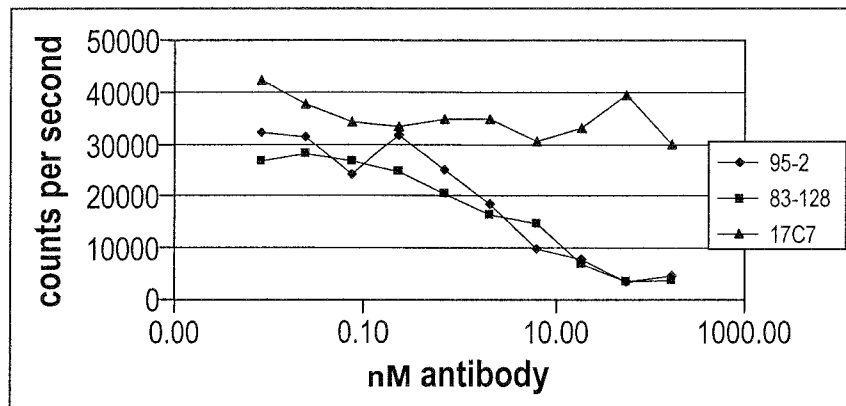
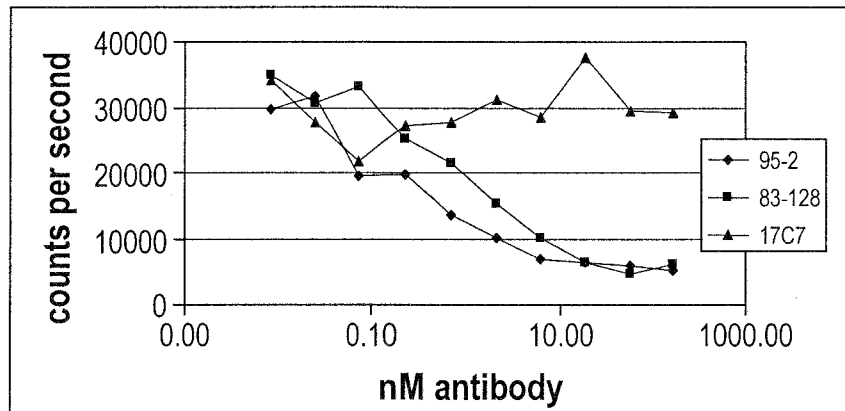
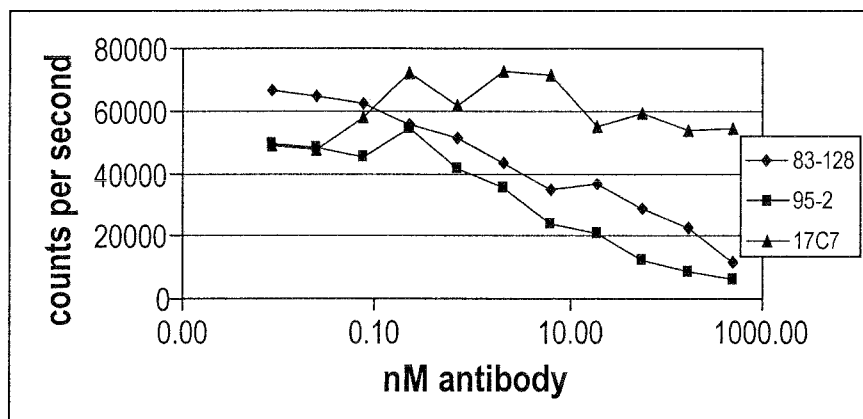
Genotype 1 soluble E2 ELISA. ELISA plates were coated with 2 $\mu\text{g/ml}$ of 1a E2-660 (triangles) or 1b E2-661 (squares) and probed with HuMabs 95-2 and 83-128 in two-fold dilutions. Bound antibody was detected with goat-anti-human secondary antibody conjugated to alkaline phosphatase and PNPP substrate.

Fig. 1

1a pseudovirus**1b pseudovirus**

HCV genotype 1a and 1b pseudovirus neutralization. Five-fold dilutions of HuMabs 95-2, 83-128, and 17C7 (rabies HuMab, negative control) were incubated with HCV pseudovirus for 1 hr at room temperature. The virus-antibody mixture was added to Hep3B cells followed by incubation at 37°C for 72 hrs. Infection was quantitated with Brightglo luciferase assay and read in a Victor3 plate reader for light output.

Fig. 2A

2b pseudovirus**3a pseudovirus****4a pseudovirus**

HCV genotype 2b, 3a and 4a pseudovirus neutralization. Five-fold dilutions of HuMabs 95-2, 83-128, and 17C7 (rabies HuMab, negative control) were incubated with HCV pseudovirus for 1 hr at room temperature. The virus-antibody mixture was added to Hep3B cells followed by incubation at 37°C for 72 hrs. Infection was quantitated with Brightglo luciferase assay and read in a Victor3 plate reader for light output.

Fig. 2B

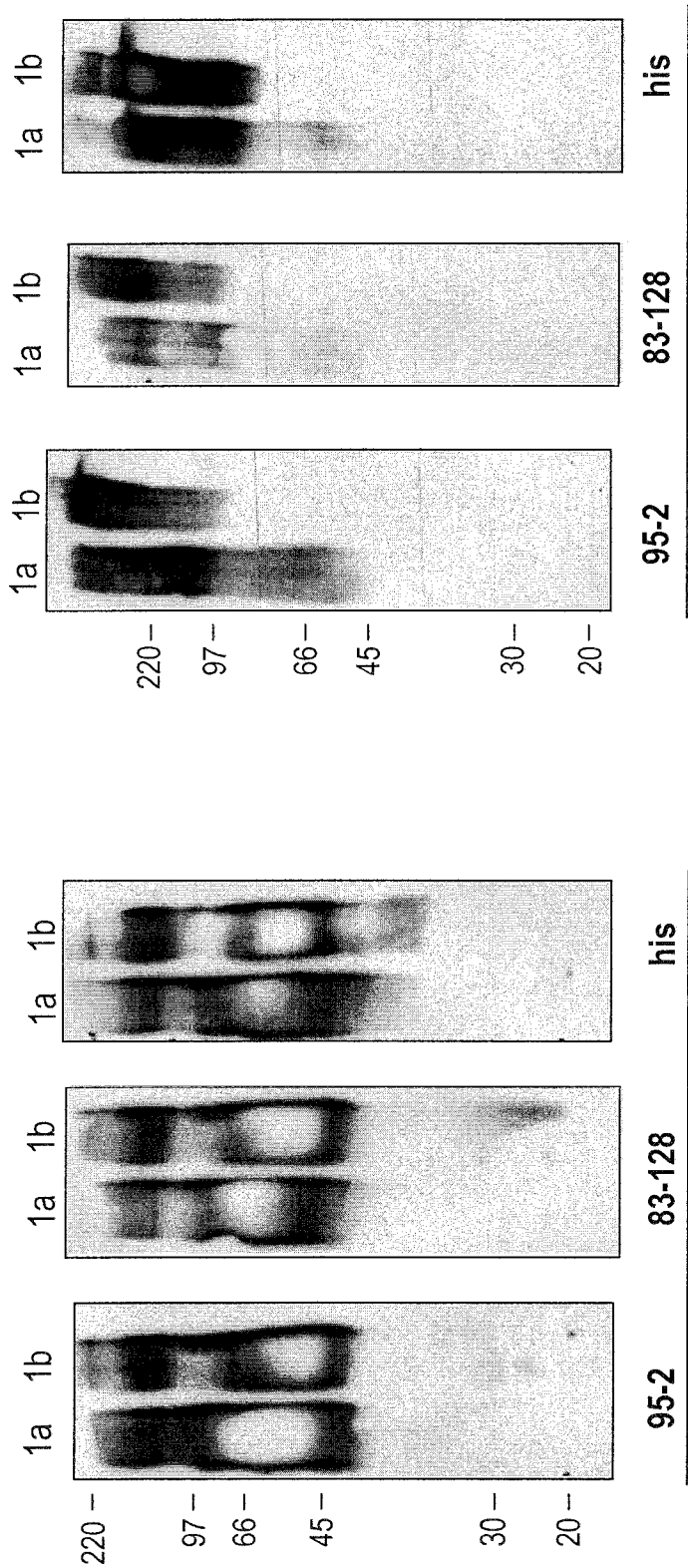


Figure 3A: Reduced

Figure 3B: Non-Reduced

Reducing and non-reducing Western blots of genotype 1a and 1b E2 soluble protein. Purified soluble mammalian expressed E2 protein (E2-661) from genotype 1a and 1b was subjected to reducing or non-reducing SDS-PAGE followed by transfer to PVDF membrane. Western blots were performed with anti-his tag monoclonal (his), 83-128 and 95-2 using anti-mouse IgG (his) or anti-human IgG (95-2 and 83-128) conjugated to HRP with enhanced chemiluminescent detection reagent. The genotype of the soluble E2 is indicated above the blots. The primary antibody used for detection is listed below the blots. The molecular weight markers in kilodaltons is listed to the left of each set of blots.

Fig. 3

	ka (1/Ms)	kd (1/s)	KD (M)
95-2	1.6e5	3.0e-4	1.9e-9
83-128	1.9e5	7.3e-4	3.8e-9

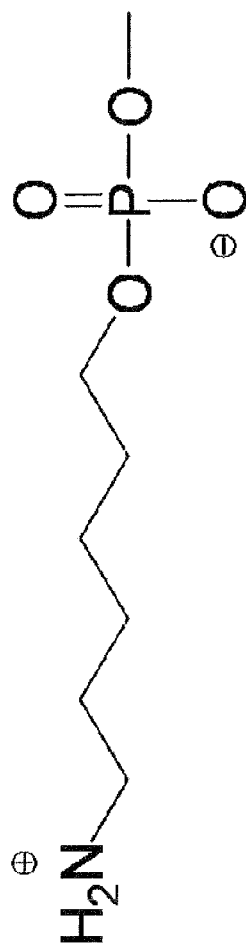
95-2 and 83-128 affinity determination for E2 412-423 epitope expressed as a bacterial fusion protein. Goat anti-human IgG Fc was amide coupled to the Biacore chip. 95-2 and 83-128 were separately captured on the chip and E2-G bacterially expressed protein containing E2 412-423 amino acids was flowed over at varying concentrations. The BIAevaluation software was used to fit the curves and calculate the affinity constants.

Fig. 4

FIG. 5

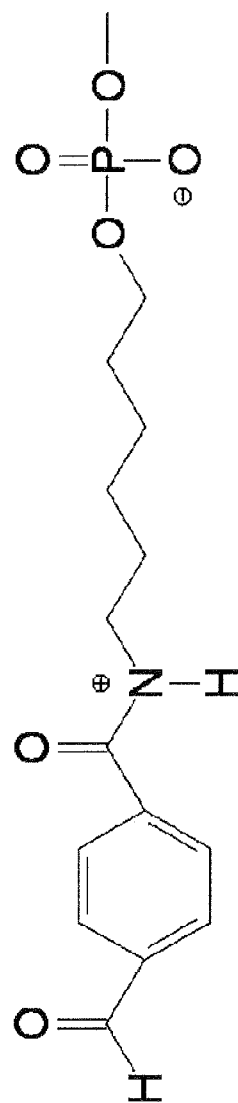
Oligo with N6 linker and

SFB



Add SFB

aldehyde



Modification of oligonucleotide with SFB

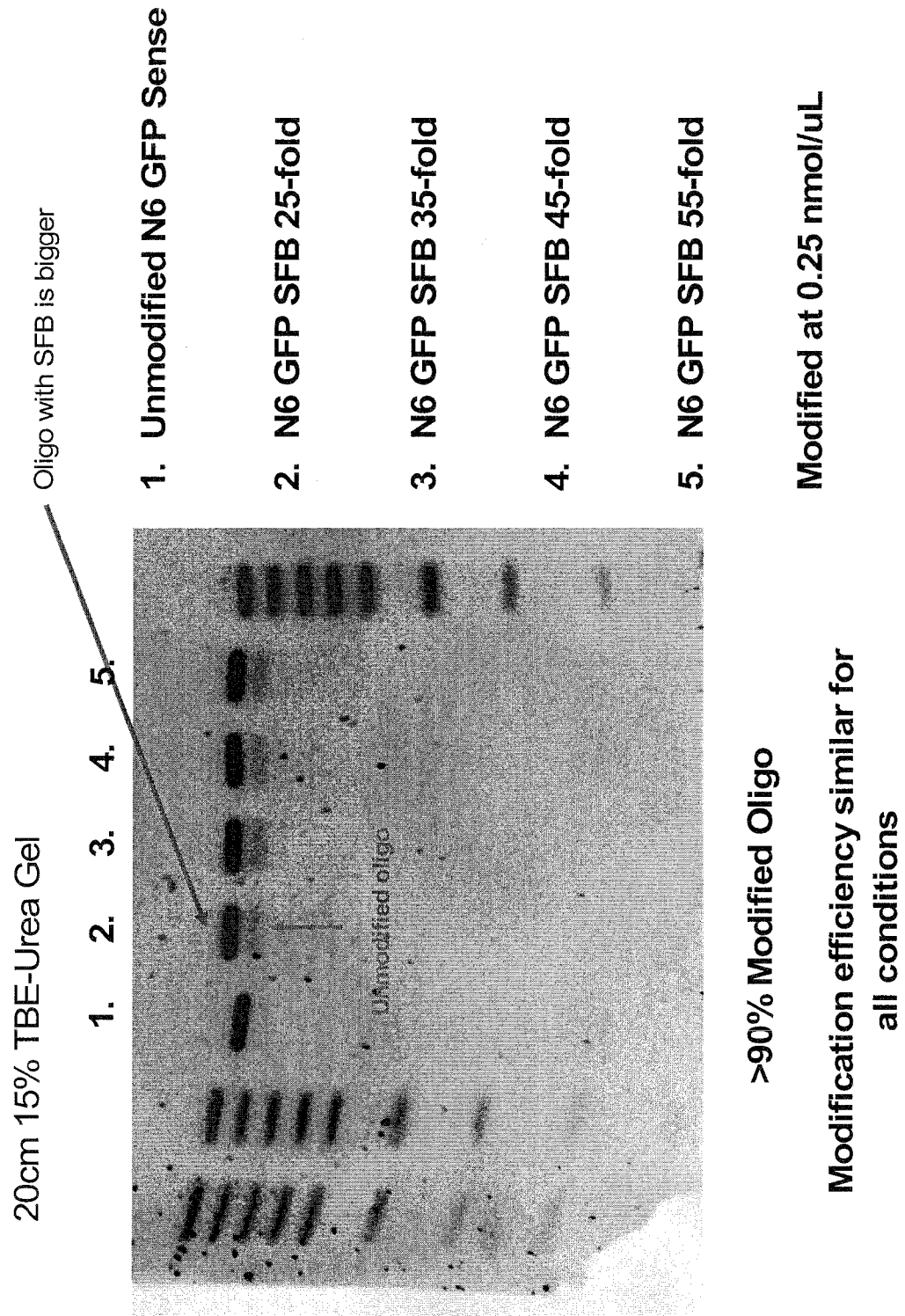


Figure 6

Comparison of Duplex vs. ssDNA Modification

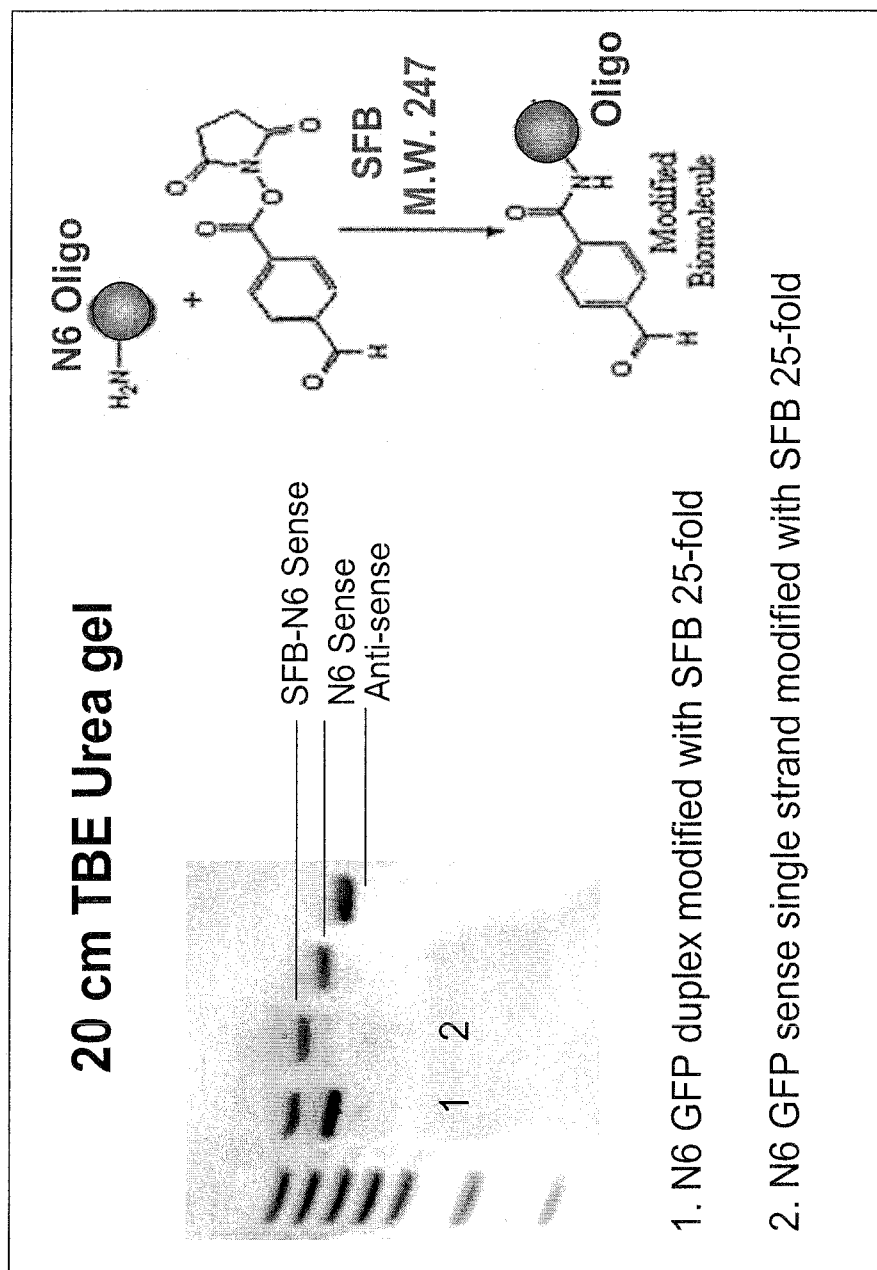
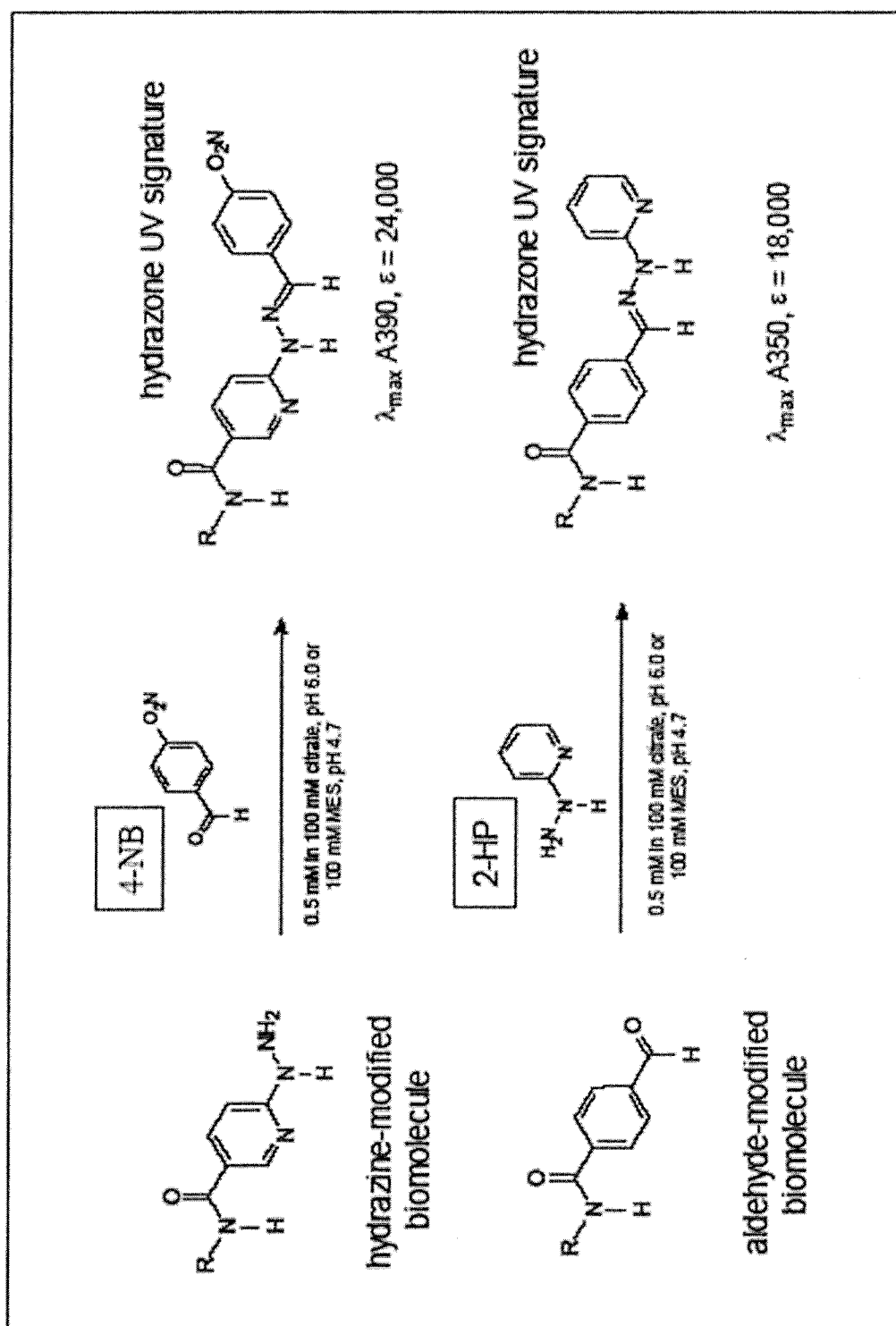


FIG. 7

□ > 90% of Oligo is modified with SFB

FIG. 8



Track SANH conjugation using 4-NB

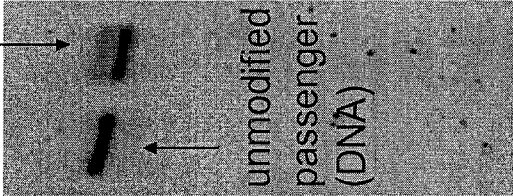
Track SFB conjugation using 2-HP

Modification of siRNA (N6-GFPz Stable2) with SFB

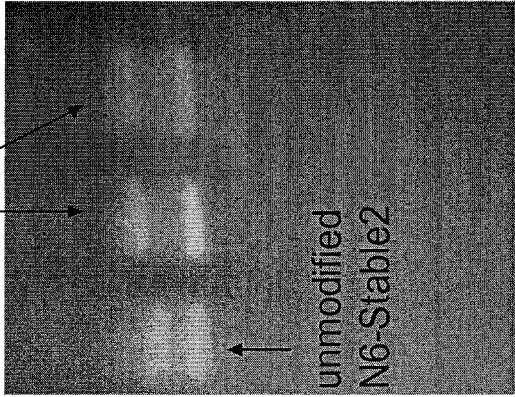
2-HP method confirms >90% modification of siRNA

- Dilute sample into 2-HP and incubate 1 hour at 37°C
- Use spec to measure OD₂₆₀ and 350nm reading
- Use Solulink calculator to determine % oligo modified
- 2-HP results are comparable to results visualized on gel.

partially modified
passenger (DNA)
not dialyzed



SFB-siRNA duplex
(dialyzed)



oligo MW	6821
OD/ μ mol	197
OD/ μ L	0.0239
μ mol/OD	0.005076
μ mol/ μ L	0.000121
oligo name	XXXX
OD/ μ L	0.0239
μ mol/OD	0.005076
μ mol/ μ L	0.000121
sample (μ L)	20
total OD	0.478
μ mol	0.002426
reagent (μ L)	500
μ M	4.85E-06
A350	0.0252
μ M [hyd]	1.4E-06
MSR*	28.8%

oligo MW	13526	oligo MW	13526
OD/ μ mol	393	OD/ μ mol	393
OD/ μ L	0.0253	OD/ μ L	0.0332
μ mol/OD	0.002545	μ mol/OD	0.002545
μ mol/ μ L	6.44E-05	μ mol/ μ L	8.45E-05
oligo name	XXXX	oligo name	XXXX
OD/ μ L	0.0253	OD/ μ L	0.0332
μ mol/OD	0.002545	μ mol/OD	0.002545
μ mol/ μ L	6.44E-05	μ mol/ μ L	8.45E-05
sample (μ L)	20	sample (μ L)	20
total OD	0.506	total OD	0.664
μ mol	0.001288	μ mol	0.00169
reagent (μ L)	500	reagent (μ L)	500
μ M	2.58E-06	μ M	3.38E-06
A350	0.0437	A350	0.0575
μ M [hyd]	2.43E-06	μ M [hyd]	3.19E-06
MSR*	94.3%	MSR*	94.5%

FIG. 9

IC₅₀ of SFB modified GFPZ Stable 6

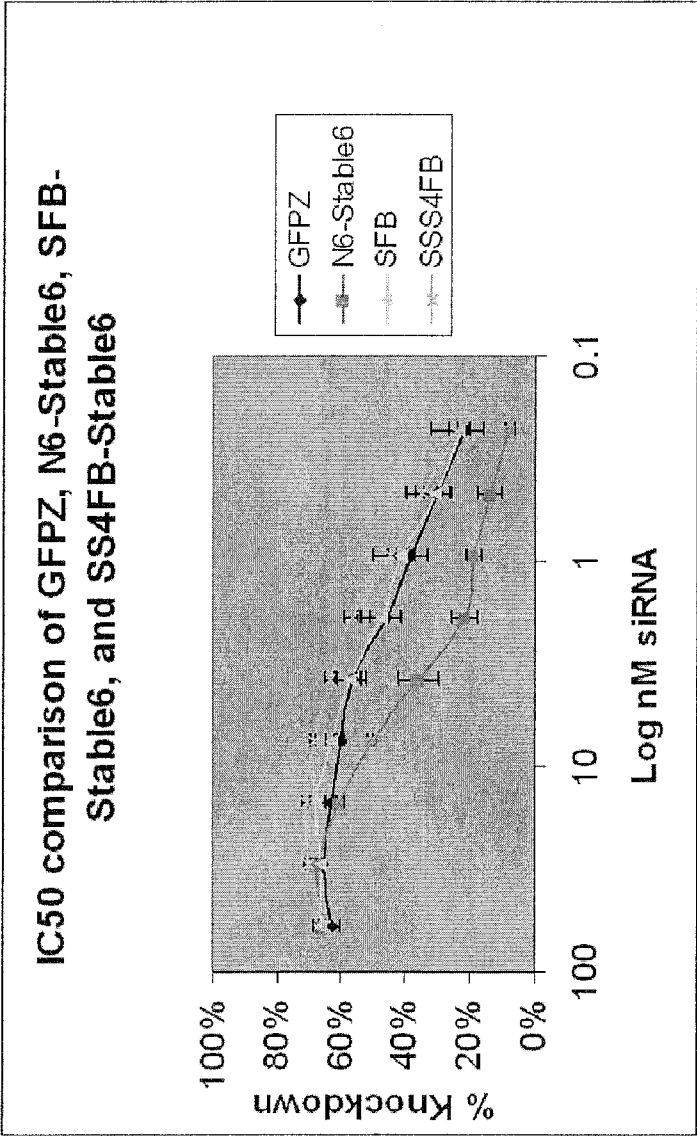


FIG. 10

GFPZ	Guide:	5' UAACUUCAGGGUCAGCUUGCCG 3'
	Passenger:	3' UAAUUGAAGUCCAGUCGAACG 5'
N6GFPZ Stable 6	Guide:	5' UAACUUCAGGGUCAGCUUGCmCmG 3'
	Passenger:	3' GmAmAmUmUGAAGUCCAGUCGAACG-N6 5'
SFB-GFPZ Stable 6	Guide:	5' UAACUUCAGGGUCAGCUUGCmCmG 3'
	Passenger:	3' GmAmAmUmUGAAGUCCAGUCGAACG-N6-SFB 5'
SSS4FB-GFPZ Stable 6	Guide:	5' UAACUUCAGGGUCAGCUUGCmCmG 3'
	Passenger:	3' GmAmAmUmUGAAGUCCAGUCGAACG-N6-SSS4FB 5'

Ab-DNA/RNA duplex conjugation

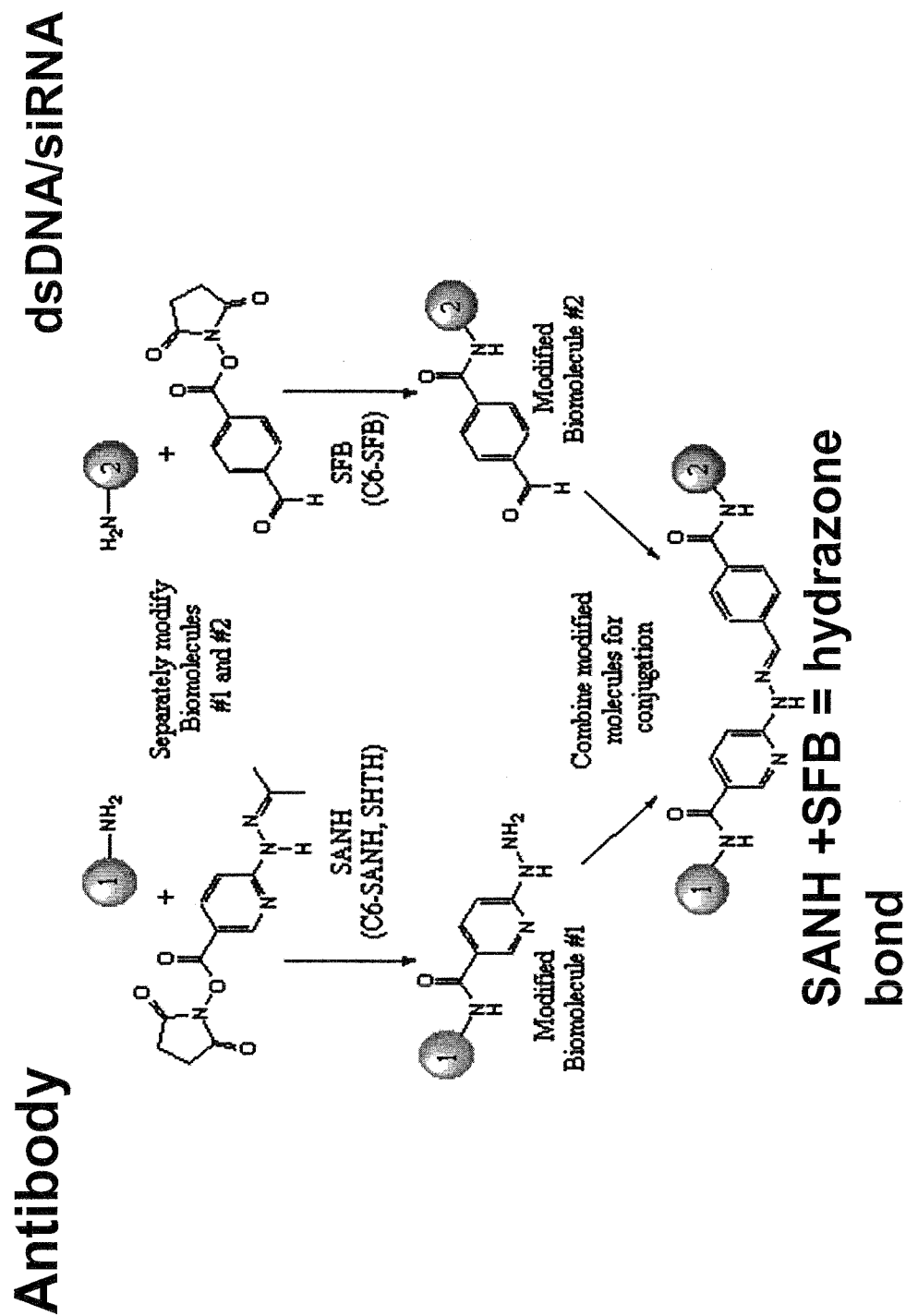


FIG. 11

2-Day Conjugation Protocol

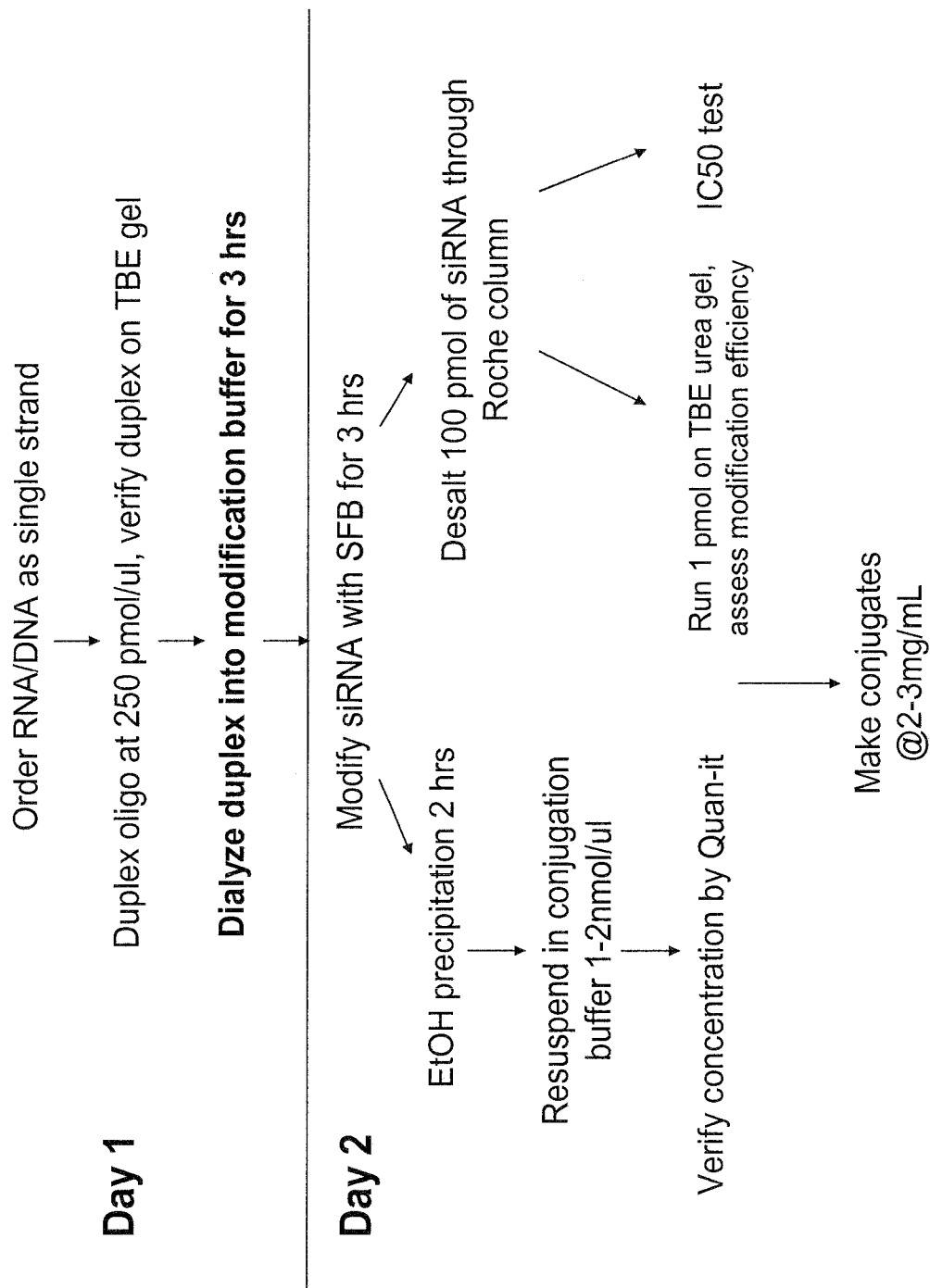
**FIG. 12**

FIG. 13

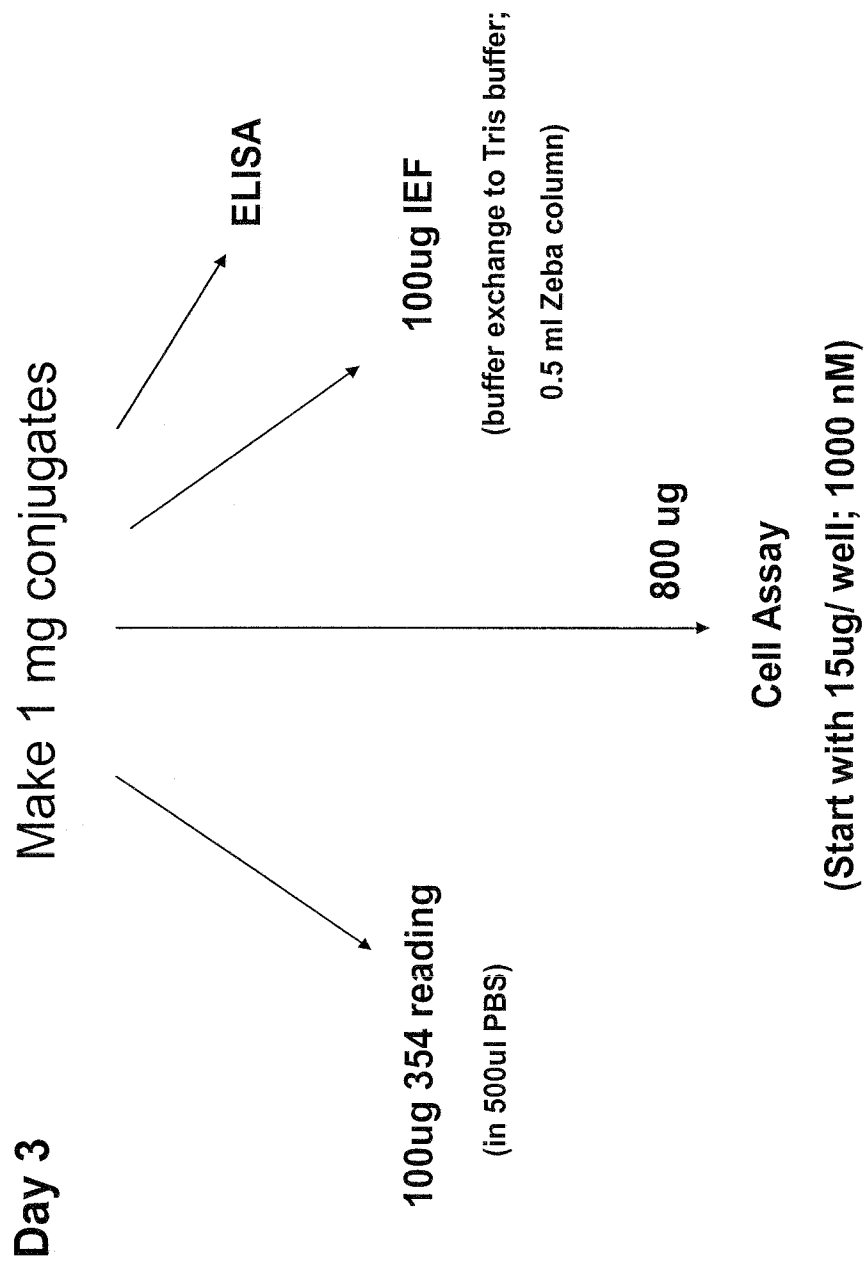


FIG. 14

Ab-DNA/RNA duplex conjugation – early non-optimized data

Isoelectric Focusing gel – adding oligo with neg charge changes pI of antibody

□ Problem--- Variation in conjugation efficiency

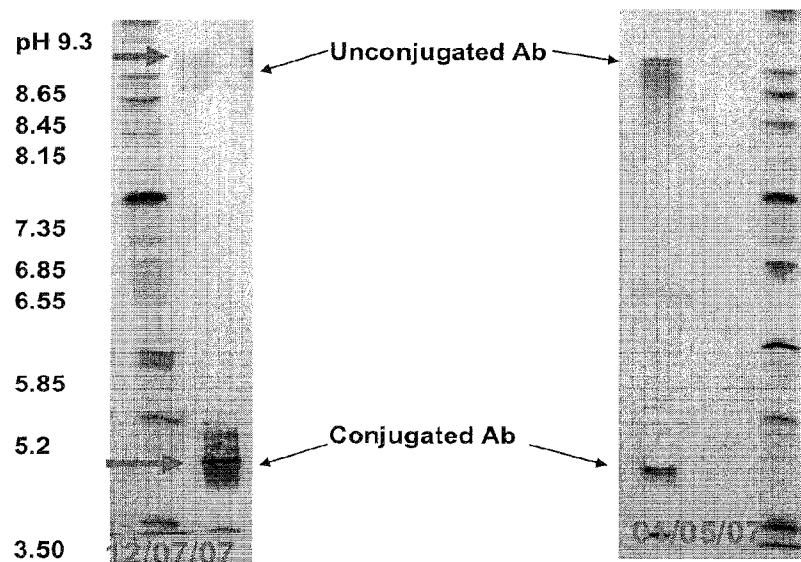
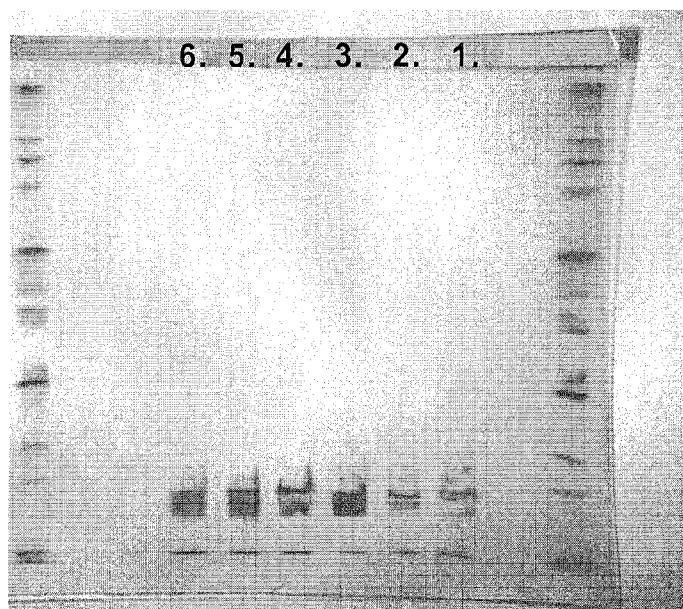


FIG. 15

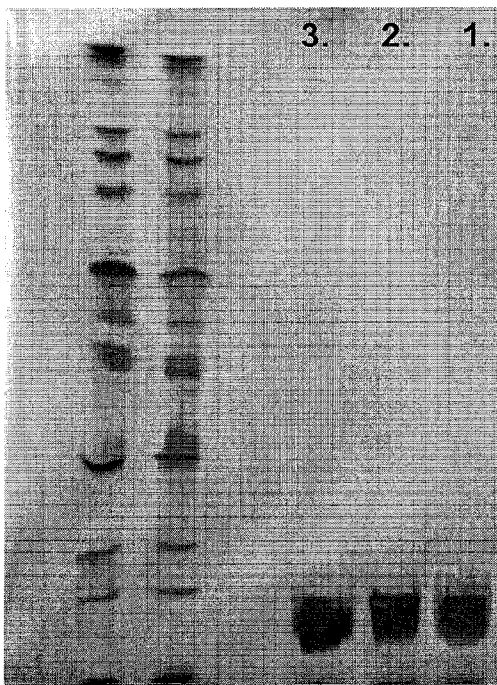
Improved Conjugation Efficiency With Fully Modified DNA-SFB



Performed 4/22/08

Sample	# of SANH/Ab	Molar Ratio of Oligo to Ab- SANH
1	4	4
2	4	6
3	7	2
4	7	4
5	7	6
6	7	10

75ug Conjugations

FIG. 16**Optimal Conjugation with Modified Oligo**

Performed 4/2/08

Sample	# of SANH/Ab	Molar Ratio of Oligo to Ab-SANH
1	7	6
2	7	10*
3	7	15

*50nmol oligo needed for 100ug (0.67nmol Ab)
conjugation

100% shift with 6 fold excess oligo

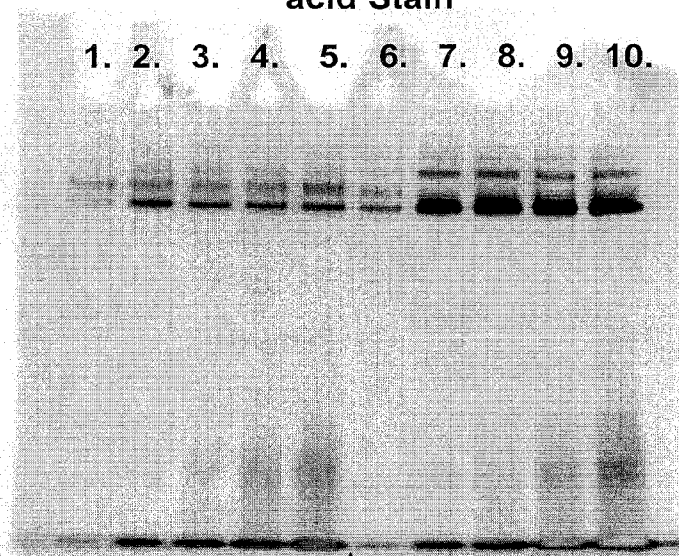
FIG. 17**14% Tris-Glycine**

Antibody alone will not run on this gel

Sybr Gold nucleic
acid Stain

1ug Ab loaded per lane

1. 2. 3. 4. 5. 6. 7. 8. 9. 10.



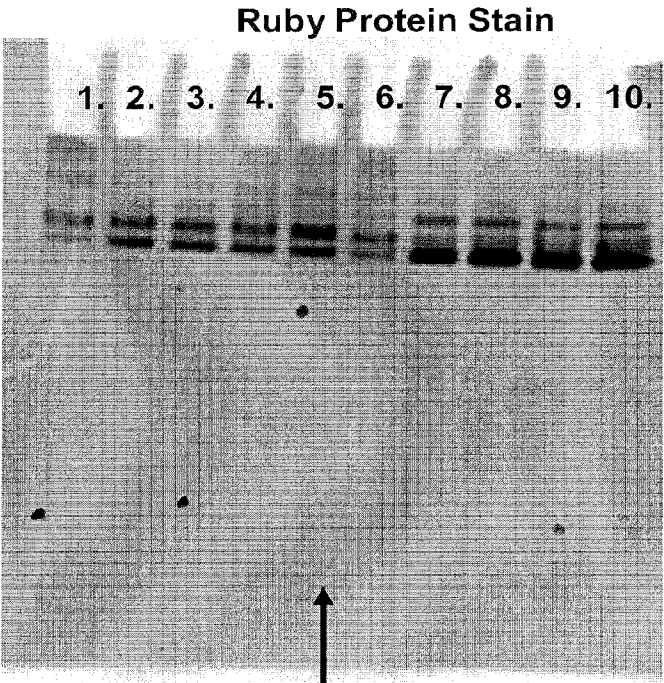
Sample	# of SANH/Ab	Molar Ratio of Oligo to Ab- SANH
1	4	0.2
2	4	1
3	4	2
4	4	4
5	4	6
6	7	0.2
7	7	1
8	7	2
9	7	4
10	7	6

Various excess
unconjugated oligo

6 fold excess oligo not run on IEF

FIG. 18

Tris-Glycine

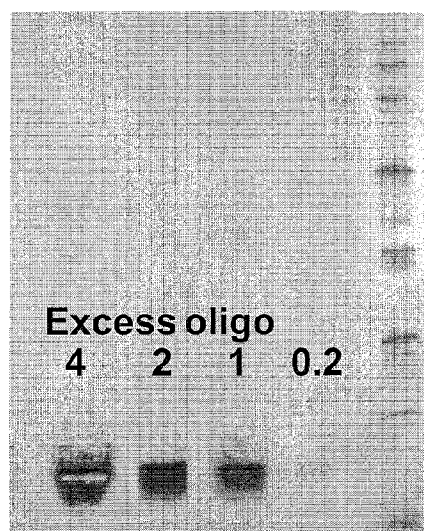
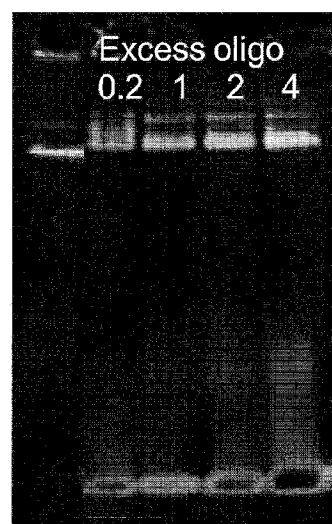
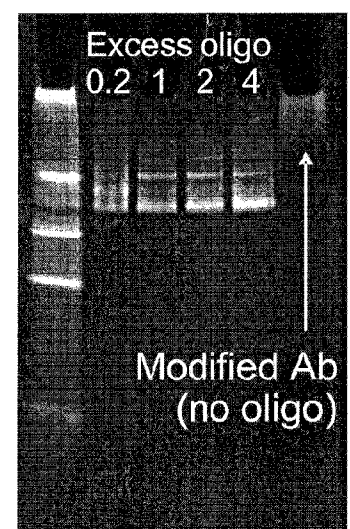


Sample	# of SANH/Ab	Molar Ratio of Oligo to Ab-SANH
1	4	0.2
2	4	1
3	4	2
4	4	4
5	4	6
6	7	0.2
7	7	1
8	7	2
9	7	4
10	7	6

FIG. 19

AB - GFPz stable 2 siRNA Conjugation

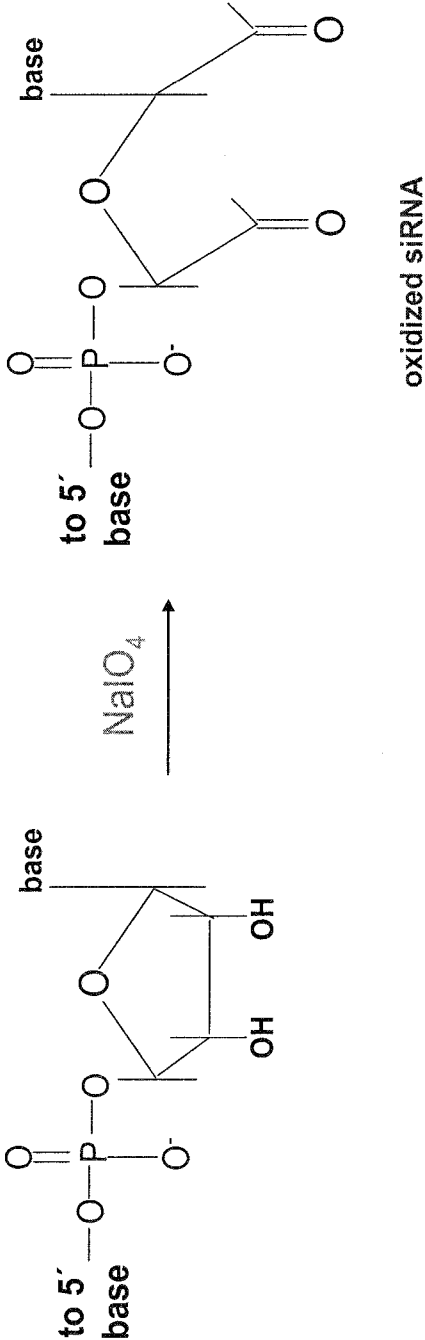
- Average of 7 SANH linkers per Ab
- Conjugated at Ab concentration of 2.5mg/ml

**IEF****Sybr Gold****Ruby Protein Stain**

3' oxidation of siRNA

Treat siRNA with sodium periodate (NaIO_4) to oxidize hydroxyls (-OH) at the 2' and 3' positions of the ring to generate aldehydes.

FIG. 20



Optimization of oxidation reaction

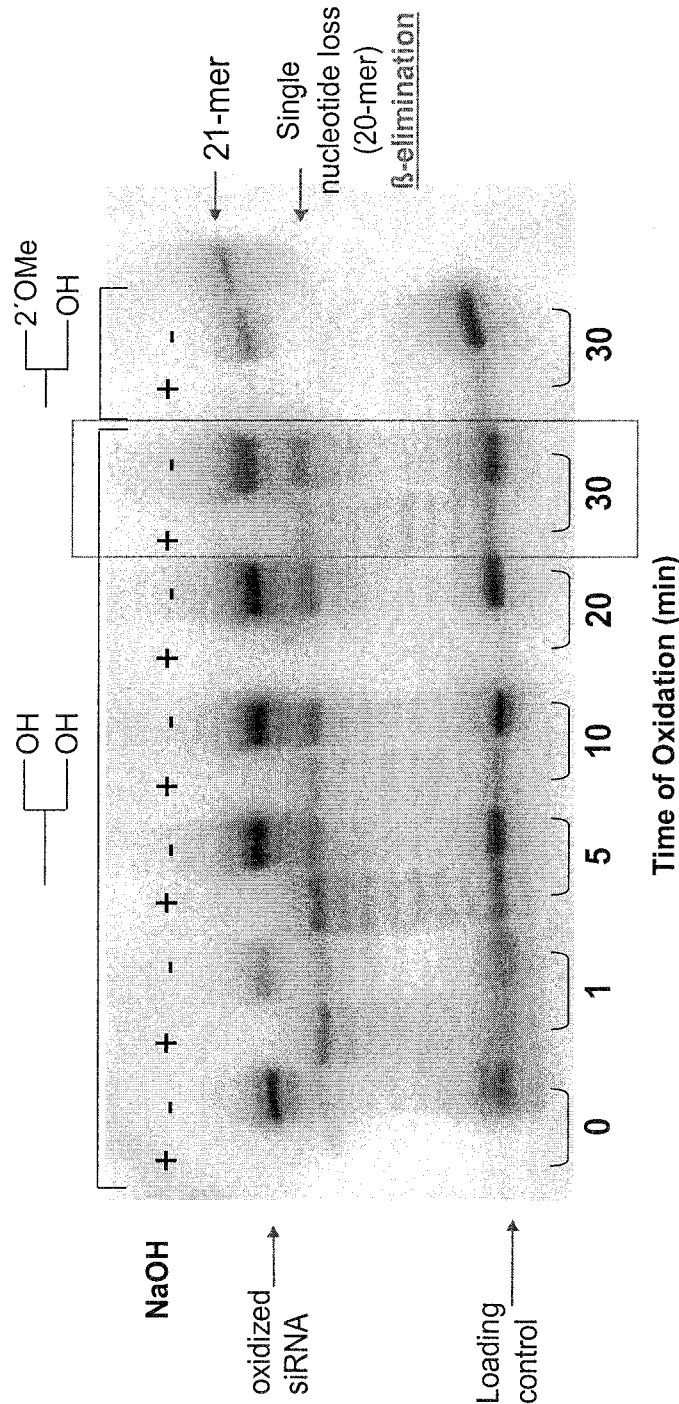
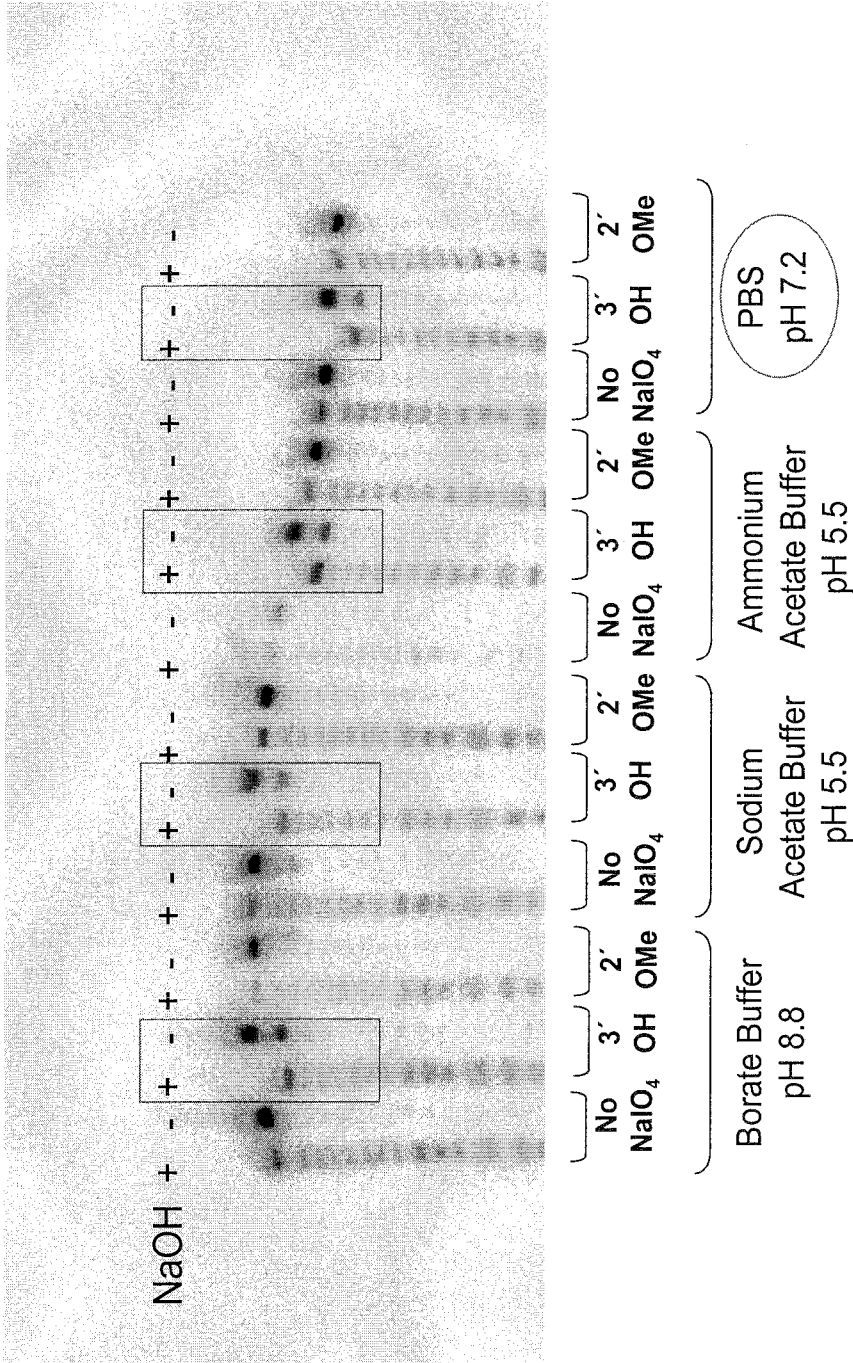


FIG. 21

If the siRNA is completely oxidized, β -elimination will lead to single nucleotide loss.

3' oxidation of siRNA under varying pH conditions

FIG. 22



Oxidation is complete in each buffer tested.

25 mM NaIO4 is sufficient to oxidize up to at least 100 μ M siRNA

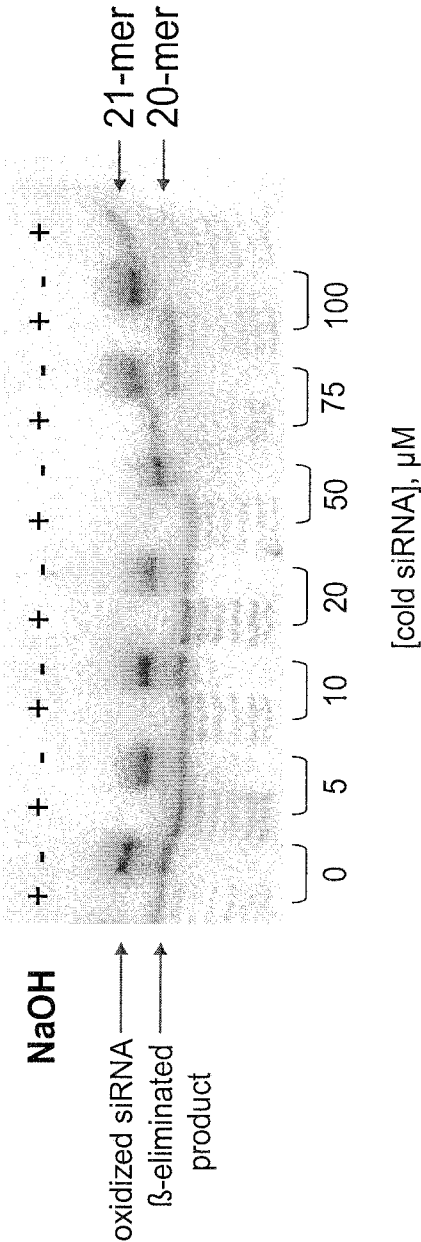
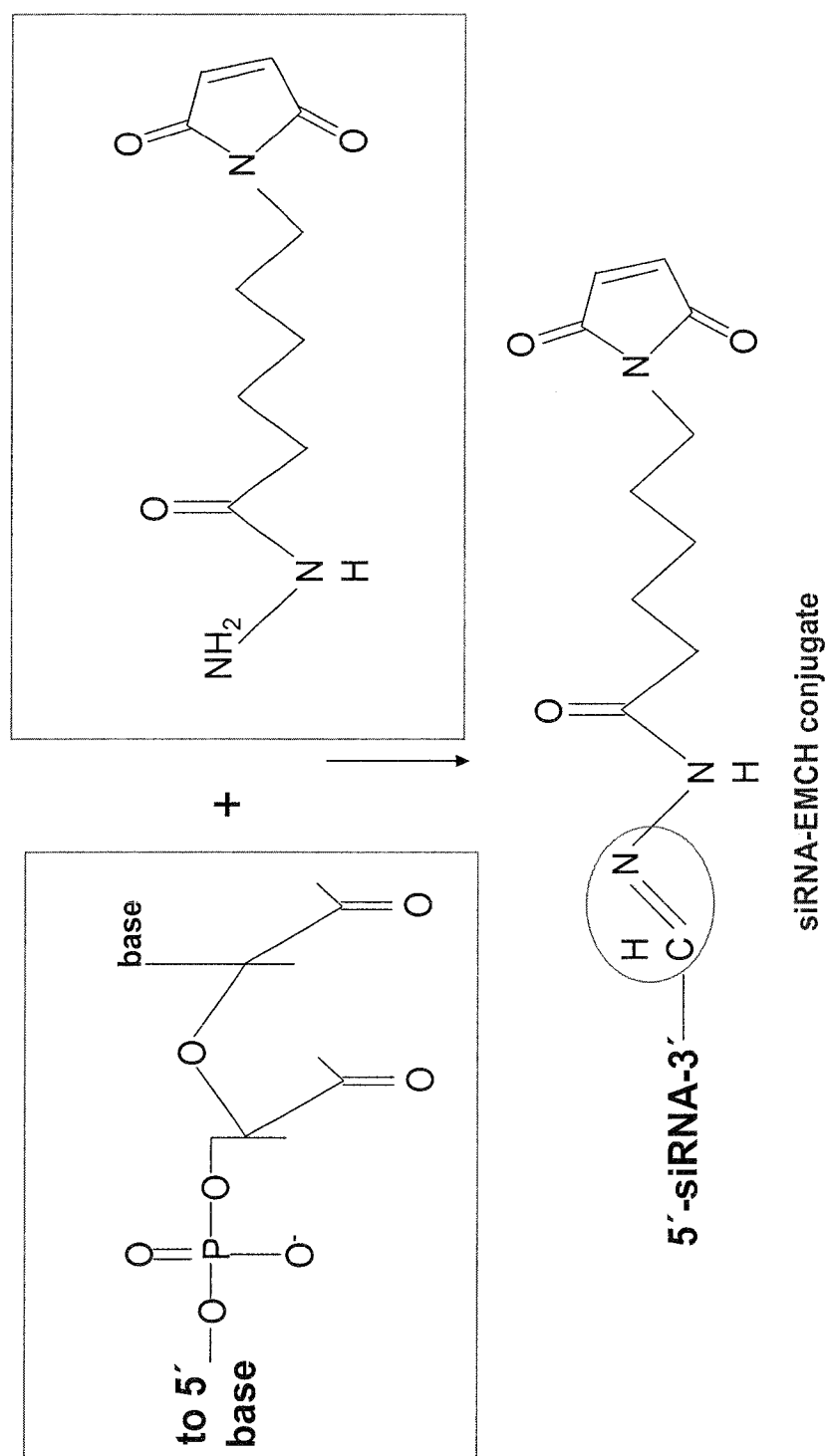


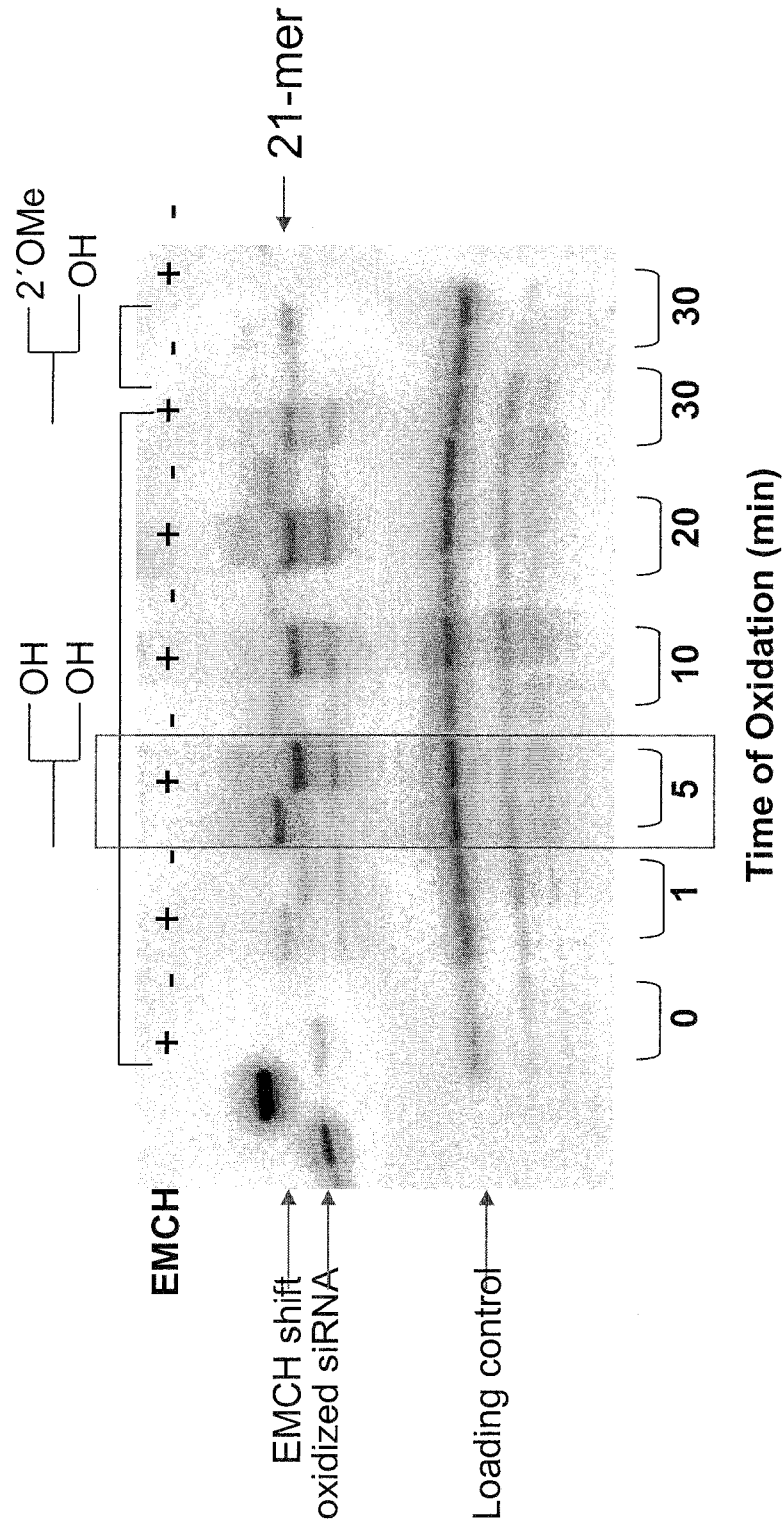
FIG. 23

Reaction of oxidized siRNA with the linker, EMCH

FIG. 24



Reaction of Oxidized siRNA with EMCH



Oxidized siRNA reacts almost completely with EMCH.

FIG. 26

Optimization of oxidation of siRNA and reaction with EMCH in a single tube

	[NaIO ₄]	time of ox	cysteine quench?	complete oxidation?	EMCH shift?
Experiment 1	25 mM	1 min	no (MOPS gel)	yes	no
Experiment 2	25 mM	1 min	yes (10-fold excess)	no	no
Experiment 3	2.5 mM	1 min	yes (10-fold excess)	no	yes
Experiment 4	25 mM	30 min	yes (10-fold excess)	yes	no
Experiment 5	2.5 mM	30 min	yes (10-fold excess)	yes	yes

Conclusions:

1. Oxidation is not complete after 1min in NaIO₄ (25 mM or 2.5 mM). The reaction continues during the overnight precipitation. The addition of EMCH prevents the completion of the oxidation. A 30 min (or longer) oxidation is necessary.
2. 25 mM NaIO₄ "kills" the EMCH but 2.5 mM NaIO₄ is "tolerated."

HPLC purification of the siRNA-EMCH conjugate

Goal: use HPLC to separate siRNA from the
siRNA-EMCH conjugate

Buffer A: 0.1M Ammonium Acetate
Buffer B: 0.1M Ammonium Acetate in
50% Acetonitrile

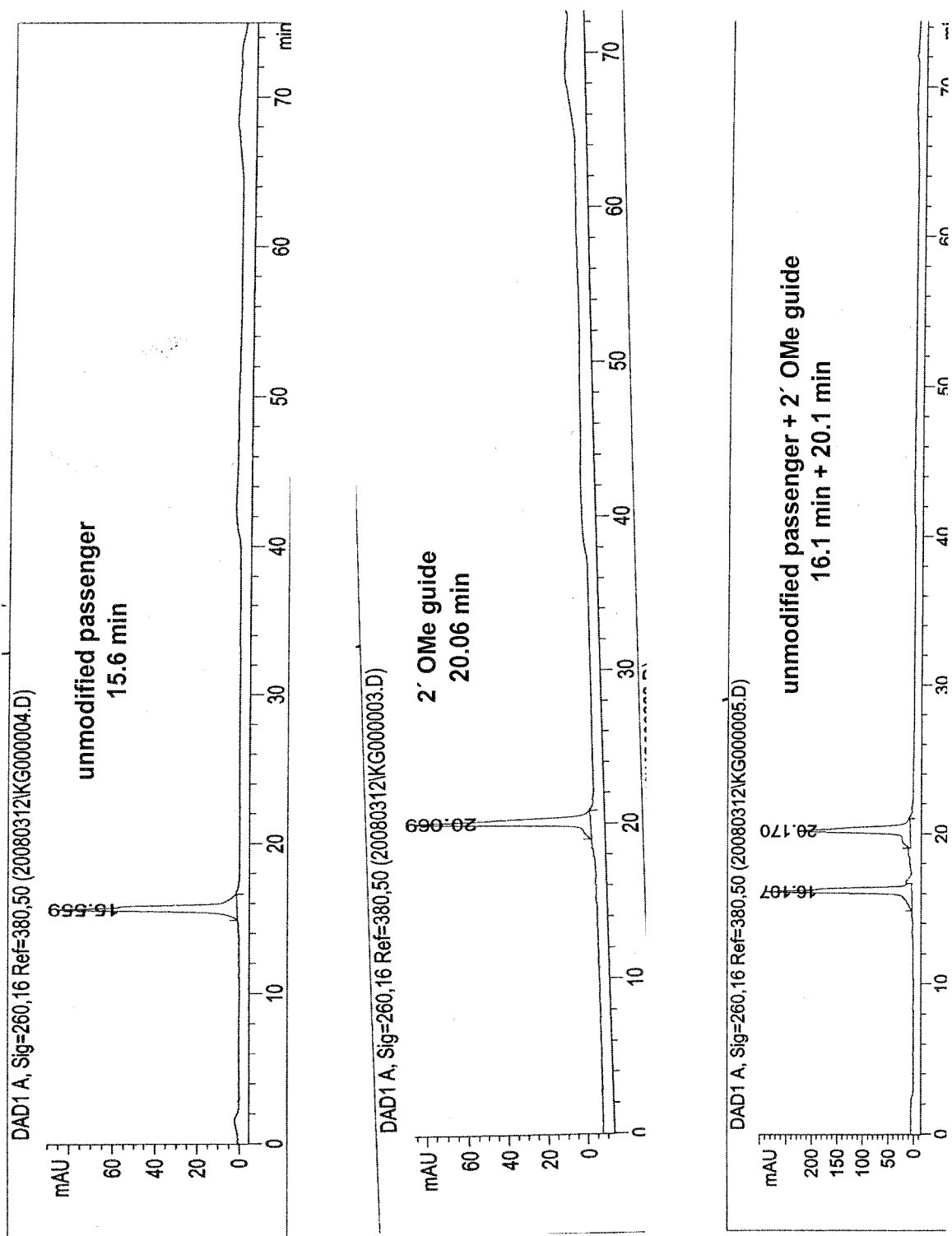
FIG. 27

Time	% Buffer B
0 min - 5 min	10%
5 min - 60 min	10% - 65%
60 min -65 min	65% - 100%
65 min - 70 min	100%
70 min -75 min	100% - 10%

FIG. 28

Sample	Retention Time (min)	Comments
unmodified passenger	15.6	the siRNA strand that will get oxidized
2'OMe guide	20	the siRNA strand that is modified so oxidation cannot occur
unmodified passenger + 2'OMe guide	16 and 20.1	mixture of the two strands, not annealed
3'Bio passenger	18.5	passenger strand modified with a biotin at the 3' end
unmodified passenger + 3'Bio passenger	16 and 18.5	mixture of the two strands, not annealed
oxidized passenger	14.1	passenger treated with NaIO ₄ to generate aldehydes
oxidized passenger - EMCH	14 and 16.5	passenger treated with NaIO ₄ and reacted with EMCH
oxidized passenger - biotin-hydrazide	14 and 27	passenger treated with NaIO ₄ and reacted with biotin-hydrazide

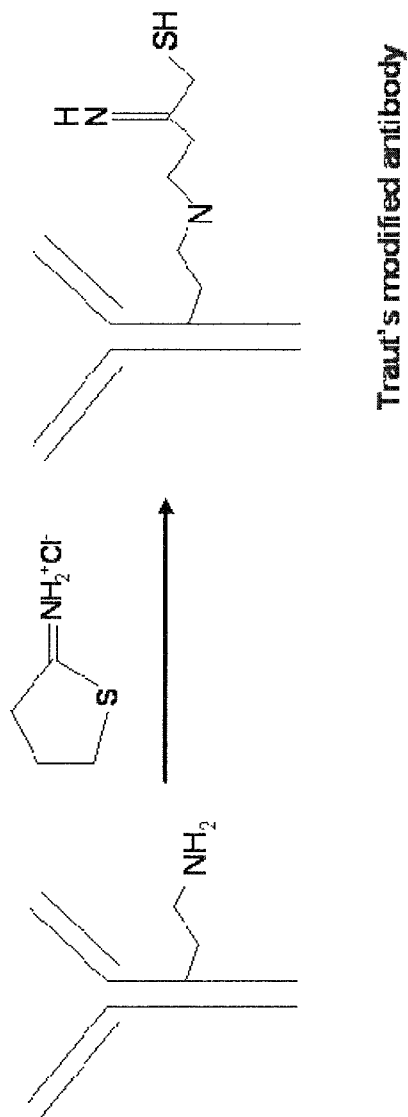
FIG. 29



Modification of lysine residues on the antibody

Modify lysine residues on the antibody with Traut's reagent to generate sulfhydryls (-SH).

FIG. 30



Modification of lysine residues on the antibody

FIG. 31

	25-fold excess Traut's	50-fold excess Traut's	200-fold excess Traut's
mAb expt 1	0.6	0.8	6.1
mAb expt 2	0.4	1.6	6.7

Antibody modification does not significantly alter
antigen recognition

FIG. 32

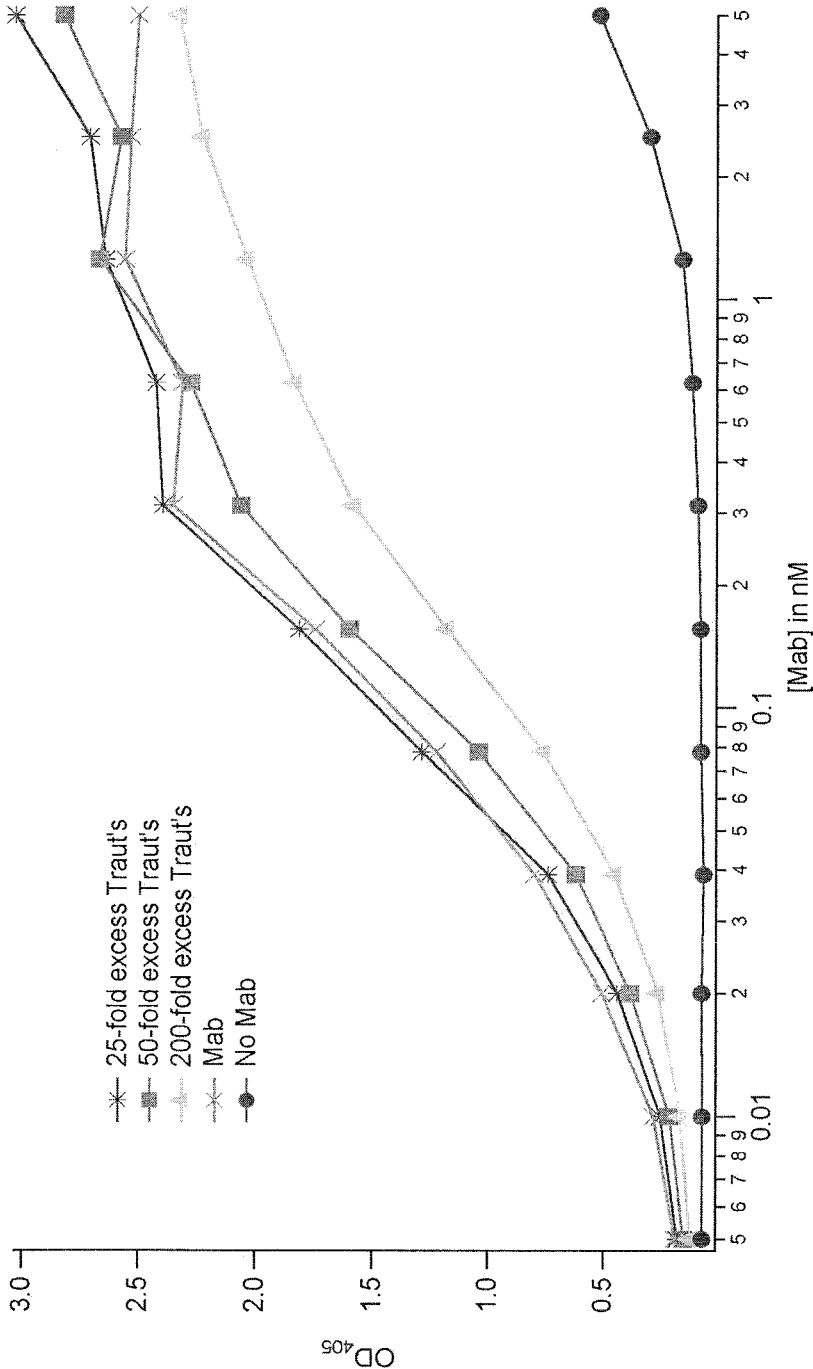


FIG. 33

Conjugation of siRNA to a human monoclonal antibody

React Traut's modified antibody with siRNA-EMCH conjugate.

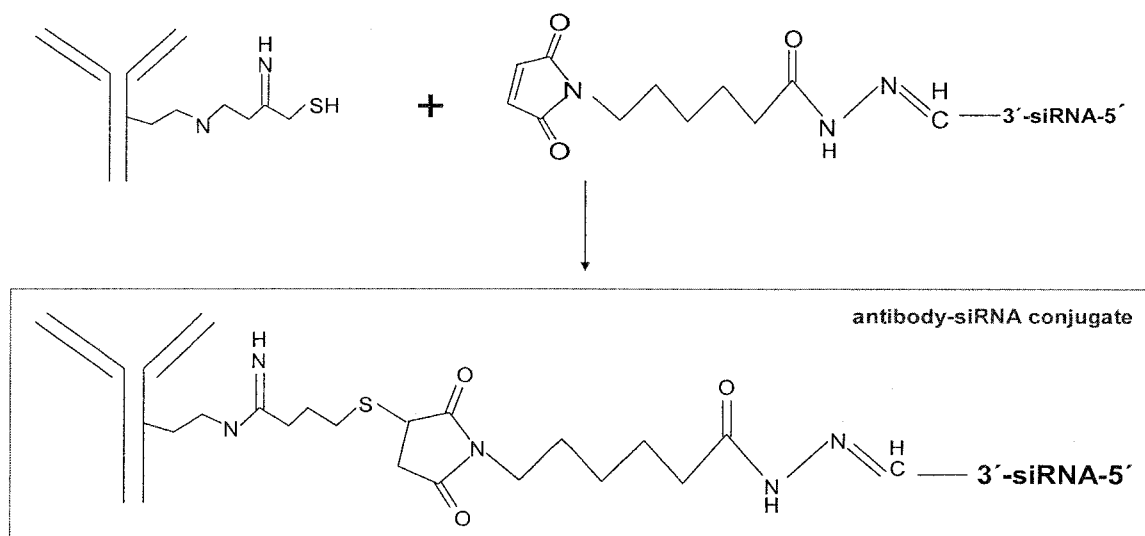


FIG. 34

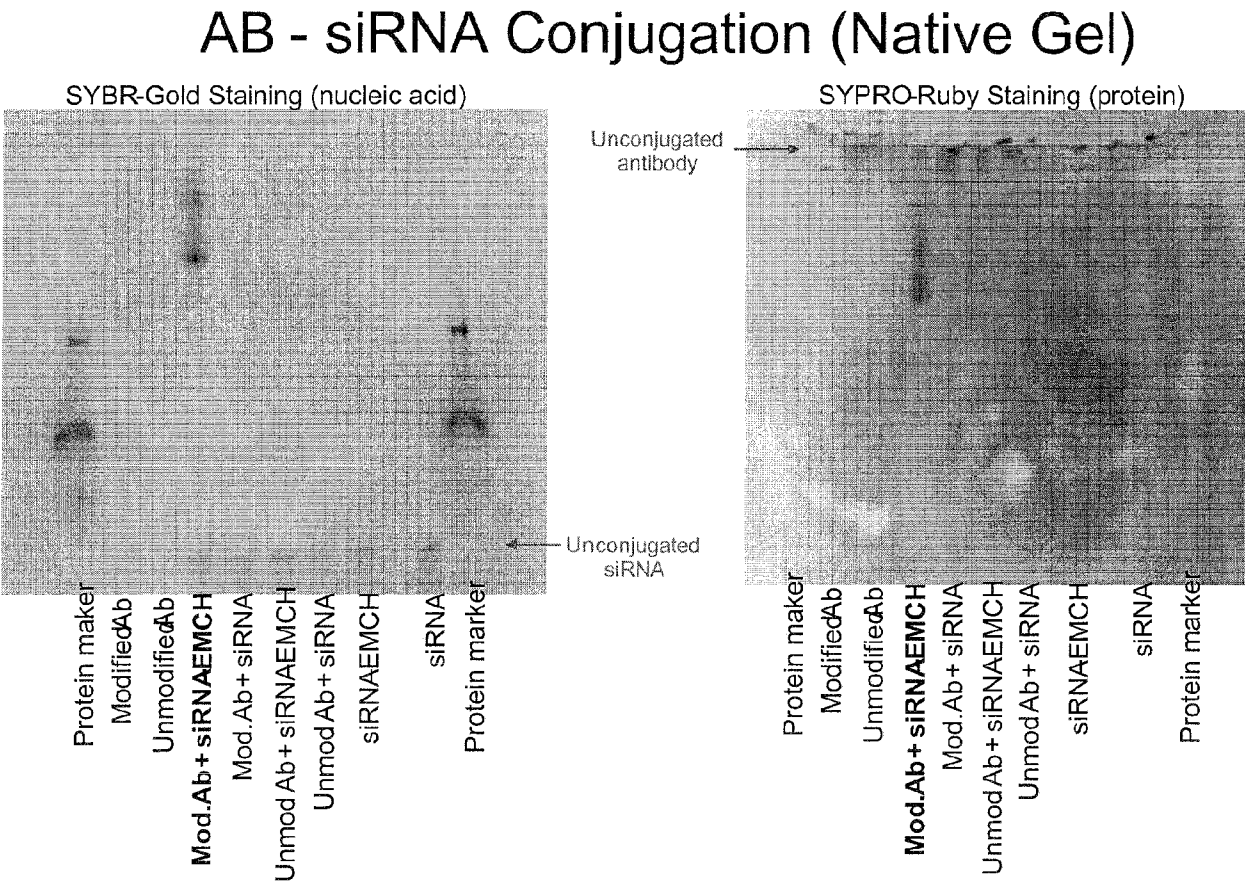
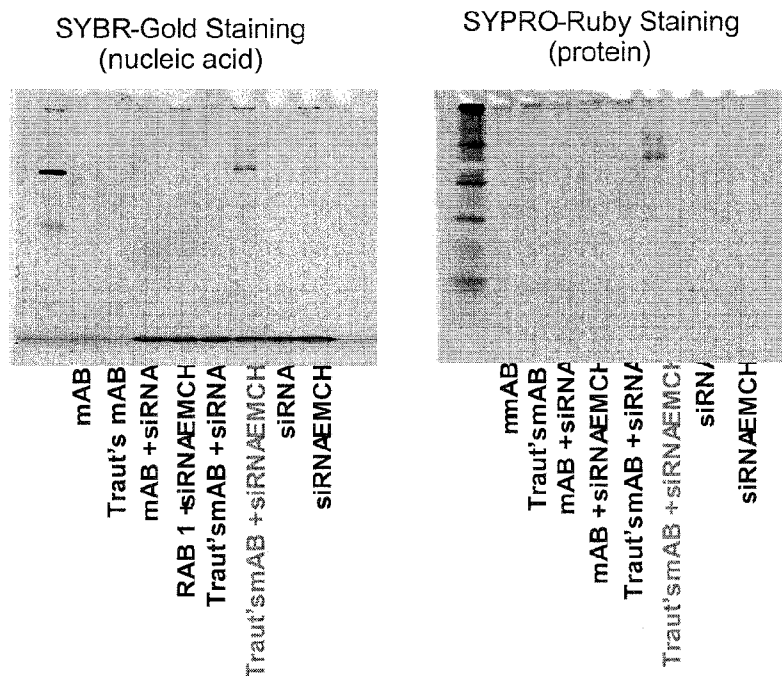


FIG. 35

Antibody-siRNA conjugation

Traut's modified mAb (Traut's mAb; 5 free sulfhydryls per mAb quantitated with Ellman's reagent), and unmodified mAb (mAb) were reacted with 3' oxidized siRNA reacted with the EMCH linker (siRNA-EMCH), or siRNA only as a control. (~ 2:1 molar ratio, siRNA:free sulfhydryls on the mAb)



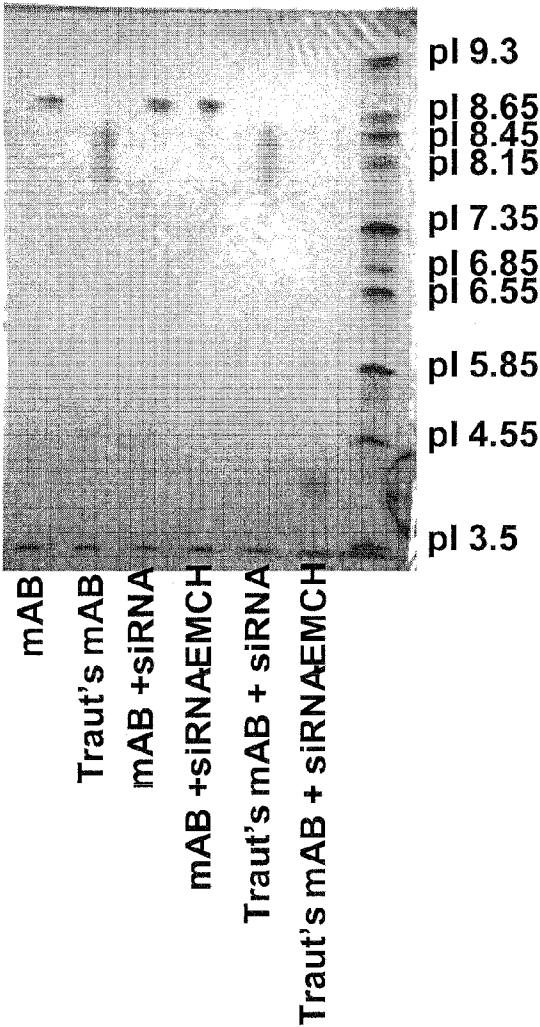
12% Native TRIS-Glycine Gels

FIG. 36

Antibody-siRNA conjugation

Same samples subjected to isoelectric focusing and stained with Gel Code Blue for total protein stain.

IEF Gel



ALIGNMENT AND CONSENSUS SEQUENCE FOR MONOCLONAL ANTIBODIES REACTIVE AGAINST EPITOPE
412-423 OF E2-660 HCV GLYCOPROTEIN Germ line: VH3-33, D3-10, JH4; Vk-L6, Jk5

Heavy Chain Amino Acid Alignment

95-43 (SEQ ID NO:36)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWFDGNNKYY
95-54 (38)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWFDGNNKYY
95-25 (34)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS NYGMHWVRQAPGKGLEWVAVIWFDGNNKYY
95-14 (32)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWYDGNKYY
95-49 (37)	QVQLVESGGGVVQPGRSLRLSCAASGFTLS SYGMHWVRQAPGKGLEWVAVIWYDGNKYY
95-62 (40)	QVQLVESGGGVVQPGRSLRLSCAASGFTLS SYGMHWVRQAPGKGLEWMAVIWYDGNKYY
95-42 (35)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWYDESNKYY
95-58 (39)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWYDENNKYY
95-2 (3)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWFDGNNQYY
95-38 (33)	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMHWVRQAPGKGLEWVAVIWLDGSNTYY
83-128 (1)	QVQLVESGGGVVQPGRSLRLSCTASGFTFN NYGMHWVRQTPGKGLEWLAVIWFDENNKYY
	*****:*****:..*****:*****:***** * . * **
95-43	ADSMKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-54	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-25	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMDRGVFIYFDYWGQGT
95-14	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-49	ADSVKGRFTISRDN SKNTLYLQMDSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-62	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-42	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-58	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTMVRGVFIYFDYWGQGT
95-2	ADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARDIFTVVRGVFIYFDYWGQGT
95-38	ADSVKGRFTISRDN SKNTLFLQMNSLRAEDTAVYYCARDIFTVARGVFIYFDYWGQGT
83-128	ADSVRGRFTISRDN SKNTLFLQMNSLKTEDTAMYYCARDI-SLVRDAFIYFDYFWGLGT
	:**:*****:*****:***** :..*..:*****:*** **
95-43	LVTVSS
95-54	LVTVSS
95-25	LVTVSS
95-14	LVTVSS
95-49	LVTVSS
95-62	LVTVSS
95-42	LVTVSS
95-58	LVTVSS
95-2	LVTVSS
95-38	LVTVSS
83-128	LVTVSS

Fig. 37

Light Chain Amino Acid Alignments

073-1	(SEQ ID NO: 6)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRRATGIPA
95-38	(53)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRRATGIPA
95-2	(4)	EIVLTQSPATLSLSPGERATLSCRASQSVGSYLAWYQQKPGQAPRLLIYDASNRRATGIPA
95-14	(44)	EIVLTQSPATLSLSPGERATLSCRASQSVGSYLAWYQQKPGQAPRLLIYDASNRRATGIPA
83-128	(2)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIYDASNRRATGIPA
		*****.*****

073-1		RFSGSGGTDFTLTIISLEPEDFAVYYCQQRSNWVTFGQGTREIK
95-38		RFSGSGGTDFTLTIISLEPEDFAVYYCQQRSNWVTFGQGTREIK
95-2		RFSGSGGTDFTLTIISLEPEDFAVYYCQQRSNWVTFGQGTREIK
95-14		RFSGSGGTDFTLTIISLEPEDFAVYYCQQRSNWVTFGQGTREIK
83-128		RFSGSGGTDFTLTIISLEPEDFAVYYCQQRSNWITFGQGTREIK
		*****;*****

Fig. 38