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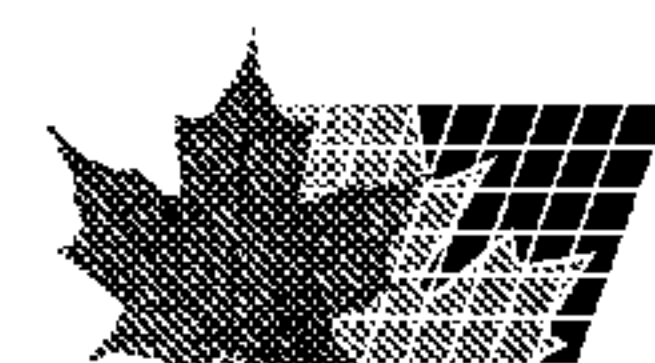
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(54) Title: LIBRARIES AND METHODS FOR ISOLATING ANTIBODIES

(57) **Abrégé/Abstract:**

The disclosure features methods for identifying antibodies that bind to a carbohydrate moiety. Libraries coding for antibodies that bind to a carbohydrate moiety are provided. The libraries can be provided by modifying a pre-existing nucleic acid library. Antibodies that bind to a carbohydrate moiety are described.



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(54) Title: LIBRARIES AND METHODS FOR ISOLATING ANTIBODIES

(57) Abstract: The disclosure features methods for identifying antibodies that bind to a carbohydrate moiety. Libraries coding for antibodies that bind to a carbohydrate moiety are provided. The libraries can be provided by modifying a pre-existing nucleic acid library. Antibodies that bind to a carbohydrate moiety are described.



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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
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THIS IS VOLUME __1__ OF __2__

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LIBRARIES AND METHODS FOR ISOLATING ANTIBODIES

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 60/649,065, filed on February 1, 2005.

10 BACKGROUND

This application relates to identifying antibodies that bind to a carbohydrate moiety.

It has been reported that it is difficult to raise antibodies to polysaccharides and molecules such as heparan sulfate by conventional immunization. There are, however,
15 several disease states in which sulfated carbohydrates are found.

SUMMARY

In one aspect, the disclosure features a method for identifying an antibody that binds to a carbohydrate moiety. The method includes: providing a protein library that includes a plurality of diverse antibodies, each antibody including a heavy chain
20 variable domain sequence and/or a light chain variable domain sequence, contacting members of the protein library to a target molecule that includes a carbohydrate moiety; and identifying one or more members that interact with the target molecule. The method can be used to identify an antibody to an epitope that includes a negatively charged carbohydrate moiety and may further include confirming that the antibody
25 interacts with (e.g., binds to) the target molecule and/or epitope.

In one embodiment, the heavy chain variable domain sequence includes diversity in HC CDR3, but at least one or two amino acid positions are restricted to a basic amino acid. Restricted positions can be, independently, completely invariant, or can be allowed to vary among basic amino acids. For example, one basic amino acid
30 position is an invariant arginine, and another basic amino acid position is allowed to vary between arginine and lysine. At least two of the basic amino acid positions can be separated by at least one amino acid position that is allowed to vary among at least ten

different amino acids. For example, for each antibody of the plurality, HC CDR3 includes X-B-B-X-B-X (SEQ ID NO:1) (wherein B is a varied or invariant basic amino acid); or X-(R/K)-X-(K/R)-X (SEQ ID NO:2); or (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO:3). In one embodiment, X is any amino acid. In another embodiment, X represents
5 positions that are varied among at least two, three, four, five, six, ten, twelve, thirteen, fifteen, sixteen, or nineteen different amino-acid types. For example, X can represent, independently, a position that is varied among non-cysteine amino acids (e.g., all nineteen non-cysteine amino acids or a subset thereof), a position that is varied among all possible amino acids, a position that is varied among a set of amino acids that
10 exclude basic amino acids, or a position that allows K, R, or H.

In one embodiment, the HC CDR3 region is less than 10, 8, or 7 amino acids in length, e.g., about 5, 6, or 7 amino acids in length.

In one embodiment, the antibodies of the plurality include diverse light chain variable domains. At least some of which can be light chain variable domains other
15 than DPL16 or and/or a light chain variable domain sequence that has a canonical structure other than that of a DPL16 light chain. The library may or may not also further include a DPL16 light chain or a variable domain sequence that has a canonical structure of a DPL16 light chain.

For example, the light chain variable domain sequence of each antibody of the
20 plurality is encoded by nucleic acids derived from transcripts of human hematopoietic cells.

In one embodiment, the heavy chain variable domain sequences of the antibodies of the plurality have the same canonical structure. In another embodiment, the heavy chain variable domain sequences of the antibodies of the plurality include a
25 plurality of different canonical structures. In one embodiment, the canonical structure is that of the 3-23 heavy chain VH segment.

In one embodiment, the heavy chain variable domain sequences of the antibodies of the plurality include one or more frameworks from a human germline sequence, e.g., : DP-1, DP-8, DP-12, DP-2, DP-25, DP-15, DP-7, DP-4, DP-31, DP-32,
30 DP-33, DP-35, DP-40, 7-2, hv3005, hv3005f3, DP-46, DP-47, DP-58, DP-49, DP-50, DP-51, DP-53, and DP-54, or a framework sequence that differs by two or fewer differences (e.g., substitutions, deletions, or insertions), e.g., one difference, relative to a human germline sequence.

In one embodiment, the antibodies of the plurality include diverse HC CDR1 and/or CDR2 regions. For example, CDR1 and CDR2 of the heavy chain variable domain sequence are encoded by sequences derived from diverse synthetic oligonucleotides.

5 The step of identifying one or more members that interact with the target molecule can include physically separating members of the library that bind to the target molecule from members that do not bind. The target molecule can be immobilized on a insoluble support, e.g., a solid support, prior to, during, or after the step of contacting. In one embodiment, the insoluble support is an immunotube or a
10 membrane (e.g., a nitrocellulose, nylon membrane, magnetic bead, or non-magnetic bead).

 In one embodiment, the protein is a member of a phage display library. In this case, identifying one or more members that interact with the target molecule may include recovering one or more phage that encodes an antibody that binds to the target
15 molecule.

 In one embodiment, the protein library is a collection of proteins disposed on an array. In this case, step of identifying one or more members that interact with the target molecule may include imaging the array or otherwise obtaining qualitative or quantitative indications of binding interactions.

20 The antibodies in the library can be in a variety of forms, e.g., as a Fab, a Fab2', a domain antibody (dAb), a single chain antibody, or a full-length antibody. Not every antibody in the protein library need be fully functional. Further, the protein library may include members other than members of the plurality. In one embodiment, the members of the plurality constitute at least 10, 25, 50, 75, 80, 90, 95, 99, or 100% of
25 the members in the protein library.

 In one embodiment, the protein library is depleted of members that interact with a non-target molecule, for example, a non-sulfated version of a sulfated carbohydrate.

 The target molecule can be a glycosaminoglycan, e.g., a sulfated carbohydrate, e.g., heparan sulfate, dermatansulfate, Sulfated Sialyl Lewis X (sLex) moieties (for a
30 review see, e.g., S. Hemmerich, S. Rosen (2000), *GlycoBiology* 10, 849-856) or other synthetic sulfated carbohydrate moieties. The target can be a carbohydrate having negatively charged or electronegative groups, such as carboxylic acid, sulfate,

phosphate, arsonate, fluoro, or nitro groups. Still other target molecules are described herein.

The method can further include evaluating interaction between (i) one or more antibodies that include CDRs from a library member identified as interacting with the target molecule and (ii) a molecule other than the target molecule. This evaluation can be used to identify antibodies that preferentially bind to the target molecule relative to another molecule, e.g., another related molecule. For example, the target molecule and the non-target molecule can include a carbohydrate moiety, e.g., different carbohydrate moieties, or the same carbohydrate moiety in different contexts (e.g., on different proteins, with a sulfate removed, or with the sulfate in a different site).

The method can further include identifying a plurality of antibodies that interact with the target molecule, and selecting a subset of antibodies from the plurality that preferentially interacts with the target molecule relative to a non-target molecule. A subset of antibodies can include one or more antibodies.

The method can further include evaluating the ability of one or more antibodies that include CDRs from a library member identified as interacting with the target molecule to modulate interaction between a cognate ligand of the target molecule and the target molecule.

The method can further include preparing a pharmaceutical composition that includes an antibody that includes CDRs from a library member identified as interacting with the target molecule. For example, the antibody in the composition includes human or effectively human framework regions and constant regions. The antibody in the composition can be humanized or deimmunized. The composition can be substantially free of sugar compounds.

An antibody coding library can be provided by modifying a pre-existing nucleic acid library or nucleic acid components, e.g., to provide a library described herein. For example, one method for providing an antibody coding library includes: providing an antibody coding library that includes a plurality of members, each member including a sequence encoding a heavy chain variable domain sequence; providing nucleic acids that encode a diverse population of HC CDR3 sequences (such as a diverse population of HC CDR3 sequences described herein), e.g., on which at least one amino acid position in CDR3 is restricted to a basic amino acid, or complements of such nucleic acids; and modifying the HC variable domain coding sequences to include the HC

CDR3 coding sequence. Prior to the modifying, the HC variable domain coding sequences can be diverse among members of the plurality. The modifying can include one or more of PCR, restriction digestion and ligation.

In one embodiment, the nucleic acids that encode a diverse population of HC CDR3 sequences include oligonucleotide, e.g., synthetic oligonucleotides. Synthetic oligonucleotides can be made, for example, by chemically attaching oligonucleotide building blocks (e.g., mono- or tri-nucleotides), e.g., using phosphoramidite chemistry. In one embodiment, each oligonucleotide includes a sequence with a FR3::CDR3- coding sequence: ARG|RGT|NNK|ARG| NNK|ARG|NNK (SEQ ID NO:4), wherein positions designated by N are varied by inclusion of one of the four nucleotides (A/G/C/T) and positions designated by R are varied by inclusion one of two nucleotides (A/G) and positions designated by K are varied by inclusion one of two nucleotides (T/G), or oligonucleotides complementary to such varied oligonucleotides. In another embodiment, each oligonucleotide includes a sequence that encodes a CDR3 that includes: X-B-B-X-B-X (SEQ ID NO:1) (wherein B is a varied or invariant basic amino acid); or X-(R/K)-X-(K/R)-X (SEQ ID NO:2); or (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO:3); or oligonucleotides complementary to such varied oligonucleotides. In addition, the nucleic acid may encode variation in framework residues adjacent to HC CDR3, for example residue 94. For example, the oligonucleotide encodes: Y-Y-C-A-(R/K)-G-X-R-X-(K/R)-X-W-G (SEQ ID NO:5) (underscored amino acids are part of FR3 or FR4) or Y-Y-C-A-(R/K)-(G/S)-X-(R/K)-X-(K/R)-X-W-G (SEQ ID NO:6).

In certain implementations, the oligonucleotide can exclude sequences that encode other CDRs, e.g., CDR1 or CDR2. The oligonucleotides can be less than 200, 150, 100, 80, or 60 nucleotides in length. The oligonucleotides can be isolated, e.g., in a preparation that is at least 10%, e.g., free of other nucleic acid species.

The method can further include expressing the members of the antibody coding library to obtain a library of proteins.

In another aspect, the disclosure features a protein library that includes a plurality of diverse antibodies, each antibody including a heavy chain variable domain sequence and a light chain variable domain sequence. In one embodiment, the heavy chain variable domain sequence of each antibody of the plurality includes diversity in HC CDR3, but at least one amino acid position is restricted to a basic amino acid.

In one embodiment, the library has a theoretical diversity of at least 10^7 , 10^9 , 10^{10} , or 10^{11} different antibodies and/or fewer than 10^{18} , 10^{16} , 10^{14} , 10^{12} , 10^{11} , or 10^{10} different antibodies. In one embodiment, the theoretical diversity is between 10^5 - 10^{12} , 10^3 - 10^{15} or 10^8 - 10^{16} antibodies. The theoretical diversity refers to the total number of distinct amino acid sequences that could be encoded by the library in its completely represented form, regardless of an actual implementation. Theoretical diversity is generally the product of the number of variations at each position. For example, the theoretical diversity of varying only two positions among all twenty amino acids is 20×20 , or 400. The actual library size is determined by the number of actual antibodies present in the library, e.g., the number of transformants of a display library or the number of distinct addresses on a protein array. The actual library diversity can be smaller than the theoretical diversity, e.g., between 10^5 - 10^{11} or between 10^3 and 10^{15} antibodies, and may be larger, e.g., due to random mutations introduced during cloning.

The protein library can include one or more features described herein.

The disclosure also features an antibody coding library that includes a plurality of diverse nucleic acids, e.g., an antibody coding library described herein. Each nucleic acid can include a sequence that encodes at least a heavy chain variable domain of an antibody in a protein library described herein. The antibody coding library can include one or more features described herein.

In another aspect, this disclosure features an antibody described herein or identified by a process disclosed herein. For example, the antibody includes a HC CDR3 that includes (G/S) X-(R/K)-X-(K/R)-X (SEQ ID NO:7), wherein X is any amino acid or other motif described herein. In one embodiment, the antibody binds a molecule that includes a negatively charged carbohydrate moiety, e.g., a glycosaminoglycan, e.g., heparan sulfate or dermatan sulfate or chondroitin sulfate or a Sulfated Sialyl Lewis X moiety. For example, the antibody binds to a sulfate group in the carbohydrate, or binding is dependent on the compound being sulfated.

In one embodiment, the antibody binds to sulfated sialyl Lewis X moiety. For example, the antibody preferentially binds to a sulfated sialyl Lewis X moiety relative to a moiety that is otherwise the same, but non-sulfated (Rosen S.D. 2004, *Annu. Rev. Immunol.* 22, 129-156; Uchimura et al., 2005, *Nature Immunology* 6:1105-13; Kawashima et al, 2005, *Nature Immunology*, 6:1096-1104; Dube D.H. et al., 2005, *Nature Drug Discovery reviews* 4:477-88; Pratt et al. 2004, *Org. Lett.* 6(14):2345-48).

The antibody can include other features described herein.

In one aspect, the disclosure features an antibody that includes a heavy chain variable domain sequence and/or a light chain variable domain sequence. The antibody has an antigen binding site that interacts with the target molecule, e.g., binds with a K_d of less than 10^{-7} , 10^{-8} , 10^{-9} , or 10^{-10} molar. The antibody can bind, e.g., an epitope that includes a negatively charged moiety, e.g., a negatively charged carbohydrate moiety, e.g., a moiety described herein. The antibody can include other features described herein.

In another aspect, the disclosure features an antibody (e.g., an isolated antibody) that binds to heparan sulfate and/or chemically desulfated N-sulfated heparin (CDSNS) and has (a) a HC immunoglobulin variable domain sequence comprising one or more CDRs that are at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR of a HC variable domain described herein; (b) a LC immunoglobulin variable domain sequence comprising one or more CDRs that are at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR of a LC variable domain described herein; (c) a LC immunoglobulin variable domain sequence is at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a LC variable domain described herein; or (d) a HC immunoglobulin variable domain sequence is at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a HC variable domain described herein. In some embodiments, such antibodies will have a HC CDR3 domain that is at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR3 of a HC variable domain of a HS-binding antibody described herein.

In a further aspect, the disclosure features an antibody (e.g., an isolated antibody) that binds to sulfocore 6 and has (a) a HC immunoglobulin variable domain sequence comprising one or more CDRs that are at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR of a HC variable domain described herein; (b) a LC immunoglobulin variable domain sequence comprising one or more CDRs that are at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR of a LC variable domain described herein; (c) a LC immunoglobulin variable domain sequence is at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a LC variable domain described herein; or (d) a HC immunoglobulin variable domain sequence is at least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a HC variable domain described herein. In some embodiments, such antibodies will have a HC CDR3 domain that is at

least 85, 88, 90, 92, 94, 95, 96, 97, 98, 99, or 100% identical to a CDR3 of a HC variable domain of a SC6-binding antibody described herein.

In another aspect, this disclosure features a pool of oligonucleotides that includes a plurality of different oligonucleotides. Each oligonucleotide of the plurality
5 includes a sequence with a CDR3-coding sequence. In one embodiment, the CDR3 coding sequence encodes (G/S)-X-(R/K)-X-(K/R)-X (SEQ ID NO:7), wherein X can be any amino acid, and (K/R) can be either lysine or arginine and G and S can either be a serine or glycine. In another embodiment, the DNA sequence includes RGT|NNK|ARG|NNK|ARG|NNK (SEQ ID NO:8), wherein positions designated by N
10 are varied by inclusion of one of the four nucleotides (A/G/C/T) and positions designated by R are varied by inclusion one of two nucleotides (A/G) and positions designated by K are varied by inclusion one of two nucleotides (T/G), or oligonucleotides complementary to such varied nucleotides. The CDR3 coding sequence can be flanked by one or more of a sequence encoding at least a portion of
15 FR3 and a sequence encoding at least a portion of FR4.

In another aspect, the disclosure features a method for identifying an antibody that binds to a polypeptide with a negatively charged modification, e.g., a phosphorylated polypeptide or phosphocarbohydrate. The method includes: providing a protein library that includes a plurality of diverse antibodies, each antibody including
20 a heavy chain variable domain sequence and/or a light chain variable domain sequence, contacting members of the protein library to a target molecule that includes a negatively charged modification; and identifying one or more members that interact with the target molecule. The method can be used to identify an antibody to target molecule having a negatively charged modification, e.g., by binding to an epitope that
25 includes the negatively charged modification, and may further include confirming that the antibody interacts with (e.g., binds to) the target molecule and/or epitope. For example, the method is used to identify an antibody to a phosphorylated intracellular polypeptide.

In one embodiment, the heavy chain variable domain sequence includes
30 diversity in HC CDR3, but at least one or two amino acid positions are restricted to a basic amino acid. Restricted positions can be, independently, completely invariant, or can be allowed to vary among basic amino acids. For example, one basic amino acid position is an invariant arginine, and another basic amino acid position is allowed to

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vary between arginine and lysine. At least two of the basic amino acid positions can be separated by at least one amino acid position that is allowed to vary among at least ten different amino acids. For example, for each antibody of the plurality, HC CDR3 includes X-B-B-X-B-X (SEQ ID NO:1) (wherein B is a varied or invariant basic amino acid); or X-(R/K)-X-(K/R)-X (SEQ ID NO:2); or (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO:3). In one embodiment, X is any amino acid. In another embodiment, X represents positions that are varied among at least four, six, ten, twelve, or sixteen different amino acids. For example, X can represent, independently, a position that is varied among non-cysteine amino acids (e.g., all nineteen non-cysteine amino acids or a subset thereof), a position that is varied among all possible amino acids, or a position that is varied among a set of amino acids that exclude basic amino acids.

In one embodiment, the HC CDR3 region is less than 10, 8, or 7 amino acids in length, e.g., about 6 or 7 amino acids in length.

The method can include other features described herein.

In another aspect, the disclosure features a method for evaluating an antibody. The method includes providing an antibody that includes a HC CDR3 that includes an amino acid sequence that conforms to a motif described herein or that includes two or more basic amino acids or that has a property otherwise described herein; and contacting the antibody to a target molecule, e.g., a polypeptide that includes a carbohydrate moiety or a polypeptide with a negatively charged modification, e.g., a phosphorylated polypeptide; and evaluating a parameter associated with binding.

In one embodiment, the HC CDR3 includes at least one basic amino acid position, e.g., arginine or lysine. For example, the HC CDR3 includes at least two of the basic amino acid positions, e.g., positions separated by at least one non-basic amino acid position s. For example, HC CDR3 includes X-B-B-X-B-X (SEQ ID NO:1) (wherein B is a varied or invariant basic amino acid); or X-(R/K)-X-(K/R)-X (SEQ ID NO:2); or (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO:3). In one embodiment, X is any amino acid, e.g., a non-basic amino acid. In one embodiment, the HC CDR3 region is less than 10, 8, or 7 amino acids in length, e.g., about 6 or 7 amino acids in length.

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Other aspects of the invention include:

- a method for identifying an antibody that binds to a negatively charged carbohydrate moiety, the method comprising: providing a protein library that comprises a plurality of diverse antibodies, each antibody comprising a heavy chain variable domain
5 sequence and a light chain variable domain sequence, wherein each heavy chain variable domain sequence comprises diversity in HC CDR3, and the HC CDR3 comprises (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO: 3), wherein each X is independently any amino acid; contacting members of the protein library to a target molecule that includes a negatively charged carbohydrate moiety; and identifying one or more members that interact with the
10 target molecule; and
- a pool of oligonucleotides comprising a plurality of different oligonucleotides, wherein each oligonucleotide of the plurality comprises a sequence with a HC CDR3-coding sequence that encodes the amino acid sequence (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO: 3), wherein each X is independently any amino acid, (K/R) can be either lysine
15 or arginine, and (G/S) can be either glycine or serine.

Other features and advantages will become more apparent from the following detailed description and claims. Embodiments can include any combination of features described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

5

Figure 1 is a schematic of the Library of Example 1. The Fab display cassette having captured kappa and lambda LC and synthetic diversity in HC CDR1 and CDR2 as described in Hoet et al. (*Nature Biotechnology*, 2005, 23(3):344-8. HC CDR3 is as described in Example 1.

10

Figure 2 is a schematic of the Library of Example 2. The Fab display cassette having captured kappa and lambda LC and synthetic diversity in HC CDR1 and CDR2 is as described in Hoet et al. (*Nature Biotechnology*, 2005, 23(3):344-8. HC CDR3 is as described in Example 2.

15

Figure 3A-3H shows the annotated Fab display cassette incorporated in the FAB-310 library. SEQ ID NO:25 is a portion of the cassette encoding nucleotide sequence of light chain variable region (VL). SEQ ID NO:26 is a portion of the cassette encoding amino acid sequence of light chain variable region (VL). SEQ ID NO:27 is a portion of the cassette encoding nucleotide sequence of light chain constant region (CL). SEQ ID NO:28 is a portion of the cassette encoding amino acid sequence of light chain constant region (CL). SEQ ID NO:29 is a portion of the cassette encoding nucleotide sequence of heavy chain variable region (VH). SEQ ID NO:30 is a portion of the cassette encoding amino acid sequence of heavy chain variable region (VH). SEQ ID NO:31 is a portion of the cassette encoding nucleotide sequence of heavy chain 1 constant region (CH1). SEQ ID NO: 32 is a portion of the cassette encoding amino acid sequence of heavy chain 1 constant region (CH1).

Figure 4 is the structure of the sulfocore 1 and sulfocore 6 carbohydrate moieties and their non-sulfated counterparts core 1 and core 6. Their respective molecular weights are mentioned below the carbohydrate structure.

DETAILED DESCRIPTION

We have discovered, *inter alia*, that certain structural properties are beneficial for immunoglobulins that interact with carbohydrates. While some antibodies specific for carbohydratase have been reported in the scientific literature (see, e.g., Dennissen et al. JBC (2002) 277(13):10982-86; Bernsen et al. *Cancer Immunol Immunother.* (2003) 52(12):780-83; Dinh et al. *J. Immunol.* 1996 157(2):732-38; Mao et al. *Proc. Nat'l Acad. Sci USA* (1999) 96(12):6953-58; Streeter et al., (1988) *J. Cell. Biol.* 107(5):1853-62; Reason et al. *Infect Immun.* (1997) 65(1):261-6; van Kuppevelt et al. *J. Biol. Chem.* (1998) 273:12960-66; Jenniskens et al. *J. NeuroSci.* (2000) 20(11):4099-111; Chen et al. *Mol. Immunol.* (2000) 37(8):455-66; van de Westerlo et al. *Blood* (2002) 99(7):2427-33; Willats et al. *Plant J.* (1999) 18(1):57-65; Chang & Siegel *Transfusion* (2001) 41(1):6-1; Lee et al. *J. Am. Chem. Soc.* (2002) 124:12439-46; Kowal et al. *Eur. J. Immunol.* (1999) 29(6):1901-11; Nguyen et al, *Nat. Struct. Biol.* (2003) 10(12):1019-25; Wang et al. *Mol. Immunol.* (1997) 34(8-9):609-18; Haidaris et al. *J. Immunol. Meth.* 2001 257(1-2):185-202; Foy et al. *J. Immunol. Meth.* (2002) 261(1-2):73-83), such antibodies are considered difficult to obtain. Libraries that include a significant number of immunoglobulins with the properties disclosed herein can be used as a source of immunoglobulins that interact with carbohydrates, especially negatively charged carbohydrates.

In one implementation, we produced a phage display library that displays antibodies in a Fab format. The library was used to identify antibodies that bind to an epitope that includes a carbohydrate moiety.

A general strategy for providing an enriched library includes identifying proteins that specifically interact with a target of interest, e.g., a target with reduced immunogenicity or an epitope of interest, e.g., an epitope with reduced immunogenicity, identifying a consensus sequence present in the proteins (e.g., some or all of the proteins), and preparing a protein library in which at least 10%, 50%, 80% or all the members include a sequence that conforms to the identified consensus. Targets with reduced immunogenicity include those with highly charged properties and those that have features that are prevalent among self-antigens. In one implementation, the method is used for immunoglobulin proteins, although it is applicable to any type of protein, particularly proteins with a conserved scaffold domain. The library need not

be a phage display library but can be another form of expression library, a protein array, a two-hybrid library, and so forth.

A number of properties are useful in antibodies that bind to carbohydrates, particularly negatively charged carbohydrates. Exemplary antibodies may have one or
5 more of the following properties:

- a small HC CDR3 sequence, e.g., less than 12, 11, or 10 amino acids, e.g., about 7-8 amino acids;
- at least one or two basic residues, e.g., two arginines, in one or more CDRs, e.g., HC CDR3;
- 10 - a motif, such as XBBXB (SEQ ID NO:1) (where B, basic amino acid residue; X, any amino acid residue or any non-cysteine amino acid) in HC CDR3; and
- a motif such as (G/S)-X-(R/K)-X-(R/K)-X (SEQ ID NO:9) (where X, any amino acid residue or any non-cysteine amino acid) in HC CDR3;
- 15 - variation of FR3 at the last residue between R and K.

An antibody library can include a plurality of proteins with one or more of these properties.

As used herein, the term “antibody” refers to a protein that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence. For
20 example, an antibody can include a heavy (H) chain variable region (abbreviated herein as VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term “antibody” encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab fragments, F(ab')₂, a Fd fragment, a Fv
25 fragments, and dAb fragments) as well as complete antibodies. Antibodies with only a single variable domain have also been described, e.g., a soluble VH domain or a camelid V domain. Davies et al. (1996) *Immunotechnology* 2:169-179, for example, describes synthetic VH domains that bind to antigen in the absence of a VL domain.

The VH and VL regions can be further subdivided into regions of
30 hypervariability, termed “complementarity determining regions” (“CDR”), interspersed with regions that are more conserved, termed “framework regions” (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., et al. (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S.

Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) *J. Mol. Biol.* 196:901-917). Kabat definitions are used herein. Each VH and VL is typically composed of three CDR's and four FR's, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2,
 5 FR3, CDR3, FR4.

The canonical structures of hypervariable loops of an immunoglobulin variable are structural properties. Typically, the canonical structure of a particular loop can be inferred from its sequence, as described in Chothia et al. (1992) *J. Mol. Biol.* 227:799-817; Tomlinson et al. (1992) *J. Mol. Biol.* 227:776-798); and Tomlinson et al. (1995)
 10 *EMBO J.* 14(18):4628-38. Further, the CDR sequences of a particular germline antibody can provide amino acid residues for a hypervariable loop that result in a particular canonical structure.

An "immunoglobulin domain" refers to a domain from the variable or constant domain of immunoglobulin molecules. Immunoglobulin domains typically contain two
 15 β -sheets formed of about seven β -strands, and a conserved disulphide bond (see, e.g., A. F. Williams and A. N. Barclay 1988 *Ann. Rev Immunol.* 6:381-405). Although highly conserved, the disulfide is not absolutely required. An "immunoglobulin variable domain sequence" refers to an amino acid sequence that can form a structure that enables the CDR regions and/or hypervariable loops to form an antigen binding
 20 structure. The sequence may include at least two or all three CDRs and sufficient framework amino acids to position the CDRs. The sequence may include all or part of the amino acid sequence of a naturally-occurring variable domain. For example, the sequence may omit one, two or more N- or C-terminal amino acids, internal amino acids, may include one or more insertions or additional terminal amino acids, or may
 25 include other alterations. In one embodiment, a polypeptide that includes immunoglobulin variable domain sequence can associate with another immunoglobulin variable domain sequence to form a target binding structure (or "antigen binding site").

The comparison of sequences and determination of percent identity between two sequences can be performed using BLAST (Altschul et al. (1990) *J. Mol. Biol.* 215:403-
 30 410), particularly BLAST 2 Sequences as described by Tatusova and Madden (1999, *FEMS Microbiol. Lett.* 174:247-250) and as implemented by the National Center for Biotechnology Information. Parameters for comparison of two

nucleotide sequences (*e.g.*, BLASTN) are Reward for a match: 1; Penalty for a mismatch:-2; Open gap penalty: 5; extension penalty gap: 2; gap x_dropoff: 50; expect: 10.0; word size: 11. Parameters for comparison of two amino acid sequences (*e.g.*, BLASTP) are Matrix: BLOSUM62; Open gap penalty: 11; extension gap penalty: 1; gap x_dropoff: 50; expect: 10.0; and word size 3.

Carbohydrate Targets

Exemplary carbohydrate molecules that can serve as target molecules include glycosaminoglycans, negatively charged glycoproteins, and, generally, any molecule that includes at least one negatively charged saccharide moiety. Saccharides can have negative charge or high negative partial charges due to the presence of groups such as (without limitation) carboxylic acid, sulfate, phosphate, arsonate, nitrate, fluoride, or chloride groups.

Some examples of carbohydrate molecules include heparin and heparan sulfates. Heparan sulfate (HS) refers to one heterogeneous class of glycosaminoglycans. exemplary forms of heparan sulfates include dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate. Examples of synthetic heparan sulfate oligosaccharide moieties include: GlcNac6S-GlcUA α Me, IdoUA-GlcNAc-GlcUA Me; GlcNS6S-GlcUA-GlcNS6S Me; GlcN-GlcUA-GlcN Me; IdoUA2S-GlcNS6S-IdoUA2S-GlcNS6S Me; GlcUA-GlcNS-GlcUA-GlcNS-IdoUA2S Me; IdoUA-GlcNS-GlcUA-GlcNS-IdoUA2S Me; GlcUA-GlcNS-IdoUA-GlcNS-IdoUA2S Me; GlcNS6S-GlcUA-GlcNS36diS-IdoUA2S-GlcNS36diS Me; GlcNS6S-GlcUA-GlcNS36diS-IdoUA2S-GlcNS6S Me; GlcNS6S-GlcUA-GlcNS36diS-IdoUA-GlcNS Me; and GlcNS6S-GlcUA-GlcNS36diS-IdoUA-GlcNS6S Me.

Other exemplary carbohydrate targets include dextran sulfate, hyaluronate, and K5 polysaccharide from *E. coli*. In some embodiments, the target molecule is a complex, *e.g.*, of a protein and a carbohydrate molecule, *e.g.*, heparin bound by anti-thrombin III, or heparan sulfate and basic fibroblast growth factor (bFGF).

Heparan sulfates can be synthesized in vitro or obtained from a tissue source, *e.g.*, kidney, aorta, lung, or intestine, *e.g.*, from an animal, *e.g.*, a human source or other mammal.

To evaluate whether an antibody recognizes a structure that depends on a sulfate group, the target molecule can be modified, e.g., chemically. It is possible to strip target molecules of their *O*- and *N*-sulfate groups. For example, HNO₂ at pH 1.5 can be used to cleave at *N*-sulfated glucosamine residues (GlcNs) and at rare *N*-unsubstituted glucosamine residues. See, e.g., van Kuppevelt (1998) *J. Biol. Chem.* 273:12960-12966.

Also, one can modify the target molecule using enzymes, e.g., a heparinase (e.g., heparinase III), a chondroitinase (e.g., chondroitinase ABC). An antibody that only interacts with the target molecule prior to modification is likely to specifically recognize a structure affected by the modification.

Further using appropriate binding and/or functional assays, it is possible to obtain antibodies that modulate biological interactions, e.g., an interaction between a carbohydrate molecule and a cognate protein ligand that recognizes a moiety on the carbohydrate molecule. For example, antibodies can be identified that modulate (e.g., increase or decrease) the binding interaction between heparin and anti-thrombin III or between heparan sulfate and bFGF. HS is involved in the binding of growth factors such as neuregulin (see, e.g., Fischbach and Rosen, *Ann Rev Neurosci* 20 (429-458)) midkine (see, e.g., Zhou et al., (1997) *Neurosci* 10:56-70), and heparin-binding epidermal factor-like growth factor (see, e.g., Chen et al. 1995, *J Biol Chem.*, 270:18285-94)

HS is also involved in molecules involved in leucocyte adhesion and function has been implicated in tumor cell invasion and angiogenesis (e.g., Vlodavsky I. et al. (1990) *Cancer Metastasis Rev.* 9 203, Iozzo R.V. et al. (2001), *J. Clin. Invest.* 108: 349-355. The different functional features of HS seem to be linked to the amount and location of its sulfate groups (e.g., Salmivirta M. et al. (1996), *FASEB J.* 10: 1270). Accordingly, an antibody described herein can be used to treat or prevent a neoplastic disorder, particularly one that is associated with angiogenesis or tumor cell invasion.

Other examples of biological relevant interactions include those mediated by endothelial ligands for L-Selectin (sulfated carbohydrate moieties), for example present on GlyCAM-1, CD34, Sgp200, Podocalyxin. See, e.g., Rosen, 1999, *Am J. Pathology*, 155:1013-20; Rosen, 2004, *Annu. Rev. Immunol.* 22:129-56; Dube et al., 2005, *Nature Drug Discovery reviews*, 4:477-488; Pratt et al., 2004, *Org Lett.* 6(14):2345-48.

Antibodies that decrease a binding interaction can, for example, block a binding site on the carbohydrate molecule recognized by the cognate protein ligand. Antibodies that increase a binding interaction can, for example, stabilize an interaction between the carbohydrate molecule and the cognate protein ligand.

5 Other useful targets include ones in which the target is associated with, produced by, or presented by a pathogen, e.g., a bacterial pathogen, e.g., as mentioned herein.

Still another class of targets includes targets that are present on tumor cells or tumor-associated structures. For example, the target is an antigen present on a tumor
10 cell in at least 2, 5, or 10 fold greater abundance than on a normal cell, an antigen the glycolipid class, including acidic glycolipid such as, for example, gangliosides GD2, GD3 and GM3 (melanoma) and neutral glycolipids such as, for example, the Lewis^y (Le^y) (breast, prostate, ovary) and the Globo H (breast, prostate, ovary) antigens, and/or sialylated derivatives thereof; O-glycosyl peptides (or aminoacid) class such as, for
15 example, the Tn antigen (α GalNAc-Ser or α Gal NAc-Thr), T* antigen (β Gal-(1-3)- α -GalNAc-Ser or β Gal(1-3) α Cal-NAc-Thr) (see, e.g., Springer G. F. Science 224, 1198-1206 (1984)) (ovary, breast, lung), or di-Tri (α GalNAc-Ser/Thr)₂, tri-Tn(α GalNAc-Ser/Thr)₃ or hexa-Tn(α GalNAc-Ser/Thr)₆.

Phospho-polypeptide targets

20 Another class of targets include proteins that have a phosphate group, e.g., as a result of phosphorylation. Exemplary proteins contain one or multiple negatively charged phosphate groups, for example Phospho-Tyrosine or Phospho-Serine. Examples of phosphorylated proteins include kinases, transcription factors, tyrosine kinase receptors, and adaptor proteins. Antibodies to such proteins can be used for a
25 variety of purposes, including detecting the presence of the phosphorylated form of such proteins.

Antibody Libraries

An antibody library is a collection of proteins that include proteins that have at least one immunoglobulin variable domain sequence. For example, camelized variable
30 domains (e.g., VH domains) can be used as a scaffold for a library of proteins that include only one immunoglobulin variable domain sequence. In another example, the

proteins include two variable domains sequences, e.g., a VH and VL domain, that are able to pair. An antibody library can be prepared from a nucleic acid library (an antibody-coding library) that includes antibody-coding sequences.

In cases where a display library is used, each member of the antibody-coding
5 library can be associated with the antibody that it encodes. In the case of phage display the antibody protein is physically associated (directly or indirectly) with a phage coat protein. A typical antibody display library displays a polypeptide that includes a VH domain and a VL domain. The display library can display the antibody as a Fab fragment (e.g., using two polypeptide chains) or a single chain Fv (e.g., using a single
10 polypeptide chain). Other formats can also be used.

As in the case of the Fab and other formats, the displayed antibody can include one or more constant regions as part of a light and/or heavy chain. In one embodiment, each chain includes one constant region, e.g., as in the case of a Fab. In other embodiments, additional constant regions are included. It is also possible to add one or
15 more constant regions to a molecule after it is identified as having useful antigen binding site. See, e.g., US 2003-0224408.

Antibody libraries can be constructed by a number of processes (see, e.g., de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20, Hoogenboom *et al.* (2000) *Immunol Today* 21:371-8, and
20 Hoet *et al.* (2005) *Nat Biotechnol.* 23(3):344-8. Further, elements of any appropriate known process can be combined with those of other processes. Variation can be introduced into a single immunoglobulin domain (e.g., VH or VL) or into multiple immunoglobulin domains (e.g., VH and VL). The variation can be introduced into an immunoglobulin variable domain, e.g., in the region of one or more of CDR1, CDR2,
25 CDR3, FR1, FR2, FR3, and FR4, referring to such regions of either and both of heavy and light chain variable domains. In one embodiment, variation is introduced into HC CDR3. In one embodiment, variation is introduced into all three CDRs of a given variable domain. In another preferred embodiment, the variation is introduced into CDR1 and CDR2, e.g., of a heavy chain variable domain. Any combination is feasible.

30 Antibody-coding libraries can be constructed by a variety of methods. In one exemplary process, antibody-coding libraries are constructed by inserting diverse oligonucleotides that encode CDRs into the corresponding regions of an antibody-coding nucleic acid. A population of diverse oligonucleotides can be synthesized using

pools of different monomeric nucleotides or pools of different trinucleotides. The inclusion of a given element (e.g., a given nucleotide) is random with respect to the distribution and may only depend on the ratio of subunits in the pool. One example of a degenerate source of synthetic diversity is an oligonucleotide that includes NNN
5 wherein N is any of the four nucleotides in equal proportion or some other desired proportion.

Synthetic diversity can also be more constrained, e.g., to limit the number of codons in a nucleic acid sequence at a given trinucleotide to a distribution that is smaller than NNN. For example, such a distribution can be constructed using less than
10 four nucleotides at some positions of the codon. Mixtures of mononucleotides can be used to vary a codon, e.g., between two, three, four, eight, or other numbers of amino acid types.

In addition, trinucleotide addition technology can be used to obtain more particularized distributions. So-called "trinucleotide addition technology" is described,
15 e.g., in Wells *et al.* (1985) *Gene* 34:315-323, Knappik *et al.* (2000) *J. Mol. Biol.* 296:57-86; U.S. Patent No. US 4,760,025 and 5,869,644. Oligonucleotides are synthesized on a solid phase support, one codon (i.e., trinucleotide) at a time. The support includes many functional groups for synthesis such that many oligonucleotides are synthesized in parallel. The support is first exposed to a solution containing a
20 mixture of the set of codons for the first position. The unit is protected so additional units are not added. The solution containing the first mixture is washed away and the solid support is deprotected so a second mixture containing a set of codons for a second position can be added to the attached first unit. The process is iterated to sequentially assemble multiple codons. Trinucleotide addition technology enables the synthesis of a
25 nucleic acid that at a given position can encode a number of amino acids. The frequency of these amino acids can be regulated by the proportion of codons in the mixture. Further the choice of amino acids at the given position is not restricted to blocks of the codon table as is the case if mixtures of single nucleotides are added during the synthesis.

30 These and other methods can be used prepare oligonucleotides that include one or more codons that are constrained to a basic amino acid (e.g., arginine or lysine), to potentially positively charged amino acids (e.g., histidine, arginine, or lysine), or to vary among such combinations of codons. The oligonucleotide can include other

positions that are more varied, e.g., varied among all possible amino acids, non-cysteine amino acids, aliphatic amino acids, hydrophilic amino acids, and so on. The oligonucleotide can include a pattern of variation described herein.

5 The oligonucleotides in a population of diverse oligonucleotide can also include positions at which all the oligonucleotides have the same nucleotide. For example, the termini typically include at least 5, 10, 15, 18, or 20 nucleotides that are invariant.

Components of antibody coding libraries can be obtained from nucleic acid amplified from naïve germline immunoglobulin genes or from mRNA expressed by antibody producing cells. The amplified nucleic acid includes nucleic acid encoding
10 the VH and/or VL domain or a region thereof, e.g., a framework or constant region. Sources of immunoglobulin-encoding nucleic acids are described below. Amplification can include PCR, e.g., with one or more primers that anneal to one or more conserved regions, or another amplification method. Nucleic acid encoding immunoglobulin domains can be obtained from the immune cells of, e.g., a human, a primate, mouse,
15 rabbit, camel, or rodent. In one example, the cells are selected for a particular property. B cells at various stages of maturity can be selected. In another example, the B cells are naïve.

In one embodiment, fluorescent-activated cell sorting (FACS) is used to sort B cells that express surface-bound IgM, IgD, or IgG molecules. Further, B cells
20 expressing different isotypes of IgG can be isolated. In another preferred embodiment, the B or T cell is cultured *in vitro*. The cells can be stimulated *in vitro*, e.g., by culturing with feeder cells or by adding mitogens or other modulatory reagents, such as antibodies to CD40, CD40 ligand or CD20, phorbol myristate acetate, bacterial lipopolysaccharide, concanavalin A, phytohemagglutinin or pokeweed mitogen.

25 In still another embodiment, the cells are isolated from a subject that has an immunological disorder, e.g., systemic lupus erythematosus (SLE), rheumatoid arthritis, vasculitis, Sjogren syndrome, systemic sclerosis, or anti-phospholipid syndrome. The subject can be a human, or an animal, e.g., an animal model for the human disease, or an animal having an analogous disorder. In yet another embodiment,
30 the cells are isolated from a transgenic non-human animal that includes a human immunoglobulin locus.

In one preferred embodiment, the cells have activated a program of somatic hypermutation. Cells can be stimulated to undergo somatic mutagenesis of

immunoglobulin genes, for example, by treatment with anti-immunoglobulin, anti-CD40, and anti-CD38 antibodies (see, e.g., Bergthorsdottir et al. (2001) *J Immunol.* 166:2228). In another embodiment, the cells are naïve.

The nucleic acid encoding an immunoglobulin variable domain can be isolated
5 from a natural repertoire by the following exemplary method. First, RNA is isolated from the immune cell. Full length (i.e., capped) mRNAs are separated (e.g. by degrading uncapped RNAs with calf intestinal phosphatase). The cap is then removed with tobacco acid pyrophosphatase and reverse transcription is used to produce the cDNAs. The reverse transcription of the first (antisense) strand can be done in any
10 manner with any suitable primer. See, e.g., de Haard et al. (1999) *J. Biol. Chem* 274:18218-30. The primer binding region can be constant among different immunoglobulins, e.g., in order to reverse transcribe different isotypes of immunoglobulin. The primer binding region can also be specific to a particular isotype of immunoglobulin. Typically, the primer is specific for a region that is 3' to a
15 sequence encoding at least one CDR. In another embodiment, poly-dT primers may be used (and may be preferred for the heavy-chain genes). A synthetic sequence can be ligated to the 3' end of the reverse transcribed strand. The synthetic sequence can be used as a primer binding site for binding of the forward primer during PCR amplification after reverse transcription. The use of the synthetic sequence can obviate
20 the need to use a pool of different forward primers to fully capture the available diversity. The variable domain-encoding gene is then amplified, e.g., using one or more rounds. If multiple rounds are used, nested primers can be used for increased fidelity. The amplified nucleic acid is then cloned into a display library vector.

Antibody libraries are particularly useful, for example for identifying human or
25 "humanized" antibodies that recognize human antigens. Such antibodies can be used as therapeutics to treat human disorders. Antigen binding sites identified from antibody libraries can be modified, for example, fused to human constant regions or modified human constant regions. Since the constant and framework regions of the antibody are human, these therapeutic antibodies may avoid themselves being recognized and
30 targeted as antigens. The constant regions may also be optimized to recruit effector functions of the human immune system. The *in vitro* display selection process surmounts the inability of a normal human immune system to generate antibodies against self-antigens. Other types of antibody expression libraries can be used,

including, e.g., protein arrays of antibodies (see, e.g., De Wildt et al. (2000) *Nat. Biotechnol.* 18:989-994), lambda gt11 libraries, and so forth.

One exemplary antibody library is described in US 2002102613. A pre-existing antibody coding library can be modified, e.g., to replace CDR3-coding sequences with
5 oligonucleotides designed to encode CDR3 sequences that are biased for carbohydrate binding. For example, the oligonucleotides can encode CDR3 sequences of a particular length, e.g., less than ten, nine, or eight amino acids in length, e.g., about seven or six amino acids in length. The oligonucleotides can include one or positions that are preferentially basic (e.g., have at least a 20% likelihood of being basic) or which are
10 invariant (e.g., always arginine, always lysine, or allowed to vary between arginine and lysine).

Antibodies isolated from the libraries of the present disclosure are analyzed to determine the type of the LC and the closest germline gene. In a preferred embodiment, non-germline framework residues are changed back to the germline
15 amino acid so long as binding affinity and specificity are not adversely affected to an unacceptable extent. The substitutions may be done as a group or singly.

Display Libraries

In one embodiment, a display library can be used to screen a collection of proteins that includes proteins biased for a particular property, e.g., ability to bind to
20 carbohydrates, particularly negatively charged carbohydrates.

A display library is a collection of entities; each entity includes an accessible protein component and a recoverable component that encodes or identifies the protein component. The protein component can be of any length, e.g. from one amino acids to over 400 amino acids. In a selection, the protein component is probed with a target
25 molecule and if the protein component binds to the target molecule, the display library member is identified, typically by retention on a support.

Retained display library members are recovered from the support and analyzed. The analysis can include amplification and a subsequent selection under similar or dissimilar conditions. For example, positive and negative selections can be alternated.
30 The analysis can also include determining the amino acid sequence of the protein component and purification of the protein component for detailed characterization. A variety of formats can be used for display libraries. Examples include the following.

Phage Display. One format of display utilizes viruses, particularly bacteriophages. This format is termed "phage display." The protein component is typically covalently linked to a bacteriophage coat protein. The linkage results from translation of a nucleic acid encoding the protein component fused to a gene fragment encoding a functional portion of a coat protein of the phage. The linkage can include a flexible peptide linker, a protease site, or an amino acid incorporated as a result of suppression of a stop codon. Phage display is described, for example, in Ladner *et al.*, U.S. Patent No. 5,223,409; Smith (1985) *Science* 228:1315-1317; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20; Hoogenboom *et al.* (2000) *Immunol Today* 2:371-8; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Rebar *et al.* (1996) *Methods Enzymol.* 267:129-49; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; Barbas *et al.* (1991) *PNAS* 88:7978-7982; Hoet *et al.* (2005) *Nat Biotechnol.* 23(3):344-8.

The filamentous phage display systems typically use fusions to a minor coat protein, such as gene III protein or a domain of the gene III protein, e.g., the anchor domain or "stump" (see, e.g., U.S. Patent No. 5,658,727 for a description of the gene III protein anchor domain). It is also possible to physically associate the protein being displayed to the coat using a non-peptide linkage, e.g., a non-covalent bond or a non-peptide covalent bond. For example, a disulfide bond and/or heterodimerization domains can be used for physical associations (see, e.g., Crameri *et al.* (1993) *Gene* 137:69 and WO 01/05950). Phage display can use virus with a complete phage genome, but also phagemids which are prepared with helper phage.

Other types of protein display include cell-based display (see, e.g., WO 03/029,456.); ribosome display (see, e.g., Mattheakis *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:9022 and Hanes *et al.* (2000) *Nat Biotechnol.* 18:1287-92); protein-nucleic acid fusions (see, e.g., US 6,207,446); and immobilization to a non-biological tag (see, e.g., US 5,874,214).

Iterative Selection. In one preferred embodiment, display library technology is used in an iterative mode. A first display library is used to identify one or more ligands for a target. These identified ligands are then varied using a mutagenesis method to form a second display library. Higher affinity ligands are then selected from the second library, e.g., by using higher stringency or more competitive binding and washing conditions.

In some implementations, the mutagenesis is targeted to regions known or likely to be at the binding interface. If, for example, the identified ligands are antibodies, then mutagenesis can be directed to the CDR regions of the heavy or light chains as described herein. Further, mutagenesis can be directed to framework regions near or adjacent to the CDRs. In the case of antibodies, mutagenesis can also be limited to one or a few of the CDRs, e.g., to make precise step-wise improvements. Likewise, if the identified ligands are enzymes, mutagenesis can be directed to the active site and vicinity.

Some exemplary mutagenesis techniques include: error-prone PCR (Leung *et al.* (1989) *Technique* 1:11-15), recombination, DNA shuffling using random cleavage (Stemmer (1994) *Nature* 389-391; termed "nucleic acid shuffling"), RACHITTTM (Coco *et al.* (2001) *Nature Biotech.* 19:354), site-directed mutagenesis (Zoller *et al.* (1987) *Nucl Acids Res* 10:6487-6504), cassette mutagenesis (Reidhaar-Olson (1991) *Methods Enzymol.* 208:564-586) and incorporation of degenerate oligonucleotides (Griffiths *et al.* (1994) *EMBO J* 13:3245).

In one example of iterative selection, the methods described herein are used to first identify an antibody from a display library that binds a target molecule with at least a minimal binding specificity for a target or a minimal activity, e.g., an equilibrium dissociation constant for binding of less than 100 nM, 50 nM, 10 nM, 5 nM, or 1 nM. The nucleic acid sequences encoding the initially identified antibodies are used as template nucleic acids for the introduction of variations, e.g., to identify a second antibody that has enhanced properties (e.g., binding affinity, kinetics, or stability) relative to the initial antibody.

One preferred embodiment involves diversification of an antibody selected from a library of the present disclosure having a desired specificity but either insufficient affinity or less than perfect specificity. One preferred embodiment involves preparing a secondary library in which HC is held constant and a diversity of LC is provided, e.g.

the diversity of FAB-310. One preferred embodiment involves preparing a secondary library in which HC CDR3 and LC are held constant and a diversity of HC CDR1 & CDR2 is provided, e.g. the CDR1 & 2 diversity of FAB-310. One preferred embodiment involves preparing a secondary library in which HC CDR1 & CDR2 and LC are held constant and a diversity of HC CDR3 is provided, e.g. a diversity based on the selected CDR3.

Off-Rate Selection. Since a slow dissociation rate can be predictive of high affinity, particularly with respect to interactions between polypeptides and their targets, the methods described herein can be used to isolate ligands with a desired kinetic dissociation rate (i.e. reduced) for a binding interaction to a target.

To select for slow dissociating antibodies from a display library, the library is contacted to an immobilized target. The immobilized target is then washed with a first solution that removes non-specifically or weakly bound antibodies. Then the bound antibodies are eluted with a second solution that includes a saturating amount of free target, i.e., replicates of the target that are not attached to the particle. The free target binds to antibodies that dissociate from the target. Rebinding of the eluted antibodies is effectively prevented by the saturating amount of free target relative to the much lower concentration of immobilized target.

The second solution can have solution conditions that are substantially physiological or that are stringent (e.g. low pH, high pH, or high salt). Typically, the solution conditions of the second solution are identical to the solution conditions of the first solution. Fractions of the second solution are collected in temporal order to distinguish early from late fractions. Later fractions include antibodies that dissociate at a slower rate from the target than biomolecules in the early fractions. Further, it is also possible to recover antibodies that remain bound to the target even after extended incubation. These can either be dissociated using chaotropic conditions or can be amplified while attached to the target. For example, phage bound to the target can be contacted to bacterial cells.

Selecting or Screening for Specificity. The display library screening methods described herein can include a selection or screening process that discards antibodies that bind to a non-target molecule. Examples of non-target molecules include, e.g., a carbohydrate molecule that differs structurally from the target molecule, e.g., a carbohydrate molecule that has a different biological property from the target molecule.

In the case of a sulfated carbohydrate, a non-target may be the same carbohydrate without the sulfate or with the sulfate in a different position. In the case of a phosphopeptide, the non-target may be the same peptide without the phosphate or a different phosphopeptide.

5 In one implementation, a so-called “negative selection” step is used to discriminate between the target and related non-target molecule and a related, but distinct non-target molecules. The display library or a pool thereof is contacted to the non-target molecule. Members that do not bind the non-target are collected and used in subsequent selections for binding to the target molecule or even for subsequent
10 negative selections. The negative selection step can be prior to or after selecting library members that bind to the target molecule.

In another implementation, a screening step is used. After display library members are isolated for binding to the target molecule, each isolated library member is tested for its ability to bind to a non-target molecule (e.g., a non-target listed above).
15 For example, a high-throughput ELISA screen can be used to obtain this data. The ELISA screen can also be used to obtain quantitative data for binding of each library member to the target. The non-target and target binding data are compared (e.g., using a computer and software) to identify library members that specifically bind to the target.

20 **Other Expression Libraries**

Other types of collections of antibodies (e.g., expression libraries) can be used to identify antibodies with a particular property. Such other examples include, e.g., protein arrays of antibodies (see, e.g., De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994), lambda gt11 libraries, two-hybrid libraries and so forth.

25

Assays

Methods for evaluating antibodies for carbohydrate binding include ELISA, immunohistochemistry, immunoblotting, and fluorescence-activated cell sorting.

These methods can be used to identify antibodies which have a K_D of better
30 than a threshold, e.g., better than 0.1 μ M, 50 nM, 10 nM, 5 nM, 1 nM, or 0.5 nM.

ELISA. Proteins encoded by a display library can also be screened for a binding property using an ELISA assay. For example, each protein is contacted to a

microtitre plate whose bottom surface has been coated with the target, e.g., a limiting amount of the target. The plate is washed with buffer to remove non-specifically bound polypeptides. Then the amount of the protein bound to the plate is determined by probing the plate with an antibody that can recognize the polypeptide, e.g., a tag or
5 constant portion of the polypeptide. The antibody is linked to an enzyme such as alkaline phosphatase, which produces a colorimetric product when appropriate substrates are provided. The protein can be purified from cells or assayed in a display library format, e.g., as a fusion to a filamentous bacteriophage coat. Alternatively, cells (e.g., live or fixed) that express the target molecule, e.g., a target that contains a
10 carbohydrate moiety, can be plated in a microtitre plate and used to test the affinity of the peptides/antibodies present in the display library or obtained by selection from the display library.

In another version of the ELISA assay, each polypeptide of a diversity strand library is used to coat a different well of a microtitre plate. The ELISA then proceeds
15 using a constant target molecule to query each well.

Cell Binding Assays. Antibodies can be evaluated for their ability to interact with one or more cell types, e.g., a hematopoietic cell. Fluorescent activated cell sorting (FACS) is one exemplary method for testing an interaction between a protein and a cell. The antibody is labeled directly or indirectly with a fluorophore, before or
20 after, binding to the cells, and then cells are counted in a FACS sorter.

Other cell types can be prepared for FACS by methods known in the art.

Homogeneous Binding Assays. The binding interaction of candidate polypeptide with a target can be analyzed using a homogenous assay, i.e., after all components of the assay are added, additional fluid manipulations are not required. For
25 example, fluorescence resonance energy transfer (FRET) can be used as a homogenous assay (see, for example, Lakowicz *et al.*, U.S. Patent No. 5,631,169; Stavrianopoulos, *et al.*, U.S. Patent No. 4,868,103). A fluorophore label on the first molecule (e.g., the molecule identified in the fraction) is selected such that its emitted fluorescent energy can be absorbed by a fluorescent label on a second molecule (e.g., the target) if the
30 second molecule is in proximity to the first molecule. The fluorescent label on the second molecule fluoresces when it absorbs to the transferred energy. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a

situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. A binding event that is configured for monitoring by FRET can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter). By
5 titrating the amount of the first or second binding molecule, a binding curve can be generated to estimate the equilibrium binding constant.

Another example of a homogenous assay is Alpha Screen (Packard Bioscience, Meriden CT). Alpha Screen uses two labeled beads. One bead generates singlet oxygen when excited by a laser. The other bead generates a light signal when singlet
10 oxygen diffuses from the first bead and collides with it. The signal is only generated when the two beads are in proximity. One bead can be attached to the display library member, the other to the target. Signals are measured to determine the extent of binding.

The homogenous assays can be performed while the candidate polypeptide is
15 attached to the display library vehicle, e.g., a bacteriophage.

Surface Plasmon Resonance (SPR). The binding interaction of a molecule isolated from a display library and a target can be analyzed using SPR. SPR or Biomolecular Interaction Analysis (BIA) detects biospecific interactions in real time, without labeling any of the interactants. Changes in the mass at the binding surface
20 (indicative of a binding event) of the BIA chip result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)). The changes in the refractivity generate a detectable signal, which are measured as an indication of real-time reactions between biological molecules. Methods for using SPR are described, for example, in U.S. Patent No. 5,641,640;
25 Raether (1988) *Surface Plasmons* Springer Verlag; Sjolander and Urbaniczky (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705 and on-line resources provide by BIAcore International AB (Uppsala, Sweden).

Information from SPR can be used to provide an accurate and quantitative measure of the equilibrium dissociation constant (K_d), and kinetic parameters, including
30 K_{on} and K_{off} , for the binding of a biomolecule to a target. Such data can be used to compare different biomolecules. For example, proteins encoded by nucleic acid selected from a library of diversity strands can be compared to identify individuals that have high affinity for the target or that have a slow K_{off} . This information can also be

used to develop structure-activity relationships (SAR). For example, the kinetic and equilibrium binding parameters of matured versions of a parent protein can be compared to the parameters of the parent protein. Variant amino acids at given positions can be identified that correlate with particular binding parameters, e.g., high affinity and slow K_{off} . This information can be combined with structural modeling (e.g., using homology modeling, energy minimization, or structure determination by crystallography or NMR). As a result, an understanding of the physical interaction between the protein and its target can be formulated and used to guide other design processes.

10 **Protein Arrays.** Proteins identified from the display library can be immobilized on a solid support, for example, on a bead or an array. For a protein array, each of the polypeptides is immobilized at a unique address on a support. Typically, the address is a two-dimensional address. Methods of producing polypeptide arrays are described, e.g., in De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res.* 28, e3, I-VII; 15 MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 01/40803 and WO 99/51773A1. Polypeptides for the array can be spotted at high speed, e.g., using commercially available robotic apparatus, e.g., from Genetic Microsystems or BioRobotics. The array substrate can be, for example, nitrocellulose, plastic, glass, 20 e.g., surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

Pharmaceutical Compositions

An antibody identified by a method described herein can be formulated as a composition, e.g., a pharmaceutically acceptable composition, for example with a pharmaceutically acceptable carrier. The carrier can be suitable for intravenous, 25 intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g., by injection or infusion) to a subject, e.g., a human subject. The composition can be administered in an amount and for a time effective to ameliorate a condition, e.g., a condition described herein.

30 Compositions that include a protein described herein 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,

tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for administration of humans with antibodies. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular).

The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, the ligand) 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 preferred 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 antibodies can be administered by a variety of methods known in the art. For many applications, the route of administration is intravenous injection or infusion. The route and/or mode of administration can vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

Pharmaceutical compositions can be administered with medical devices known in the art, e.g., an implantable pump, a needleless hypodermic injection device, or a stent.

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 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 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 disclosure 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 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 an antibody is 0.1-20 mg/kg, more preferably 1-10 mg/kg. An antibody can be administered by intravenous infusion at a rate of less than 30, 20, 10, 5, or 1 mg/min to reach a dose of about 1 to 100 mg/m² or about 5 to 30 mg/m². It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens can 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.

The pharmaceutical compositions of the disclosure may include a “therapeutically effective amount” or a “prophylactically effective amount” of an antibody. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the composition may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the protein ligand to elicit a desired response in the individual. A therapeutically effective amount

is also one in which any toxic or detrimental effects of the composition is outweighed by the therapeutically beneficial effects. A “therapeutically effective dosage” preferably inhibits a measurable parameter, e.g., tumor growth rate by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 5 60%, and still more preferably by at least about 80% relative to untreated subjects. Even more preferably, the “therapeutically effective dose” causes tumor stasis or regression. The ability of a compound to inhibit a measurable parameter, e.g., cancer, can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability 10 of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner.

A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the 15 prophylactically effective amount will be less than the therapeutically effective amount.

An antibody described herein can be used in a method of targetting a cell, tissue, or biological structure, e.g., a carbohydrate moiety-containing matrix. For example, the method can be used to prevent binding of a cell or protein to the carbohydrate-moiety displaying cell, tissue, or biological structure. For example, the 20 antibody can be used to treat a leukocyte-migration disorder, e.g., an inflammatory disorder, or an endothelial cell-based disorder, e.g., a disorder associated with excessive angiogenesis, e.g., a cancer (e.g., a metastatic cancer), or a cardiovascular disorder, e.g., atherosclerosis. In one embodiment that relates to a cancerous disorder, the antibody binds to an epitope of a tumor (cancer) glycosidic antigens, e.g., an antigen present on a 25 tumor cell in at least 2, 5, or 10 fold greater abundance than on a normal cell, an antigen the glycolipid class, including acidic glycolipid such as, for example, gangliosides GD2, GD3 and GM3 (melanoma) and neutral glycolipids such as, for example, the Lewis^y (Le^y) (breast, prostate, ovary) and the Globo H (breast, prostate, ovary) antigens, and/or sialylated derivatives thereof; O-glycosyl peptides (or 30 aminoacid) class such as, for example, the Tn antigen (α GalNAc-Ser or α Gal NAc-Thr), T* antigen (β Gal-(1-3)- α -GalNAc-Ser or β Gal(1-3) α Cal-NAc-Thr)- (see, e.g., Springer G. F. *Science* 224, 1198-1206 (1984)) (ovary, breast, lung), or di-Tri (α GalNAc-Ser/Thr)₂, tri-Tn(α GalNAc-Ser/Thr)₃ or hexa-Tn(α GalNAc-Ser/Thr)₆. An

antibody can be conjugated to a toxin, e.g., a cytotoxin or a radionucleoside, to kill, ablate, or otherwise inhibit a tumor cell. The antibody can be administered to a subject, e.g., a human subject, in need of such treatment, e.g., in amount effective and/or for a time effective to ameliorate such a disorder, e.g., to reduce tumor size and/or migration.

5 An antibody to a carbohydrate moiety that is presented by a bacterial cell can be used, e.g., to treat or prevent a disorder associated with a bacterial infection. For example, the epitope recognized by the antibody can be an epitope of a pathogenic bacteria, e.g., *Salmonella*. One *Salmonella* antigen is the serogroup B O-chain polysaccharide. Other exemplary epitopes include those on capsular bacterial
10 polysaccharides selected from the group consisting of *Neisseria meningitis*, *Haemophilus influenza*; *Streptococcus pneumoniae* and other *Streptococcus* species. Accordingly, the antibody can be used to treat infections or other disorders associated with a bacterial pathogen, e.g., a bacteria of such species.

15 Diagnostic Uses

Antibodies identified by the methods described herein (e.g., antibodies that bind to carbohydrates) can be used in a diagnostic method. For example, such an antibody can be used to detect a target molecule (e.g., a carbohydrate) *in vitro* (e.g., a biological sample, such as tissue, biopsy, e.g., a cancerous tissue) or *in vivo* (e.g., *in vivo* imaging
20 in a subject). For example, it may be useful to locate regions within a subject where a particular carbohydrate or other moiety is present.

An exemplary method includes: (i) contacting a sample with the antibody; and (ii) detecting formation of a complex between the antibody and the sample. The method can also include contacting a reference sample (e.g., a control sample) with the
25 antibody, and determining the extent of formation of the complex between the protein and the sample relative to the same for the reference sample. A change, e.g., a statistically significant change, in the formation of the complex in the sample or subject relative to the control sample or subject can be indicative of the presence of the target molecule in the sample.

30 Another method includes: (i) administering the antibody to a subject; and (iii) detecting the protein in the subject, for example using *in vivo* imaging or other

monitoring. The detecting can include determining localization of the protein in the subject.

The antibody can be directly or indirectly labeled with a detectable substance to facilitate detection. Exemplary detectable substances include various enzymes,
5 prosthetic groups, fluorescent materials, luminescent materials, MRI contrast agents (such as chelates that can attach paramagnetic substances), and radioactive materials.

The following examples, which should not be construed as further limiting, illustrate further embodiments. The contents of all references, pending patent
10 applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1. Library construction:

An antibody library that includes antibodies with properties biased for interaction with charged carbohydrates is constructed as follows. The library is constructed by modifying the antibody coding nucleic acids from a more general antibody library, the "FAB-310 library" (Hoet et al. 2005). The Fab-display cassette of FAB-310 is shown in Figure 3. Figure 3 illustrates a representative Fab with the useful restriction sites: ApaLI, AscI, SfiI, XbaI, BstEII, and NotI. The FAB-310 antibody library includes synthetic diversity at HC CDR1 and HC CDR2 in a VH3-23 framework (at HC positions 31, 33, 35, 56 and 58, any amino-acid type except cysteine was allowed; at HC positions 50 and 52, Y, R, W, G, V, and S were allowed; and at position HC 52a, P and S were allowed). It includes natural diversity at HC CDR3 and throughout the LC region (kappa and lambda light chains (LC) from 35 donors who had one or more autoimmune conditions were used). Antibody coding nucleic acids from the FAB-310 antibody library are modified by replacing the segment encoding HC FR3::CDR3 with synthetic oligonucleotides with the following design:

For the (R/K)-(G/S)-X-(R/K)-X-(K/R)-X (SEQ ID NO:10) motif the following codons were used:

ARG|RGT|NNK|ARG|NNK|ARG|NNK (SEQ ID NO:4) .

where K=T&G, N=A,G,C,T, and R= A&G.

The region in which the HC CDR3 resides is diagrammed as follows:

```

      Y   L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C
      |tac|ttg|cag|atg|aac|agC|TTA|AGg|gct|gag|gac|aCT|GCA|Gtc|tac|tat|tgt|
30  FR3-----
      A  R/K G/S  X  R/K  X  R/K  X  W  G  Q  G  T  L  V  T  V  S
      gct|aRg|Rgt|NNK|ARG|NNK|ARG|NNK|TGG|GGC|CAA|GGT|ACC|CTG|GTC|ACC|gtc|tc
      t
35  FR3---- <-----CDR3----->

      S (SEQ ID NO:12)
      Agt| (SEQ ID NO:11)

```

The following primers are used:

Primer 1 (to amplify the first PCR product, anneals in FR4 of HC)

5'AGC|ACA|ATA|GTA|GAC|TGC|AGT|GTC|CTC|AGC|CCT|TAA|GCT|GTT|CAT|C
TG|CAA|GTA 3' (SEQ ID NO:18)

5

Primer 2 (anneals in FR4 of HC, brings the HC-CDR3 diversity)

5'ACT|AGA|GAC|GGT|GAC|CAG|GGT|ACC|TTG|GCC|CCA|MNN|CYT|MNN|CYT
10 |MNN|ACY|CYT|AGC|ACA|ATA|GTA|GAC|TGC|AGT 3' (SEQ ID NO:19)

The library is assembled using two rounds of PCR. In the first reaction, a
nucleic acid segment encoding synthetic CDR1-CDR2 is amplified from plasmid DNA
15 using pUC reverse (5'-AGC GGA TAA CAA TTT CAC ACA GG-3' (SEQ ID
NO:20), beyond *Bst*EII in pMID21) and primer #1 for 15 cycles. In a second reaction,
the products of the first reaction are amplified with a C_{kappa} forward primer (5'- CCA
TCT GAT GAG CAG TTG AAA TCT - 3' (SEQ ID NO:21), shown in Table 50) and
primer #2 for 15 cycles. The product of the second reaction is digested with restriction
20 enzymes *Sfi*I-*Bst*EII and ligated into corresponding sites in the antibody coding library
FAB-310 kappa and lambda plasmid DNA.

This result of these manipulations is a specialized FAB-310 library that differs
in H-CDR3 sequence from the parental FAB-310 library (see Figure 1).

25 Example 2. Library construction:

An antibody library that included antibodies with properties biased for
interaction with carbohydrates was constructed. We modified antibody coding nucleic
acids from a more general antibody library, the "FAB-310 library" described in
30 Example 1. Antibody coding nucleic acids from the FAB-310 antibody library were
modified by replacing the segment encoding HC CDR3 with synthetic oligonucleotides
with the following design:

For the G X R X K/R X (SEQ ID NO:13) motif the following codons were
35 used:

GGA|MNN|CGA|MNN|ARR|MNN (SEQ ID NO:14) .

where M=A&C, N=A, G, C, T, and R= A&G.
40

The region in which the HC CDR3 resides is diagrammed as follows:

Y L Q M N S L R A E D T A V Y Y C
 |tac|ttg|cag|atg|aac|agc|TTA|AGg|gct|gag|gac|aCT|GCA|Gtc|tac|tat|tgc|
 5
 A R G X R X K/R X W G Q G T L V
gct|aga|GGA|MNN|CGA|MNN|ARR|MNN|TGG|GGC|CAA|GGT|ACC|CTG|GTC|
 <-----CDR3----->
 10 T V S S (SEQ ID NO:16)
 ACC|gtc|tct|Agt| (SEQ ID NO:15)

The MNN codons allow the amino-acid types: L, P, Q, H, R, I, M, T, N, and S.

15 The following primers were used:

Primer 1

5' TCCTCTAGCGCAATAGTAGACTGCAGTGTCTCAGCCCTTAAGCTGTTCATCTGCA
 AGTA 3' (SEQ ID NO:22)

20 Primer 2

5' ACTAGAGACGGTGACCAGGGTACCTTGGCCCCANNKYTNNKTCGNNKTCCTCTAG
 CGCAATAGTAGAC3' (SEQ ID NO:23)

25 The library, as shown in Figure 2, was assembled using two rounds of PCR. In
 the first reaction, a nucleic acid segment encoding synthetic CDR1-CDR2 was
 amplified from plasmid DNA using pUC reverse and primer #1 for 15 cycles. In a
 second reaction, the products of the first reaction were amplified with a C_{kappa} forward
 primer and primer #2 for 15 cycles. The product of the second reaction was digested
 30 with restriction enzymes SfiI-BstEII and ligated into corresponding sites in the
 antibody coding library FAB-310 kappa and lambda plasmid DNA.

This result of these manipulations was a specialized FAB-310 library that
 differs only in H-CDR3 sequence from the parental FAB-310 library. The number of
 transformants obtained for the antibody coding library was about 2×10^9 .

35 Example 3. Selection of antibodies from a designed antibody library

The library can be used to select antibodies that bind to various carbohydrate moieties (especially negatively charged carbohydrates), phosphopeptides, or any target.

The library can be selected using, for example, 1, 2, or 3 rounds of selection in which the target is a compound that includes a negatively charged carbohydrate moiety.

Three rounds of selection can be performed using 100-fold more phage than the number of library transformants.

The targets can be immobilized on beads prior to incubation with the phages. At least two selection strategies can be employed. For one exemplary selection, the
5 target is directly coated to immunotubes in $(\text{NH}_4)_2\text{SO}_4$ 90% saturated at pH5.5. For the other selection, the target is spotted on a nitrocellulose membrane which is blocked with BSA or other suitable blocker and selection is performed on this nitrocellulose membrane.

Nonspecific binders can sometimes be found. These bind many antigens and
10 are found because phage sometimes bind to the target membrane or well independent of the target material. However, inclusion of a step to deplete library members on streptavidin and high throughput screening could be used to aid finding specific antibodies.

We conclude that antibodies that bind to negatively charged carbohydrate can
15 successfully be isolated from an antibody library that includes CDR3 sequences with properties favourable for carbohydrate binding. We identified antibodies that specifically bind to certain target compound and that do not detectably interact a non-target compound or control antigen. When using heparan sulfate, the enrichment factors were 1600-fold when the heparan sulfate compound is immobilized using
20 immunotubes.

Example 4: Library construction

An antibody library that included antibodies with properties biased for interaction with carbohydrates was constructed, using the human semisynthetic
25 antibody phage display library FAB-310 (Hoet et al., 2005). The FAB-310 library uses V-gene (V3-23) as scaffold protein with natural HC-CDR3 diversity and synthetic HC-CDR1-CDR2 diversity, which are respectively 5 and 15 amino-acid residues in length. The heavy chains are combined with a natural repertoire of kappa and lambda human light chains. The library has a size of 3.5×10^{10} .

30 The carbohydrate-biased library was constructed to incorporate the sequence R/K-G/S-X-R/K-X-R/K-X (SEQ ID NO:17) into HC-CDR3. 10 ng of FAB-310 Kappa-HC library was used as a template for a PCR reaction using a Kappa constant forward primer.

The HC-CDR3 was designed with the following amino-acid sequence G/S X R/K X R/K X (SEQ ID NO:9). The oligonucleotide (HC-CDR3 primer) supporting this sequence carry the following nucleotide sequence 10ng DNA of the FAB 310 Kappa-HC repertoire were used as template of a PCR reaction using a Kappa constant forward primer (5'-CCATCTGATGAGCAGTTGAAATCT-3' (SEQ ID NO:23)) as 5' end primer and the HC-CDR3 primer (5'-ACTAGAGACGGTGACCAGGGTACCTTGGCCCCAMNNCYTMNNCYTMN NACYCYTAGCACAATAGTAGACTGCAGT-3' (SEQ ID NO:24)) as 3' end primer in order to amplify the full HC repertoire. PCR was performed for 35 cycles in a 25µl format using Advantage 2 DNA polymerase (Clontech) (1 min at 95°C, 1 min at 50°C and 2 min at 68°C). 10 µg of the 650 bp generated fragment were digested with 4U/µg of *BstEII* restriction endonuclease (NEB) followed by a cleavage with 10U/µg *XbaI*. A 140bp fragment containing the HC-CDR3 repertoire was gel purified. One and a half micrograms of the resulting DNA fragment was ligated into 6 µg of similarly cut phagemid vector pMID21 (containing both the kappa and lambda light chain repertoires) using T₄ DNA ligase (NEB) at a 1:4 vector :insert ratio. 2.5 µg of desalted λHC-ligation mixture and 2.5 µg of the κHC mixture were separately electroporated into the *E. coli* strain TG1 using 100 ng of ligation mixture per electroporation event. The Fab library has a size of 2x10⁹ representants.

The library was checked for quality by PCR amplifying the Fab antibody cassette as described in Shoonbroodt et al. (2005, *Nucl. Acids Res.* 33(9):e81), and sequencing the amplified products.

25 Example 5: Selection, screening, and sequencing of antibodies specific for carbohydrate antigens

Antibodies specific to carbohydrate antigens were selected from the library described in Example 2. Although this library has reduced diversity as compared to the libraries described in Examples 1 and 3, the library is nonetheless biased for interaction with carbohydrates.

Selection

Phagemid particles were rescued with with helper phage M13-KO7 according to Marks et al. (1991 *J. Mol. Biol.* 222(3):581-97) on a 3-liter scale. Three library selections were performed (1) sulfocore 6 (SC6), (2) sulfocore 1 (SC1), and (3) a combination of heparan sulfate (HS) and chemically desulfated N-sulfated heparin (CDSNS). The chemical structure of SC1 and SC6 and their non-sulfated counterparts are shown in Figure 4.

The 6-sulfo sialyl Lewis X glycans correspond to the L-selectin ligand "sulfoadhesin." SC6 selection was performed using 10^{13} phages which had been pre-incubated for 1 hour in 2% Marvel PBS at room temperature (RT). 500 nM SC6 antigen, which had been modified by addition of a serine residue to carbon₆ and coupling to a biotin molecule, was captured on magnetic streptavidin Dynabeads (Dyna), blocked with 2% Marvel PBS, then added to the blocked phages. After several washing steps, phages were eluted with 100 mM TEA and neutralized in 1M Tris-HCl (pH7.4). Eluted phages were used to infect *E. coli* TG1 cells. Two additional rounds of selection were performed, for a total of three rounds, under the same conditions.

SC1 selection was carried out essentially the same as the selection for SC6.

HS and CDSNS were immobilized on immunotubes (MAXISORP™, Nunc) by incubation with a 100 µg/mL mixture of HS and CDSNS in 90% saturated $(\text{NH}_4)_2\text{SO}_4$ buffer. Immunotubes and 10^{13} phage were blocked separately for 30 minutes with 1%BSA, 0.05% Tween-20 in PBS. The blocked phage preparation was then incubated with the target in the tubes for 30 minutes on a rotator followed by 90 minute incubation standing at RT. Tubes were washed 10 times with 0.05% TWEEN® 20 in PBS, followed by 10 times with PBS. Phage were eluted in 100 mM TEA, then immediately neutralized with 1M Tris, pH7.4. Eluted phages were used to infect *E. coli* TG1 cells. The selection was repeated an additional two rounds (for a total of three) under the same conditions except that wash steps were performed 20 times.

Screening

Phage-displaying Fab eluted from the third round of selection were recovered, propagated, and isolated as individual clones. Culture supernatants containing phage-displaying Fab were tested by ELISA. Fab from the SC6 selection were assayed against biotinylated SC6 indirectly captured via immobilized BSA-streptavidin

(MAXISORP, Nunc). Plates were coated with 50 ng SC6 per well (biotinylated) in PBS. 50 ng C6 (non-sulfated core 6) or 500ng streptavidin per well were used as negative controls. Fab from the SC1 selection were screened essentially as for SC6, but using SC1 as the antigen. HS-binding Fab were assayed using plates was coated with 500 ng per well of a mix of HS/CDSNS (in 90% $(\text{NH}_4)_2\text{SO}_4$) or 100 ng BSA (as negative control). Plates were coated overnight at 4°C. Plates were washed three times with PBS-TWEEN®20 0.05% (PBST), blocked with 2% Marvel in PBS (MPBS), then washed three times with PBST before use. Phages were incubated with the antigen for one hour at RT, then removed and the plates were washed six times with PBST. Anti-M13 HRP antibody (APB) diluted 5000x in PBS was added for one hour, followed by six washes with PBST. Optical density at 450nm (OD_{450}) was measured after development with TMB substrate. A number of clones which bound to SC6 or HS were identified in the screening, but no Fabs binding SC1 were identified. It is expected that selection of the libraries described in Examples 1 and 4 would identify SC1-specific Fabs, in view of the increased diversity for these libraries in comparison with the library described in Example 2.

Sequencing

Fab from clones giving a positive signal in ELISA (more than 2 times > background) were amplified using 5' and 3' backbone primers and the PCR products were sequenced for both light and heavy chains. Antibodies to SC6 did not react in ELISA with C6, SC1 and C1 (related structures), clearly showing the specificity of these antibodies to SC6.

We identified antibodies that specifically bind to heparan sulfate and that do not detectably interact with another sulfated carbohydrate or control antigen. Three distinct antibodies were identified, designated 1HS, 2HS, and 3HS. The heavy chain (HC) and light chain (LC) variable region amino acid sequences are shown in Table 1, and the CDR amino acid sequences are shown in Table 2.

TABLE 1

Fab	Heavy Chain (HC)	Light Chain (LC)
1HS	EVQLLESGGGLVQPGGSLRLSCAASGFTF SNYTMAWVRQAPGKGLEWVSSISSSGGHT PYADSVKGRFTISRDN SKNTLYLQMNSLR AEDTAVYYCARGKRN RN (SEQ ID NO:33)	QDIQMTQSPDSLAVSLGERATINCKSSQSVLYS SNNKNYLAWYQQKPGQPPKLLIYWASTRESGVP DRFSGSGSGTDFTLTISLQAEDVAVYYCQQYY STPPT (SEQ ID NO:34)
2HS	EVQLLESGGGLVQPGGSLRLSCAASGFTF SGYRMNWVRQAPGKGLEWVSSIGSSGGHT SYADSVKGRFTISRDN SKNTLYLQMNSLR AEDTAVYYCARGKRN RT (SEQ ID NO:35)	QDIQMTQSPGTLSPGERATLSCRASQSVSSS YLAWYQQKPGQAPRLLIYGASSRATGIPDRFSG SGSGTDFTLTISRLEPEDFAVYYCQQYGSSPRT (SEQ ID NO:36)
3HS	EVQLLESGGGLVQPGGSLRLSCAASGFTF SEYIMSWVRQAPGKGLEWVSYISPSGGTT KYADSVKGRFTISRDN SKNTLYLQMNSLR AEDTAVYYCARGRR TKH (SEQ ID NO:37)	QDIQMTQSPPLSLPVTLGQSASISCRSSQSLVHS DGNTYLNWFQQRPQGSPRR LIYKVSNRDSGVPD RFSGSGSGTDFTLKISRVEAEDVGVYYCMQGTH WPYT (SEQ ID NO:38)

TABLE 2

	CDR1	CDR2	CDR3
1HS HC	NYTMA (SEQ ID NO:39)	SISSSGGHTPYADSVKG (SEQ ID NO:40)	GKRN RN (SEQ ID NO:41)
2 HC	GYRMN (SEQ ID NO:42)	SIGSSGGHTSYADSVKG (SEQ ID NO:43)	GKRN RT (SEQ ID NO:44)
3HS HC	EYIMS (SEQ ID NO:45)	YISPSGGTTKYADSVKG (SEQ ID NO:46)	GRR TKH (SEQ ID NO:47)
1HS LC	KSSQSVLYSSNKNYLA (SEQ ID NO:48)	WASTRES (SEQ ID NO:49)	QQYYSTPPT (SEQ ID NO:50)
2HS LC	RASQSVSSSYLA (SEQ ID NO:51)	GASSRAT (SEQ ID NO:52)	QQYGSSPRT (SEQ ID NO:53)
3HS LC	RSSQSLVHSDGNTYLN (SEQ ID NO:54)	KVSNRDS (SEQ ID NO:55)	MQGTHWPY (SEQ ID NO:55)

- 5 At least sixteen clones showed specific binding to SC6 (as compared to binding to BSA, streptavidin, and unrelated sulfated carbohydrates). After sequencing, four distinct antibodies were identified, designated 1SC6, 2SC6, 3SC6, and 4SC6. Sequences of the HC and LC variable regions (both nucleotide and amino acid) are shown in Table 3, and CDR amino acid sequences are shown in Table 4.

TABLE 3

Antibody	Nucleotide Sequence	Amino Acid Sequence
1SC6 LC	CAGAGCGTCTTGACTCAGCCACCCTCAGTGTC AGTGGCCCCAGGAAGGACGGCCACCATTACCT GTGGGGGAAAAACATTGGAGCTAAAAGTGTC AACTGGTACCAACAGAGGCCAGGCCAGGCCCC TGTCTGGTCATCTTCTATGATACCGACCGGC CCGCAGGAATAACTGGGCGATTGTCTGGCTCC AATTCTGGGAACTCGGCCACCCTGACCATCAG CAGGGTCGAGGCCGGGGACGAGGCCGATTATT ACTGTCAGGTGTGGGGTGTGAGTGGTGATCAT CCGGTTTTTCGGCGGAGGGACTAGGCTGACCGT CCTGGGTCAGCCC (SEQ ID NO:56)	QSVLTQPPSVSVAPGRTATITCGGKNI GAKSVNWWYQQRPGQAPVLVIFYDTRP AGITGRLSGSNSGNSATLTISRVEAGD EADYYCQVWGVSGDHPVFEGGTRLTVL GQP (SEQ ID NO:57)
1SC6 HC	GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCT TGTTTCAGCCTGGTGGTTCTTTACGTCTTTCTT GCGCTGCTTCCGGATTCACTTTCTCTGGTTAC ATGATGGCTTGGGTTTCGCCAAGCTCCTGGTAA AGGTTTGGAGTGGGTTTCTTGGATCTCTCCTT CTGGTGGCTTTACTAAGTATGCTGACTCCGTT AAAGGTCGCTTCACTATCTCTAGAGACAACTC TAAGAATACTCTCTACTTGCAGATGAACAGCT TAAGGGCTGAGGACACTGCAGTCTACTATTGC GCTAGAGGAAGCCGACTCAGGCAT (SEQ ID NO:58)	EVQLLESGGGLVQPGGSLRLSCAASGF TFSGYMMAWVRQAPGKGLEWVSWISPS GGFTKYADSVKGRFTISRDNKNTLYL QMNSLRAEDTAVYYCARGSLRH (SEQ ID NO:59)
2SC6 LC	CAAGACATCCAGATGACCCAGTCTCCATCCTC CCTGTCTGCATCTGTAGGAGACAGAGTCACCA TCACTTGCCGGGCAAGTCAGGGCATTAGAAAT GATTTAGGCTGGTATCAGCAGAAACCAGGGAA AGCCCCTAAGCGCCTGATCTATGCTGCATCCA GTTTGCAAAGTGGGGTCCCATCAAGGTTACAGC GGCAGTGGATCTGGGACAGAATCACTCTCAC AATCAGCAGCCTGCAGCCTGAAGATTTTGCAA CTTATTACTGTCTACAGCATAATAGTTACCCG TACACTTTTGGCCAGGGGACCAAGCTGGAGAT CAAACG (SEQ ID NO:60)	QDIQMTQSPSSLSASVGDRTITCRAS QGIRNDLGWYQQKPKAPKRLIYAASS LQSGVPSRFSGSGSGTEFTLTISLQP EDFATYYCLQHNSYPYTFGQGTKLEIK (SEQ ID NO:61)
2SC6 HC	GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCT TGTTTCAGCCTGGTGGTTCTTTACGTCTTTCTT GCGCTGCTTCCGGATTCACTTTCTCTGGTTAC ATGATGCAGTGGGTTTCGCCAAGCTCCTGGTAA AGGTTTGGAGTGGGTTTCTTCTATCTCTCCTT CTGGTGGCTTTACTGAGTATGCTGACTCCGTT AAAGGTCGCTTCACTATCTCTAGAGACAACTC TAAGAATACTCTCTACTTGCAGATGAACAGCT TAAGGGCTGAGGACACTGCAGTCTACTATTGC GCTAGAGGACGGCGACACAGGAAT (SEQ ID NO:62)	EVQLLESGGGLVQPGGSLRLSCAASGF TFSGYMMQWVRQAPGKGLEWVSSISPS GGFTEYADSVKGRFTISRDNKNTLYL QMNSLRAEDTAVYYCARGRRHRN (SEQ ID NO:63)
3SC6 LC	CAAGACATCCATATGACCCAGTCTCCAGGCAC CCTGTCTTTGTCTCCAGGGGAAAGAGCCACCC TCTCCTGCAGGGCCAGTCAGAGTATTAGCAAC ACCTACTTAGCCTGGTACCAGCAGAAACCTGG CCAGGCTCCAGGCTCCTCATCTATGGTGCAT CCAGCAGGGCCACTGGCATCCAGACAGGTTT AGTGGCAGTGGGTCTGGGACAGACTTCACTCT CACCATCAGCAGGCTGGAGCCTGAAGATTTTG CAGTGTATTACTGTCAGCAGTATGGTAGCTCA CCCCTGTACACTTTTGGCCAGGGGACCAAGTT GGAGATCAAA (SEQ ID NO:64)	QDIHMTQSPGTLSSLSPGERATLSCRAS QSIINTYLAHYQQKPGQAPRLLIYGAS SRATGIPDRFSGSGSGTDFTLTISRLE PEDFAVYYCQYQYSSPLYTFGQGTKLE IK (SEQ ID NO:65)
3SC6 HC	GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCT TGTTTCAGCCTGGTGGTTCTTTACGTCTTTCTT	EVQLLESGGGLVQPGGSLRLSCAASGF TFSGYMMNWVRQAPGKGLEWVSSIGPS

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GCGCTGCTTCCGGATTCACTTTCTCTGGTTAC
ATGATGAATTGGGTTGCGCAAGCTCCTGGTAA
AGGTTTGGAGTGGGTTTCTTCTATCGGTCCTT
CTGGTGGCTATACTACTTATGCTGACTCCGTT
AAAGGTCACCTTCACTATCTCTAGAGACAACTC
TAAGAACACTCTCTACTTGCAGATGAACAGCT
TAAGGGCTGAGGACACTGCAGTCTACTATTGC
GCTAGAGGACTGCGAATGAAAAAG
(SEQ ID NO:66)

4SC6 LC

CAGAGCGTCTTGACTCAGGACCCTGCTGTGTC
TGTGGCCTTGGGACAGACAGTCAGGATCACAT
GCCAAGGAGACAGCCTCAGAAGCTATTATGCA
AGCTGGTACCAGCAGAAGCCAGGACAGGCCCC
TGTACTTGTCTATCTATGGTAAAAACAACCGGC
CCTCAGGGATCCCAGACCGATTCTCTGGCTCC
AGCTCAGGAAACACAGCTTCCTTGACCATCAC
TGGGGCTCAGGCGGAAGATGAGGCTGACTATT
ACTGTAACCTCCCGGGACAGCAGTGGTAACATC
TGGAAGGTATTGCGCGGAGGGACCAAGCTGAC
CGTCCTAGGTCAGCCC (SEQ ID NO:68)

4SC6 HC

GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCT
TGTTGAGCCTGGTGGTTCTTTACGTCCTTTCTT
GCGCTGCTTCCGGATTCACTTTCTCTGTTTAC
CCTATGCATTGGGTTGCGCAAGCTCCTGGTAA
AGGTTTGGAGTGGGTTTCTTATATCGGTTCTT
CTGGTGGCGAGACTATGTATGCTGACTCCGTT
AAAGGTCGCTTCACTATCTCTAGAGACAACTC
TAAGAATACTCTCTACTTGCAGATGAACAGCT
TAAGGGCTGAGGACACTGCAGTCTACTATTGC
GCTAGAGGAAAACGAAACAAAAGA
(SEQ ID NO:70)

GGYTTYADSVKGFHTISRDN SKNTLYL
QMNSLRAEDTAVYYCARGLRMKK
(SEQ ID NO:67)

QSVLTQDPAVSVALGQTVRITCQGDSL
RSYYASWYQQKPGQAPV LVIYGKNNRP
SGIPDRFSGSSSGNTASLTITGAQAED
EADYYCNSRDSSGNIWKVFGGGTKLTV
LGQP (SEQ ID NO:69)

EVQLLES GGGGLVQPGGSLRLSCAASGF
TFSVYPMHWVRQAPGKGLEWVSYIGSS
GGETMYADSVKGRFTISRDN SKNTLYL
QMNSLRAEDTAVYYCARGKRNKR
(SEQ ID NO:71)

TABLE 4

	LC - CDR1	LC - CDR2	LC - CDR3	Fam ily	HC-CDR1	HC-CDR2	HC-CDR3
1SC6	GGKNIGAKSVN (SEQ ID NO:72)	YDTDREA (SEQ ID NO:76)	QVWGVSGDHPV (SEQ ID NO:80)	VL3	GYMMA (SEQ ID NO:84)	WISPSGGFTKYADSVKG (SEQ ID NO:88)	GSRLRH (SEQ ID NO:92)
2SC6	RASQGIRNDLG (SEQ ID NO:73)	AASSLQS (SEQ ID NO:77)	LQHNSYPYT (SEQ ID NO:81)	VK1	GYMMQ (SEQ ID NO:85)	SISPSGGFTEYADSVKG (SEQ ID NO:89)	GRRHRN (SEQ ID NO:93)
3SC6	RASQISNTYLA (SEQ ID NO:74)	GASSRAT (SEQ ID NO:78)	QQYGSSPLYT (SEQ ID NO:82)	VK3	GYMMN (SEQ ID NO:86)	SIGPSGGYTTYADSVKG (SEQ ID NO:90)	GLRMKK (SEQ ID NO:94)
4SC6	QGDSIRSYIAS (SEQ ID NO:75)	GKNNRPS (SEQ ID NO:79)	NSRDSSGNIWKV (SEQ ID NO:83)	VL3	VYPMH (SEQ ID NO:87)	YIGSSGGETMYADSVKG (SEQ ID NO:91)	GKRNKR (SEQ ID NO:95)

5

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

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CLAIMS:

1. A method for identifying an antibody that binds to a negatively charged carbohydrate moiety, the method comprising:
 - providing a protein library that comprises a plurality of diverse antibodies,
 - 5 each antibody comprising a heavy chain variable domain sequence and a light chain variable domain sequence, wherein each heavy chain variable domain sequence comprises diversity in HC CDR3, and the HC CDR3 comprises (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO: 3), wherein each X is independently any amino acid;
 - contacting members of the protein library to a target molecule that includes a
 - 10 negatively charged carbohydrate moiety; and
 - identifying one or more members that interact with the target molecule.
2. The method of claim 1, wherein at least two of the basic amino acid positions (K/R) are separated by at least one amino acid position that is allowed to vary among at least ten different amino acids.
- 15 3. The method of claim 1 wherein, for each antibody of the plurality, X represents positions that are varied among at least four different amino acids.
4. The method of claim 3, wherein X represents a position that is varied among at least ten different amino acids.
5. The method of claim 4, wherein X represents a position that is varied among at
- 20 least sixteen different amino acids.
6. The method of claim 1, wherein X represents a position that is varied among non-cysteine amino acids.
7. The method of claim 5, wherein X represents a position that is varied among all possible amino acids.

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8. The method of claim 1, wherein X represents a position that is varied among a set of amino acids that exclude basic amino acids.
9. The method of claim 1 wherein the HC CDR3 region is less than 10 amino acids in length.
- 5 10. The method of claim 9 wherein the HC CDR3 region is less than 8 amino acids in length.
11. The method of claim 1 wherein the antibodies of the plurality comprise diverse light chain variable domains.
12. The method of claim 1 wherein CDR1 and CDR2 of the heavy chain variable
10 domain sequence are encoded by sequences derived from diverse synthetic oligonucleotides.
13. The method of claim 1 wherein the step of identifying one or more members that interact with the target molecule comprises physically separating members of the library that bind to the target molecule from members that do not bind.
14. The method of claim 1 wherein the protein library is a phage display library
15 and the step of identifying one or more members that interact with the target molecule comprises recovering one or more phage that encode an antibody that binds to the target molecule.
15. The method of claim 1 wherein the protein library is a collection of proteins disposed on an array.
- 20 16. The method of claim 1 wherein the target molecule is a glycosaminoglycan.
17. The method of claim 1 wherein the protein library is depleted of members that interact with a non-target molecule.
18. The method of claim 1 further comprising identifying a plurality of antibodies that interact with the target molecule, and selecting a subset of antibodies from the plurality
25 that preferentially interact with the target molecule relative to a non-target molecule.

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19. The method of claim 1 further comprising preparing a pharmaceutical composition that comprises an antibody that comprises CDRs from a library member identified as interacting with the target molecule and a pharmaceutically acceptable carrier.

20. A pool of oligonucleotides comprising a plurality of different oligonucleotides,
5 wherein each oligonucleotide of the plurality comprises a sequence with a HC CDR3-coding sequence that encodes the amino acid sequence (G/S)-X-(K/R)-X-(K/R)-X (SEQ ID NO: 3), wherein each X is independently any amino acid, (K/R) can be either lysine or arginine, and (G/S) can be either glycine or serine.

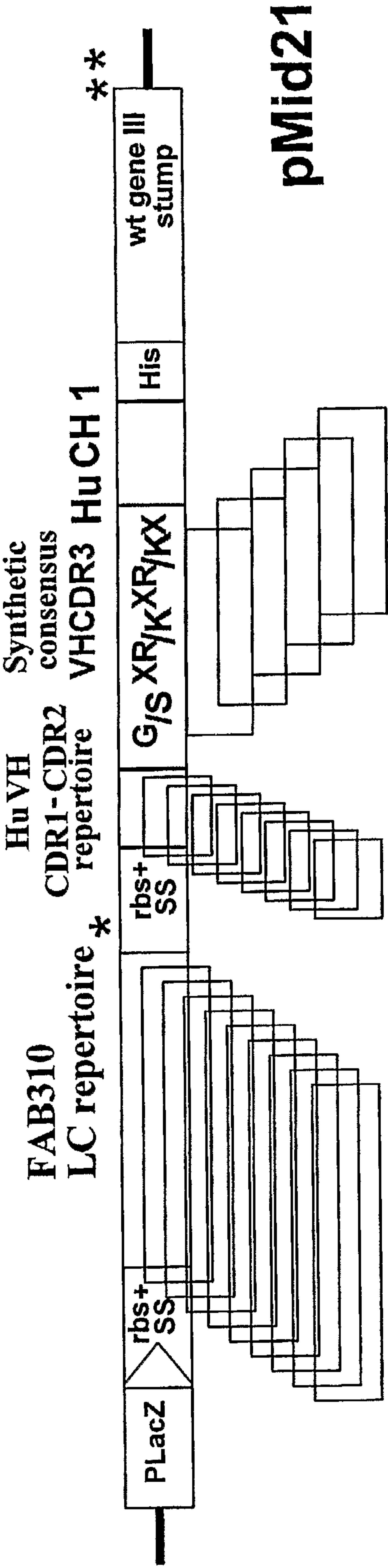


FIG. 1

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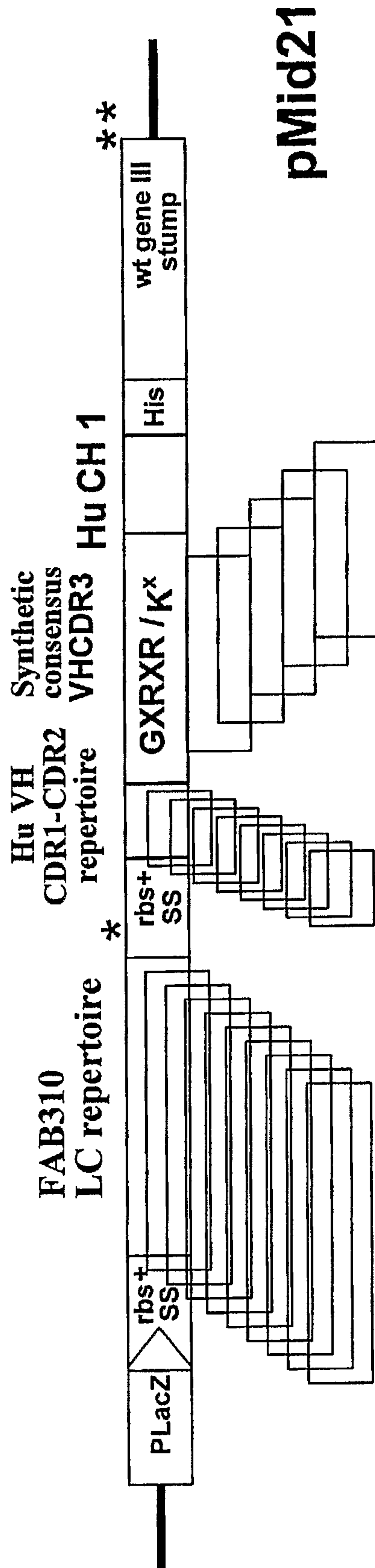


FIG. 2

```
! Fab Cassette of pMID21
! 1 GGATCC aCAC GAGcgca
! BamHI. (1/2) BssSI..(1/2)
! 18 acgcaatTAA TGTgagttag ctcaactcatt aggcacccca ggCTTTACAc tttatgcttc
! ..-35.. Plac ..-10.
! 78 cggctcgtat gttgtgtgga attgtgagcg gataacaatt tc
!
! Fab cassette
! BamHI Cassette: <promoter::signal::linker:-
! :Ckappa><VH-CH1::III-D3> trp Term
!
! 120 acaCAGGA AACAGCTATGAC
! 140 catgatta cgCCAAGCTT TGGagccttt ttttggaga ttttcaac
! PflMI.....
! Hind3. (1/3)
!
! signal::linker::CL
!
! 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
! fm K K K L L F A I P L V V P F Y
! 186 gtg aaa aaa tta tta ttc gca att cct tta gtt gtt cct ttc tat
!
! 16 17 18 19 20
! S H S A Q
! 231 tct cac aGT GCA Cag
! ApaLI.....
!
! ----- FR1 ----->
! D V V M T Q S P L S L P V A P 9D
! 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
! gat Gtt gtg atg act cag tct cca ctc tcc ctg ccc gtc Gcc cct
```

FIG. 3A

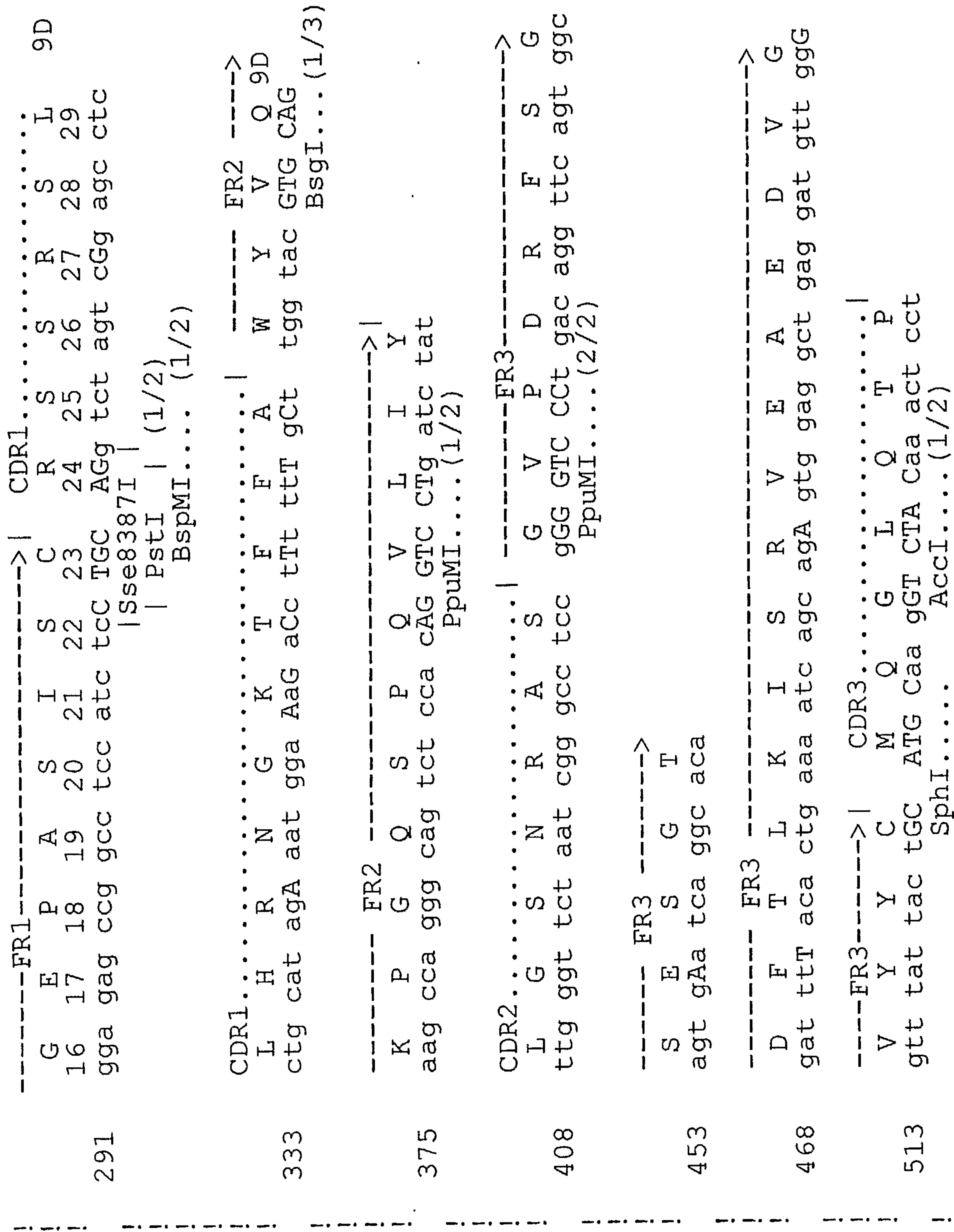


FIG. 3B

----- Fr4 ----->
Y T F G Q G T K L E I K (SEQ ID NO:26)
546 tac act ttT GGC CAg ggg acc aag ctg gag atc aaa (SEQ ID NO:25)
MscI....(2/3)

VL-CL segments will be cloned in as ApaLI-Ascl fragments. <-----

Ckappa-----
R G T V A A P S V F I F P S
582 cgt gga act gtg gCT GCA Cca tct GTC TTC atc ttc ccg cca tct
BsgI....(2/3) BbsI...(1/2) Ckappa forward

D E Q L K S G T A S V V C L L
627 gat gag cag ttg aaa tct gga act gcc tct tct gtt gtg tgc ctg ctg
Ckappa forward.....

N N F Y P R E A K V Q W K V D
672 aat aac ttc tat ccc aga gag gcc aaa gta cag tgg aag gtg.gat

N A L Q S G N S Q E S V T E Q
717 aac gcc ctc caa tcg ggt aac tcc cag gag agt gtc aca gag cag

D S K D S T Y S L S S T L T L
762 gac agc aag gac agc acc tac agc ctc agc agc acc ctg acG CTG
EspI....

S K A D Y E K H K V Y A C E V
807 AGC aaa gca gac tac gag aaa cac aaa GTC TAC gcc tgc gaa gtc
....EspI.... AccI....(2/2)

T H Q G L S S P V T K S F N R
852 acc cat cag ggc ctg agt tca CCG GTg aca aag agc ttc aac agg
AgeI....(1/2)

G E C . .
897 gga gag tgt taa taa
(SEQ ID NO:28)

FIG. 3C

912 GG CGGCGCCaatt
ASCI.....
BSSHII.

924 ctatttcaag gagacagtca ta (SEQ ID NO:27)! region upstream of Met1 preserved.

Signal for VH-CH1-IIIstump
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
M K Y L L P T A A A G L L L L L
946 atg aaa tac cta ttg cct acg gca gcc gct gga ttg tta tta ctc

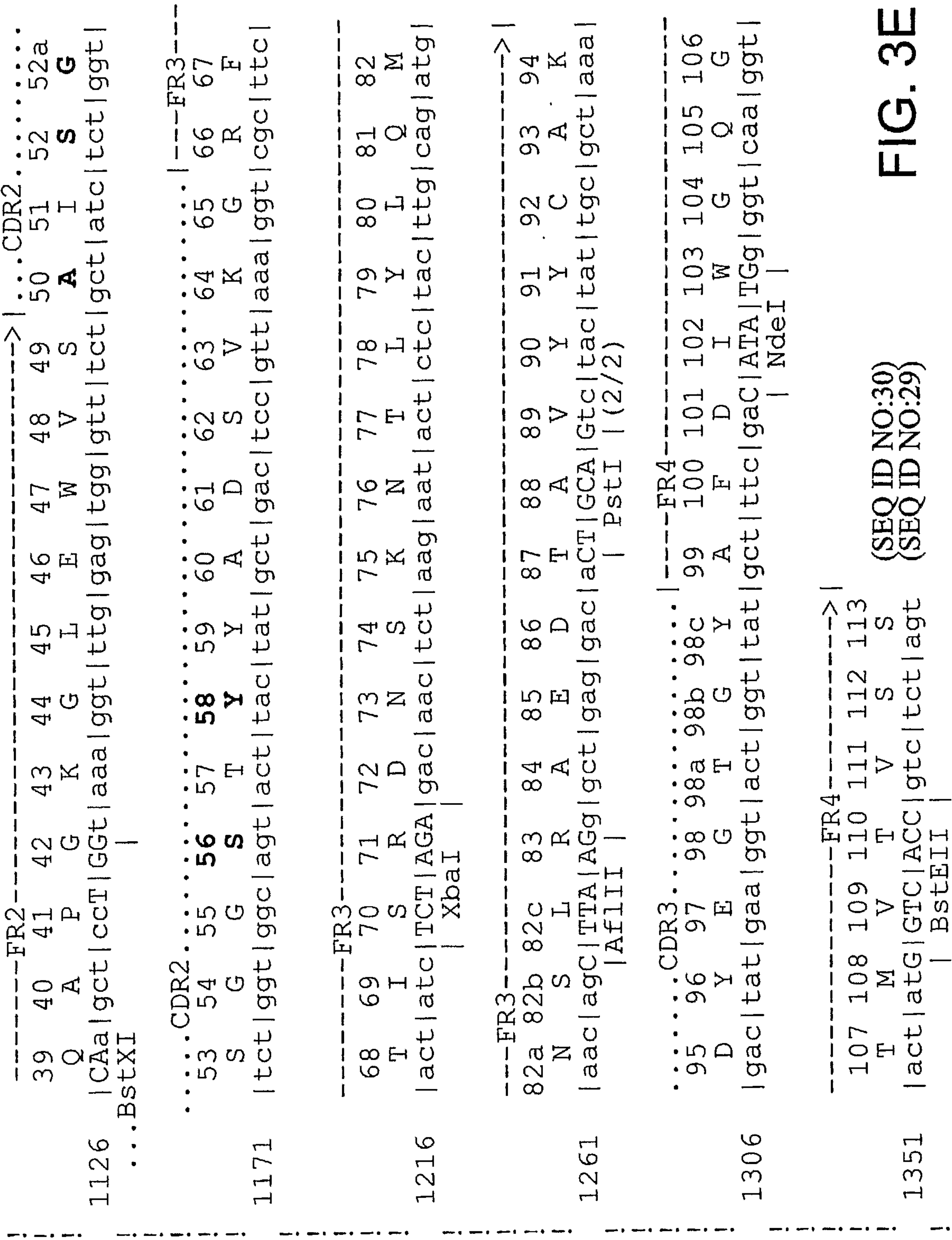
16 17 18 19 20 21 22
A A Q P A M A
991 gcG GCC cag ccG GCC atg gcc
Sfii.....
Ngomi...:(1/2)
NcoI....

VH!
FR1 (DP47/V3-23) -----
1 2 3 4 5 6 7 8
E V Q L L E S G
gaa|ggtt|CAA|TTG|tta|gag|tct|ggt|
| MfeI |

1012

-----FR1-----
9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
G G L V Q P G G S L R L S C A
1036 |ggc|ggt|ctt|gtt|cag|cct|ggt|ggt|tct|tta|cgt|ctt|tct|tgc|gct|

-----FR1----->|...CDR1.....|-----FR2-----
24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
A S G F T F S S Y A M S W V R
1081 |gct|TCC|GGA|ttc|act|tct|tct|tCG|TAC|Gct|atg|tct|tgg|gtt|cgC|
| BspEI | BsiWI | BstXI.



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FIG. 3F

```

! CH1!      A   S   T   K   G   P   S   V   F   P   L   A   P   S   S
1372      gcc tcc acc aag ggc cca tcg gtc ttc ccg CTA GCa ccc tcc tcc tcc
!                               NheI....

151 152 153 154 155 156 157 158 159 160 161 162 163 164 165
K   S   T   S   G   G   T   A   A   L   G   C   L   V   K
aag agc acc tct ggg ggc aca gcg gcc ctg ggc tgc ctg gtc aag

1417

166 167 168 169 170 171 172 173 174 175 176 177 178 179 180
D   Y   F   P   E   P   V   T   V   S   W   N   S   G   A
gac tac ttc ccc gaa ccg ggc gtc gtc acg gtg tgc tgc aac tca ggc gcc

1462

181 182 183 184 185 186 187 188 189 190 191 192 193 194 195
L   T   S   G   V   H   T   F   P   A   V   L   Q   S   S
ctg acc agc ggc ggc gtc cac acc ttc ccg gct gtc cta cag tcc tca

1507

196 197 198 199 200 201 202 203 204 205 206 207 208 209 210
G   L   Y   S   L   S   S   V   V   T   V   P   S   S   S
gga ctc tac tac tcc ctc agc agc gta gtg acc gtg ccc tCC Agc agc
1552                               BstXI.....

211 212 213 214 215 216 217 218 219 220 221 222 223 224 225
L   G   T   Q   T   Y   I   C   N   V   N   H   K   P   S
tTG Ggc acc cag acc tac atc tgc aac gtg aat cac aag ccc agc
1597                               BstXI.....

226 227 228 229 230 231 232 233 234 235 236 237 238
N   T   K   V   D   K   K   V   E   P   K   S   C
aac acc aag gtg gac aaG AAA GTT GAG CCC AAA TCT TGT

1642

139 140 141 His tag..... cMyc tag..... I
A   A   A   H   H   H   H   G   A   A   E   Q   K   L
GCG GCC GCa cat cat cac cat cac ggg gcc gca caa aaa ctc atc
1681 NotI.....
EagI....

```

```
.....
S E E D L N G A A E A S S A S N A S
1732 tca gaa gag gat ctg aat ggg GCC gca gaG GcT agt tct gct agt aAC GCG Tct
      BglI.....(3/4) MluI.....

Domain 3 (IIIstump)-----
S G D F D Y E K M A N A N K G A
1786 tcc ggt gat ttt gat tat gaa aag atg gca aac gct aat aag ggg gct

M T E N A D E N A L Q S D A K G
1834 atg acc gaa aat gcc gat gaa aac gcg cta cag tct gac gct aaa ggc

K L D S V A T D Y G A A I D G F
1882 aaa ctt gat tct gtc gct act gat tac ggt gct gct atc gat ggt ttc

I G D V S G L A N G N G A T G D
1930 att ggt gac gtt tcc ggc ctt gct aat ggt aat ggt gct act ggt gat

F A G S N S Q M A Q V G D G D N
1978 ttt gct ggc tct aat tcc caa atg gct caa gtc ggt gac ggt gat aat

S P L M N N F R Q Y L P S L P Q
2026 tca cct tta atg aat aat ttc cgt caa tat tta cct tcc ctc cct caa

S V E C R P F F V F G A G K P Y E
2074 tcg gtt gaa tgt cgc cct ttt gtc ttt ggc gct ggt aaa cca tat gaa

F S I D C D K I N L F R
2122 ttt tct att gat tgt gac aaa ata aac tta ttc cgt
      End Domain 3

G V F A F L L Y V A T F M Y V F140
2158 ggt gtc ttt gcg ttt ctt tta tat gtt gcc acc ttt atg tat gta ttt
      start transmembrane segment

S T F A N I L
2206 tct acg ttt gct aac ata ctg
```

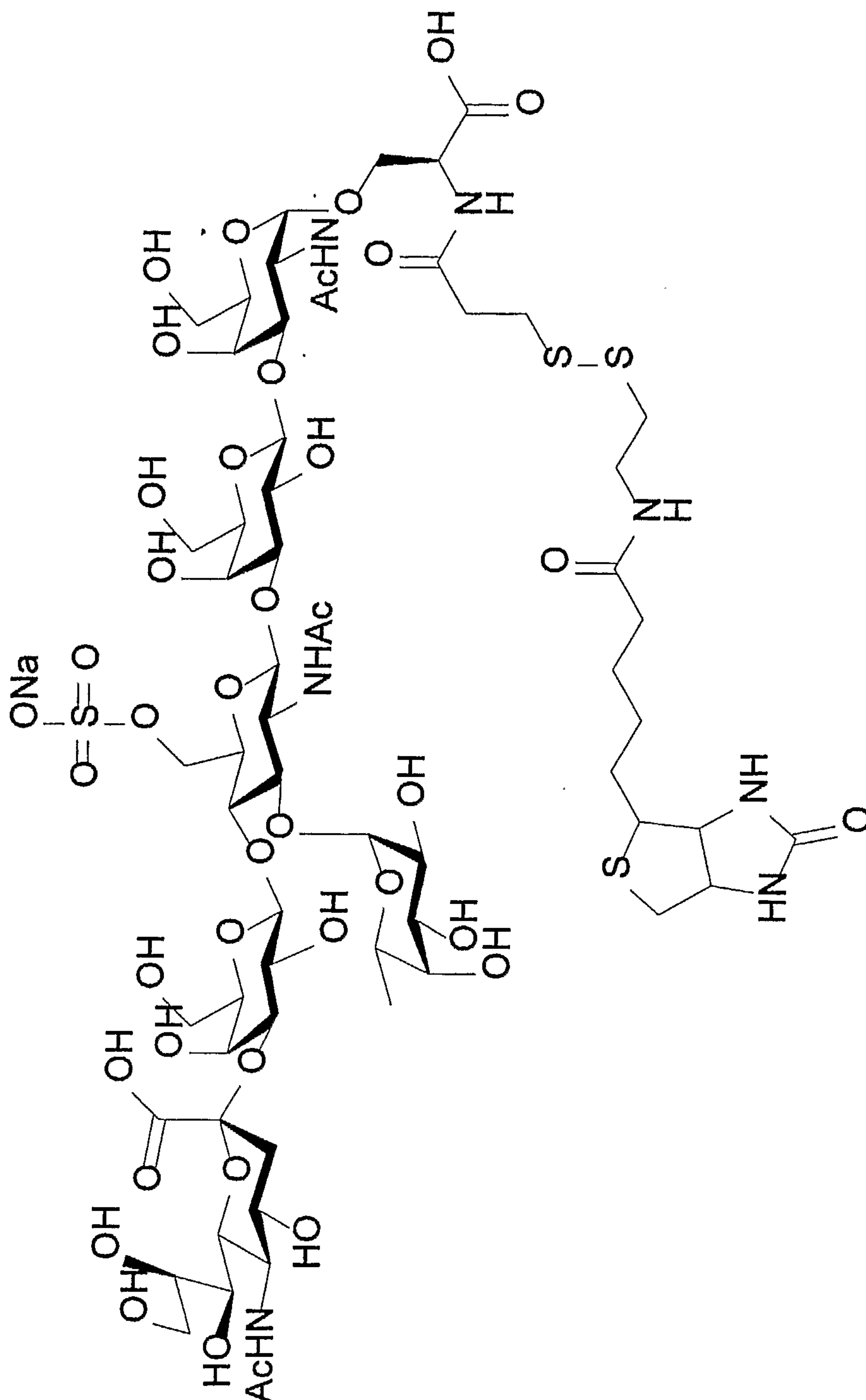
FIG. 3G

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! 2227 R N K E S .
! cgt aat aag gag tct TAA
Intracellular anchor.
tga aAC GCG Tga tga GAATTC (SEQ ID NO:32)
MluI.... EcoRI. (SEQ ID NO:31)

FIG. 3H

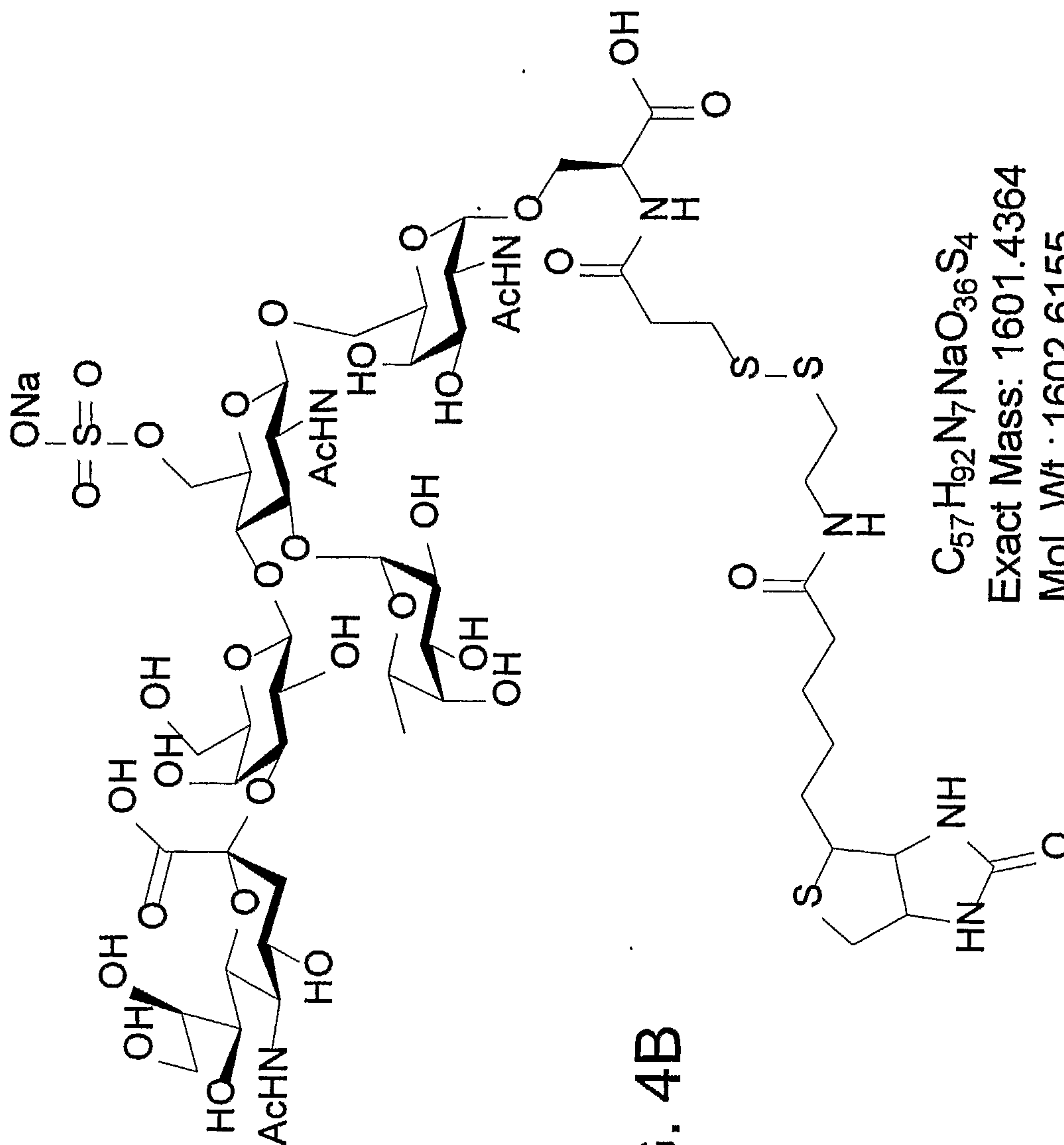
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Sulfo Core 1

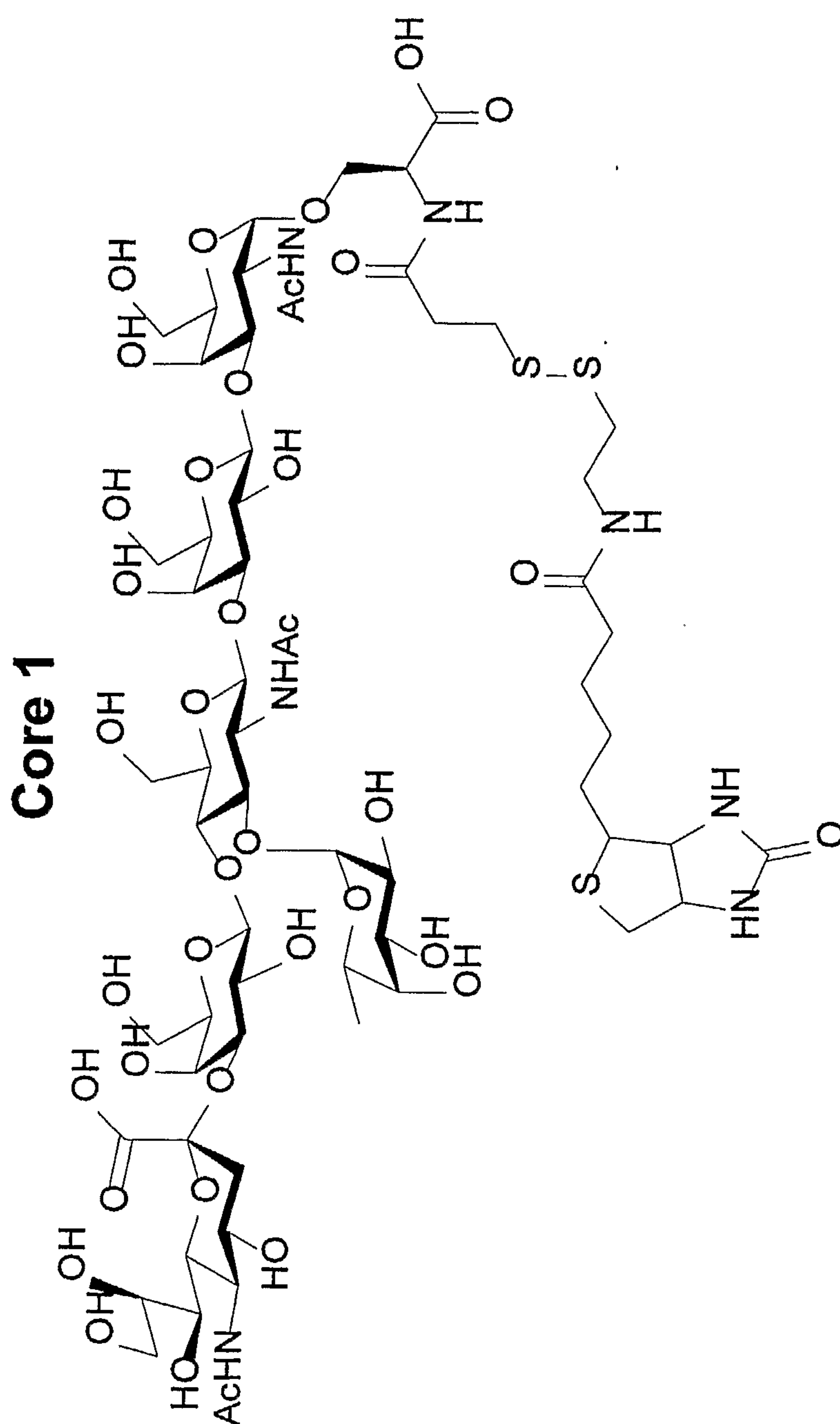
$C_{63}H_{102}N_7NaO_{41}S_4$
Exact Mass: 1763.4892
Mol. Wt.: 1764.7561

FIG. 4A

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Sulfo Core 6**FIG. 4B**

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$C_{63}H_{103}N_7O_{38}S_3$
Exact Mass: 1661.5505
Mol. Wt.: 1662.711

FIG. 4C

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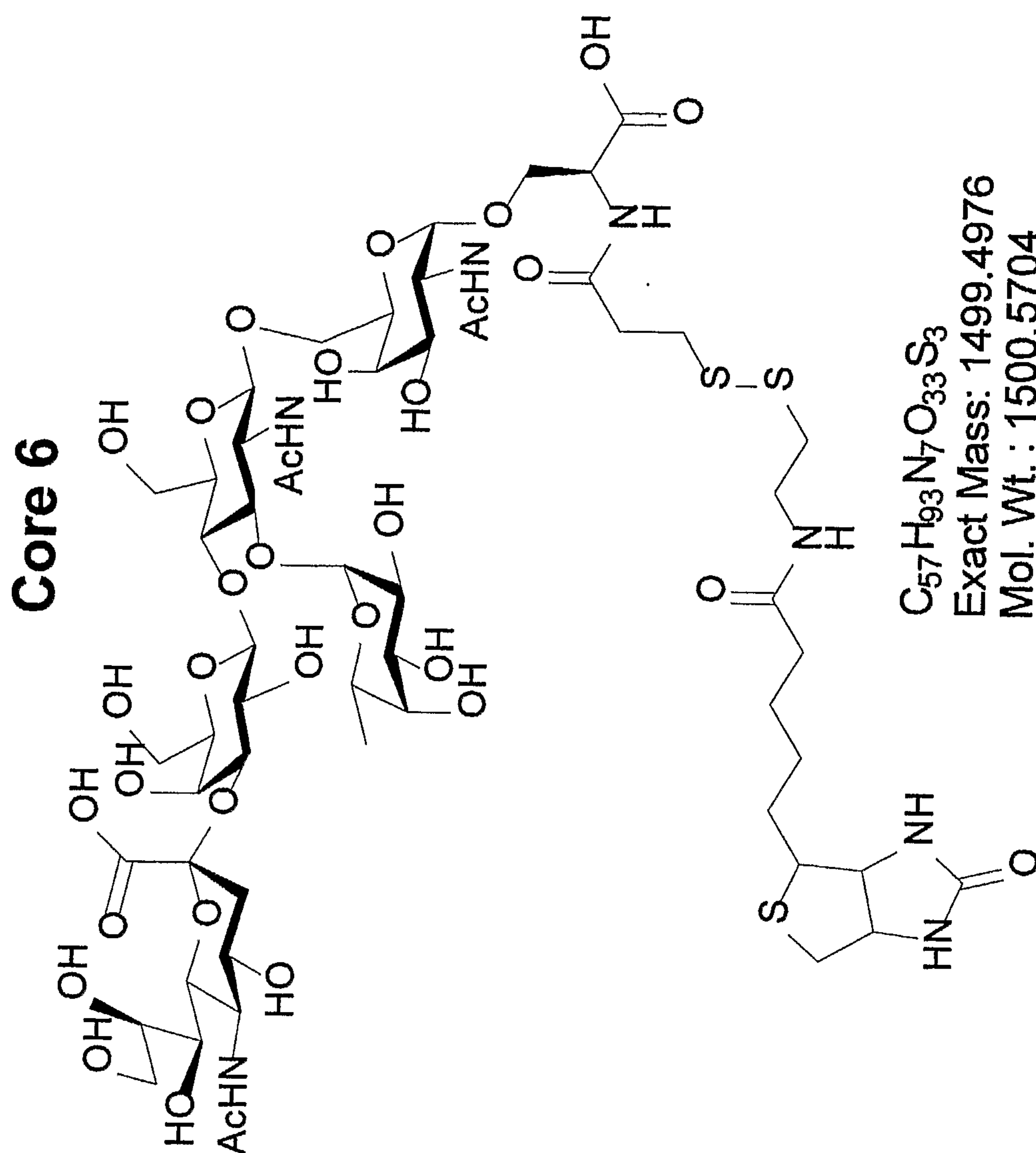


FIG. 4D