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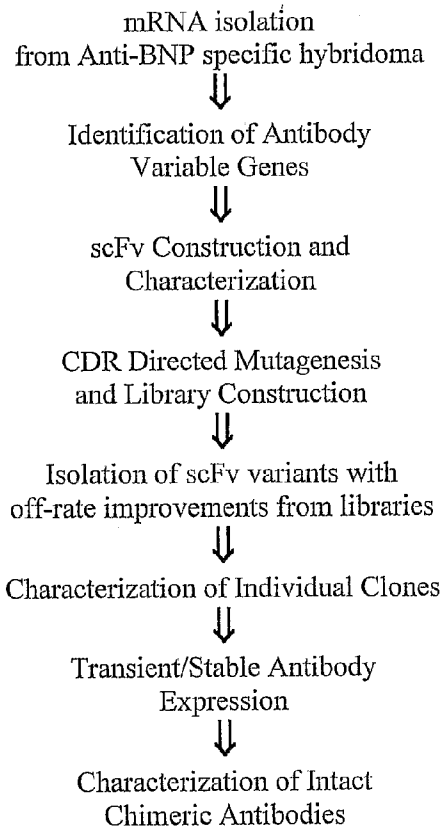

(57) Abstract: The present invention relates to antibodies that immunospecifically bind to human brain natriuretic peptide or a human brain natriuretic peptide fragment with a high binding affinity, methods for producing and selecting said antibodies, immunoassays for human brain natriuretic peptide or a human brain natriuretic peptide fragment that employ said antibodies and therapeutic compositions containing said antibodies.

WO 2007/056507 A1

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HUMAN BNP IMMUNOSPECIFIC ANTIBODIES

Field of the Invention

The present invention relates to antibodies that immunospecifically bind to human brain natriuretic peptide or a human brain natriuretic peptide fragment with a high binding affinity, methods for producing and selecting said antibodies, immunoassays for human brain natriuretic peptide or a human brain natriuretic peptide fragment that employ said antibodies and therapeutic compositions containing said antibodies.

Background of the Invention

Atrial natriuretic peptide (hereinafter referred to as "ANP"), brain natriuretic peptide (hereinafter referred to as "BNP"), C-type natriuretic peptide (hereinafter referred to as "CNP") and Dendroaspis natriuretic peptide (hereinafter referred to as "DNP") are each members of a family of hormones known as "natriuretic peptides". ANP and BNP share a wide spectrum of biological properties and belong to the cardiac natriuretic system. Both ANP and BNP are of myocardial cell origin while CNP is of endothelial cell origin. DNP was isolated from the venom of the green mamba snake and possesses structural similarity to ANP, BNP and CNP.

BNP received its name because it was first isolated from porcine brain, thus "BNP" stood for "brain natriuretic peptide". However, because BNP belongs to the cardiac natriuretic system, "brain" has been changed to "B-type". Therefore, "BNP" now refers to "B-type natriuretic peptide".

ANP is secreted by the heart in the atria. BNP is secreted by the heart through the coronary sinus, predominantly from the cardiac ventricles. BNP is secreted as a 108 amino acid polypeptide precursor (See Valli et al., *J. Lab. Clin. Med.*, 134(5):437-444 (November 1999)). The mature form of BNP is made up of 32 amino acids (representing amino acids 77-108 of the 108 amino acid polypeptide precursor) with a 17 amino acid ring closed by a disulfide bond between two cysteine residues, an amino-terminal tail of 9 amino acids, and a carboxyl-terminal tail of 6 amino acids. ANP and CNP also have a 17 amino acid ring closed by a disulfide bond between two cysteine residues. Eleven of the seventeen amino acids in the ring are conserved

between the three molecules. In addition to the 17 amino acid ring structure, ANP has an amino-terminal tail of 6 amino acids and a carboxy-terminal tail of 5 amino acids. ANP is produced as a 126 amino acid pro-ANP form that is the major storage form of ANP. After proteolytic cleavage between amino acids 98 and 99, the mature 28 amino acid peptide ANP is found in coronary sinus plasma (See Yandle, *J. Internal Med.*, 235:561-576 (1994)).

CNP is found in the brain and cerebral spinal fluid and is the most prevalent of the three peptides in the central nervous system. Little if any CNP is present in the heart. Pro-CNP is a 103 amino acid peptide that is processed into either CNP-53 (amino acids 51 to 103) or CNP-22 (amino acids 82 to 103) that are the active peptides. In addition the 17 amino acid ring structure, CNP-22 has an amino-terminal tail of 5 amino acids and contains no carboxy-terminal tail. CNP-53 is identical to CNP-22 except for a 31 amino acid extension at the amino terminal end.

As mentioned previously, DNP was isolated from the venom of the green mamba snake. The mature form of DNP is made up of 38 amino acids. DNP-like immunoreactivity (DNP-LI) has been reported in human plasma and the plasma concentration of DNP-LI has been found to be elevated in patients with congestive heart failure (See, Cataliotti, et al., *Mayo Clin. Proc.*, 76:111-1119 (2001)). Additionally, it is also known that the infusion of synthetic DNP results in marked natriuresis and diuresis in association with increased plasma and urinary cyclic guanosine monophosphate. *Id.*

One of the problems with natural human natriuretic peptides is that they are unstable in plasma and serum. Specifically, enzymes, such as proteases, cleave these peptides. For example, proteases cleave BNP (natural and synthetic) at various locations along its amino acid chain. For example, protease cleavage is known to occur at the amino terminus of BNP between amino acids 2-3 (Shimizu et al., *Clinica Chimica Acta*, 316:129-135 (2002)) and at its carboxy terminus between amino acids 30-32. Moreover, endopeptidase cleavage of BNP is also known in the art (Davidson and Struthers, *J.* Hypertension, 12:329-336 (1994)).

The measurement of mature BNP (i.e., the 32 amino acid molecule (amino acids 77-108 of the precursor polypeptide of BNP)) in humans (hereinafter referred to has "hBNP"), in the general population has been found to reflect cardiac diseases, such as congestive heart failure, ischemic heart diseases, atrial fibrillation and renal dysfunction. In fact, elevated levels of BNP in human plasma have been reported in heart disease, following acute myocardial infarction and during symptomless or subclinical ventricular dysfunction (See Mukoyama et al., *J. Clin. Invest.*, 87:11402-11412 (1991), Motwani et al., *Lancet*, 341:1109-1113 (1993), Yoshibayashi et al., *New Eng. J. Med.*, 327:434 (1992)). Increased circulating levels of ANP are seen in congestive heart failure, chronic renal failure and in severe hypertension. The presence of CNP in human plasma remains controversial with reports of its absence or presence as CNP-22 (See Yandle, *J. Internal Med.*, 235:561-576 (1994)).

A ligand binding assay is an analytical technique for measuring concentrations of substances commonly referred to as ligands that react selectively with specific binding proteins. Immunoassays that measure the concentrations of antigens that react selectively with specific antibodies are an example of a class of ligand binding assays.

Ligand binding assays, such as immunoassays, for measuring human natriuretic peptides in plasma, particularly hBNP, are well-known in the art and are commercially available. These immunoassays require the use of at least one or two specific antibodies as well as at least one calibrator and, ideally, at least one control. In addition to the calibrators and controls, immunoassays require the use of at least one test sample. Test samples are normally biological samples derived from serum, plasma, whole blood or other bodily fluids (normally from a human patient). The levels of at least one human natriuretic peptide in the test sample is quantified in the immunoassay.

For example, U.S. Patent No. 6,162,902 (hereinafter referred to as the "'902 patent") discloses isolated antibodies that are monospecifically reactive to epitopes 1-10, 5-13 and 15-25 of hBNP. More particularly, the '902 patent describes two

isolated monoclonal antibodies. The first monoclonal antibody is produced by hybridoma cell line 106.3 (ATCC Accession No. HB-12044) and is monospecifically reactive to epitopes 5-13 of hBNP. The second monoclonal antibody is produced by hybridoma cell line 201.3 (ATCC Accession No. HB 12045) and is monospecifically reactive to epitopes 1-10 of hBNP. The '902 patent also describes the use of the above antibodies in immunoassays for the purpose of quantifying the amount of hBNP in a biological sample. U.S. Patent No. 6,677,124 (hereinafter referred to as the "'124 patent") discloses a monoclonal antibody that binds to an epitope having the amino acid sequence of LYS-VAL-LEU-ARG-ARG-HIS that is found in the C-terminal region of hBNP, namely epitopes 27-32. More particularly, the '124 patent describes a monoclonal antibody produced by hybridoma cell line BC203 (FERM BP-3515). The '124 patent also describes immunoassays for hBNP using this monoclonal antibody.

It is generally known in the art that the specificity and sensitivity of the antibodies used in immunoassays, such as hBNP immunoassays, are very important. One way in which to increase both the specificity and sensitivity of one or more antibodies is to improve the binding affinity of an antibody for its intended target (i.e., an antigen). Antibodies having an improved binding affinity for their intended targets should exhibit increased specificity and sensitivity. Therefore, there is a need in the art for new antibodies that specifically bind to human BNP with a high binding affinity and thus exhibit high specificity and sensitivity when used in said hBNP immunoassays.

Brief Summary of the Invention

In one aspect, the present invention relates to an isolated antibody which immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of human brain natriuretic peptide ("hBNP") with at least about a two fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044. More specifically, the antibody of the present invention exhibits at least about a three fold improvement, at least about a five fold improvement, at least about a ten fold improvement, at least about a fifteen fold improvement, at least about a twenty fold improvement or at least about a twenty-five fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3. The isolated antibody of the present invention can be a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

In another aspect, the present invention relates to an isolated antibody which immunospecifically binds to hBNP, wherein said antibody has an association rate (k_a) of between about 5.0×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$. More specifically, the antibody of the present invention has an association rate of between about 3.3×10^4 and about 1.0×10^9 $M^{-1}s^{-1}$, between about 2.5×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$ or between about 2.4×10^4 and about 1.35×10^7 $M^{-1}s^{-1}$. The isolated antibody of the present invention can be a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof. Additionally, this isolated antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

In another aspect, the present invention relates to an isolated antibody which immunospecifically binds to hBNP, wherein said antibody has a dissociation rate (k_d) of between about 1.0×10^{-3} and about $1.0 \times 10^{-6} \cdot s^{-1}$. More specifically, the antibody of the present invention has a dissociation rate of between about 1.0×10^{-3} and about $1.0 \times 10^{-5} \cdot s^{-1}$ or between about 1.0×10^{-3} and about $1.0 \times 10^{-4} \cdot s^{-1}$. The isolated antibody of the present invention can be a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof. Additionally, this isolated antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

In another aspect, the present invention relates to an isolated antibody which immunospecifically binds to hBNP wherein said antibody has an equilibrium dissociation constant (K_D) of between about 2×10^{-11} M and about 1×10^{-15} M. More specifically, the antibody of the present invention has an equilibrium dissociation constant of between about 3.0×10^{-11} M and about 1.0×10^{-14} M, between about 4.0×10^{-11} M and about 8.0×10^{-13} M or between about 4.2×10^{-11} M and about 7.4×10^{-13} M. The isolated antibody of the present invention can be a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof. Additionally, this isolated antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

In still another aspect, the present invention relates to a Chinese hamster ovary ("CHO") cell line AM1 having A.T.C.C. Accession No. PTA-6987.

In still yet another aspect, the present invention relates to an antibody made from DNA extracted from CHO cell line AM1 having A.T.C.C. Accession No. PTA-6987.

In yet another aspect, the present invention relates to a chimeric antibody or a hBNP-epitope binding fragment thereof produced by CHO cell line AM1, wherein said cell line has A.T.C.C. Accession No. PTA-6987.

In still a further aspect, the present invention relates to an isolated antibody which immunospecifically binds to hBNP, wherein said antibody has a variable heavy domain and a variable light domain, the variable heavy domain comprising a heavy chain complementary determining region ("CDR") 1, a heavy chain CDR 2 and a heavy chain CDR 3, the variable light domain comprising a light chain CDR 1, a light chain CDR 2 and a light chain CDR 3, wherein

(a) the Heavy Chain CDR 1 has an amino acid sequence of: Gly-Tyr-Thr-Phe-Thr-His-Tyr-Gly-Ile-Asn (SEQ ID NO:6);

(b) the Heavy Chain CDR 2 has an amino acid sequence having a formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

wherein Xaa_1 is selected from the group consisting of proline and alanine;

wherein Xaa_2 is selected from the group consisting of isoleucine and tyrosine;

(c) the Heavy Chain CDR 3 has an amino acid sequence of: Ser-His-Arg-Phe-Gly-Leu-Asp-Tyr (SEQ ID NO:8);

(d) the Light Chain CDR 1 has an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

wherein Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine;

wherein Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine;

wherein Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid;

(e) the Light Chain CDR 2 has an amino acid sequence having the formula of: Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

wherein Xaa_6 is selected from the group consisting of: asparagine and cysteine;

wherein Xaa_7 is selected from the group consisting of: leucine, glycine and alanine;

wherein Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline; and

(f) the Light Chain CDR 3 has an amino acid sequence of: Gln-Gln-Ser-Asn-Glu-Asp-Pro-Phe-Thr (SEQ ID NO:11),

wherein the heavy chain CDR 2 has an amino acid sequence other than Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) when the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) and the light chain CDR 2 has the amino acid sequence of Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID

NO:10), the light chain CDR 1 has an amino acid sequence other than Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) when the heavy chain CDR 2 has the amino acid sequence Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 2 has the amino acid sequence Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:19), or the light chain CDR 2 has an amino acid sequence other than Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10) when the heavy chain CDR 2 has the amino acid sequence of Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9).

More specifically, in the above-described isolated antibody:

Xaa_1 can be alanine;

Xaa_2 can be tyrosine;

Xaa_3 can be serine;

Xaa_4 can be glutamine;

Xaa_5 can be serine;

Xaa_6 can be asparagine;

Xaa_7 can be leucine; and

Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:

Xaa_1 can be proline;

Xaa_2 can be isoleucine;

Xaa_3 can be glutamine;

Xaa_4 can be phenylalanine;

Xaa_5 can be alanine;

Xaa_6 can be asparagine;

Xaa_7 can be leucine; and

Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:

Xaa_1 can be proline;

Xaa_2 can isoleucine;

Xaa_3 can be tyrosine;

Xaa_4 can be alanine;

Xaa_5 can be serine;

Xaa_6 can be asparagine;

Xaa_7 can be leucine; and

Xaa_8 can be glutamic acid; or

In the above-described isolated antibody

Xaa_1 can be proline;

Xaa_2 can be isoleucine;

Xaa_3 can be glutamine;

Xaa_4 can be tryptophan;

Xaa_5 can be glycine;

Xaa_6 can be asparagine;

Xaa_7 can be leucine; and

Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:

Xaa_1 can be proline;

Xaa_2 can be isoleucine;

Xaa_3 can be threonine;

Xaa_4 can be tryptophan;

Xaa_5 can be aspartic acid;

Xaa_6 can be asparagine;

Xaa_7 can be leucine; and

Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:

Xaa_1 can be proline;

Xaa_2 can be isoleucine;

Xaa_3 can be arginine;
Xaa_4 can be tryptophan;
Xaa_5 can be proline;
Xaa_6 can be asparagine;
Xaa_7 can be leucine; and
Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:
Xaa_1 can be proline;
Xaa_2 can be isoleucine;
Xaa_3 can be alanine;
Xaa_4 can be tyrosine;
Xaa_5 can be glycine;
Xaa_6 can be asparagine;
Xaa_7 can be leucine; and
Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:
Xaa_1 can be proline;
Xaa_2 can be isoleucine;
Xaa_3 can be asparagine;
Xaa_4 can be tryptophan;
Xaa_5 can be proline;
Xaa_6 can be asparagine;
Xaa_7 can be leucine; and
Xaa_8 can be glutamic acid; or

In the above-described isolated antibody:
Xaa_1 can be proline;
Xaa_2 can be isoleucine;
Xaa_3 can be serine;
Xaa_4 can be glutamine;

Xaa_5 can be serine;

Xaa_6 can be cysteine;

Xaa_7 can be glycine; and

Xaa_8 can be tryptophan; or

In the above-described isolated antibody:

Xaa_1 can be proline;

Xaa_2 can be isoleucine;

Xaa_3 can be serine;

Xaa_4 can be glutamine;

Xaa_5 can be serine;

Xaa_6 can be cysteine;

Xaa_7 can be alanine; and

Xaa_8 can be proline.

The above-described antibody can have an equilibrium dissociation constant (K_D) of between about 2.0×10^{-11} M and about 1.0×10^{-15} M, between about 3.0×10^{-11} M and about 1.0×10^{-14} M, between about 4.0×10^{-11} M and about 8.0×10^{-13} M or between about 4.2×10^{-11} M and about 7.4×10^{-13} M. Additionally, the above-described antibody can have an association rate (k_a) of between about 5.0×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$. Furthermore, the above-described antibody can have a dissociation rate (k_d) of between about 1.0×10^{-3} and 1.0×10^{-6} s^{-1}. Furthermore, the above-described antibody of the present invention can be a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof. Finally, the above-described antibody can immunospecifically bind to an epitope comprising amino acid residues 5 through 13 of hBNP.

In another aspect, the present invention relates to an immunoassay for hBNP or hBNP fragment, wherein said immunoassay comprises any one of the hereinbefore described antibodies of the present invention. More specifically, said immunoassay may comprise only a single antibody that immunospecifically binds to hBNP or hBNP fragment. Moreover, said immunoassay may further comprise an additional specific binding partner for hBNP or hBNP fragment.

In another aspect, the present invention relates to a pharmaceutical composition comprising a therapeutically effective amount of any of the hereinbefore described antibodies of the present invention and a pharmaceutically acceptable carrier or excipient.

Brief Description of the Figures

Figure 1 is a flow chart showing the steps used to identify and create antibodies that immunospecifically bind to human BNP with a high binding affinity.

Figure 2 is a plasmid map for vector pYD41-40 containing the 106.3 single-chain variable fragment shown in Figure 4.

Figures 3A-3E are the nucleotide sequence of the vector shown in Figure 2.

Figure 4 is a diagram of the 106.3 single-chain variable fragment ("scFv").

Figure 5 shows the amino acid sequence of the 106.3 single-chain variable fragment ("scFv"). The solid underlined sequence represents the variable heavy chain sequence ("VH"), the double underlined sequence the linker, and the stippled underline sequence the variable light chain sequence ("VL"). Italicized and bold type indicates the complementary determining regions (CDR).

Figures 6A-6B show the nucleotide sequence of the 106.3 scFv.

Figures 7A-7B show that yeast expressing full-length 106.3 single-chain variable fragment (scFv) bind to cyclic BNP (SEQ ID NO:5) More specifically, this figure shows that 106.3 scFv expressing yeast were incubated with cyclic BNP (1-32c) (SEQ ID NO:5) or anti-V5 followed by secondary reagents streptavidin phycoerythrin (SA:PE) (Fig. 7A) and goat anti mouse-phycoerythrin (GAM:PE) (Fig. 7B). The flow cytometry histograms illustrate the full-length expression of 106.3 scFv as detected by anti-V5 and the ability of 106.3 scFv to bind to cyclic BNP peptide (1-32) (SEQ ID NO:5). PEA units (abscissa): 10^2, 10^3,10^4, and 10^5. Count units (ordinate): 0, 50, 100, 150 (Fig. 7A); 0, 25, 50, 75, 100, 125 (Fig. 7B).

Figure 8 shows the 106.3 scFv off-rate measurement. More specifically, yeast expressing 106.3 scFv were incubated with a saturating concentration of biotinylated cyclic BNP (1-32c) (SEQ ID NO:5). Cells were then washed and incubated with a saturating concentration of unlabelled BNP 1-32c (SEQ ID NO:5). At each time point, cells were transferred to ice, washed and incubated with SA:PE. After 30 minutes, cells were washed again and analyzed on the flow cytometer. A first order decay equation was used to fit the individual time points where m1 was the theoretical maximum mean fluorescence units ("MFU") at time 0, m2 was the off-rate ("koff"), m3 was the background MFU due to autofluorescence and M0, which is the time x (the x being the time that is being measured) was the time x that measurements are taken. The half-life ($t_{1/2}$) of 106.3 scFv binding to cyclic BNP (1-32c) was calculated using: $t_{1/2} = \ln 2/k_{off}$. One and a half times the half-life was the time used to sort the 106.3 CDR mutagenic libraries.

Figure 9 is a schematic depiction which shows how degenerate oligonucleotides were designed so that three amino acid positions of the complementarity determing region (9 nucleotides) were randomly mutated per library.

Figure 10 is a schematic depiction which shows how the 106.3 scFv library was constructed using yeast homologous recombination. More specifically, gapped vectors were PCR generated to exclude those nucleotides that were being mutagenized in the library. The degenerate single stranded oligonucleotides were

synthesized. Gapped vectors and single stranded degenerate oligonucleotides were transformed into S. *cerevisiae* strain EBY100. Transformed clones were selected in tryptophan deficient glucose media.

Figure 11 is a summary showing that 106.3scFv variants isolated from CDR mutagenic libraries exhibited improvements in off-rate (namely, said variants had a slower k_{off}).

Figures 12A-C show the sequence characterization of scFv 106.3 variants. More specifically, plasmid DNA was isolated from 106.3 variants and scFv genes were sequenced.

Figure 13 shows affinity measurements of selected 106.3 engineered, human-mouse chimeric antibodies and mouse 106.3 mAb using surface plasmon resonance using BIAcore.

Figures 14A-H show the fifty-four (54) oligonucleotides that were used to create the gapped pYD41 vector discussed in Example 1.

Figure 15 shows the results of testing to determine antibody AM1's ability to bind to human cyclic BNP 1-32 in a single antibody assay format as described in Example 3 (X = signal generated with given concentration of unlabelled human cyclic BNP 1-32; A = signal generated with no unlabelled human cyclic BNP 1-32; X/A = ratio of these two signals).

Figure 16 shows an anti-hBNP antibody pair evaluation using streptavidin microparticles using antibody AM1 and Fusion 3 as described in Example 4. In essence, the following were employed: M280 Streptavidin particles at 0.05% solids, 65 ng/mL conjugates, 100 µL sample volume, and a 2-step (18/4) sandwich format. Symbols & Abbreviations: diamonds, anti-BNP(106.3AM1)SA µP/anti-BNP(Fusion 3)CPSP; squares, anti-BNP(Fusion 3)SA µP/anti-BNP(106.3AM1)CPSP); RLU, Relative Light Units.

Figure 17 shows anti-hBNP antibody pair evaluation using paramagnetic microparticles (from Polymer Labs) using antibody AM1 and Fusion 3 as described in Example 4. Symbols & Abbreviations: diamonds, anti-BNP(106.3AM1)SA μP/anti-BNP(Fusion 3)CPSP; squares, anti-BNP(Fusion 3)SA μP/anti-BNP(106.3AM1)CPSP); RLU, Relative Light Units.

Figure 18 shows the displacement of antibody AM1 (used at about 0.01 μg/mL) with various hBNP peptides (used at about 181 nM).

Figure 19 shows the alanine peptide mapping of antibody AM1 using EIA.

Figure 20 shows the alanine peptide mapping of antibody AMI using BIAcore. The fold increase in k_{off} of BNP complexes comprising various BNP peptides are displayed.

Detailed Description of the Invention

I. Introduction

The present invention relates to novel antibodies that immunospecifically bind to human brain natriuretic peptide with a high binding affinity. The antibodies of the present invention are highly sensitive reagents and are useful in the qualitative and/or quantitative detection of hBNP or hBNP fragments in test samples. In another embodiment, the present invention relates to immunoassays that employ the antibodies of the present invention. In yet still a further embodiment, the present invention relates to therapeutic compositions comprising the antibodies of the present invention.

Definitions

As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies (in one aspect, a bird (for example, a duck or goose), in another aspect, a shark or whale, in yet another aspect, a mammal,

including a non-primate (for example, a cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, cat, dog, rat, mouse, etc) and a non-human primate (for example, a monkey, such as a cynomologous monkey, a chimpanzee, etc), recombinant antibodies, chimeric antibodies, single-chain Fvs (scFv), single chain antibodies, single domain antibodies, Fab fragments, $F(ab')_2$ fragments, disulfide-linked Fv (sdFv), and anti-idiotypic (anti-Id) antibodies (including, for example, anti-Id antibodies to antibodies of the present invention), and functionally active epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, namely, molecules that contain an antigen binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA and IgY), class (for example, IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) or subclass.

As used herein, the term "association rate", "k_{on}" or "k_a" as used interchangeably herein, refers to the value indicating the binding strength (degree) of an antibody to its target antigen or the rate of complex formation between mAb and antigen as shown by the below:

$$\text{Antibody (Ab)} + \text{Antigen (Ag)} \rightarrow \text{Ab-Ag}$$

Methods for determining association constants (K_A) are well known in the art. For example, a Biacore® (Sweden) assay can be used. Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyne Instruments (Boise, Idaho) can also be used.

As used herein, the term "dissociation rate", "k_{off}" or "k_d" as used interchangeably herein, refers to the value indicating the dissociation strength (degree) of an antibody from its target antigen or separation of Ab-Ag complex over time into free mAb and antigen as shown by the below:

$$\text{Antibody (Ab)} + \text{Antigen (Ag)} \leftarrow \text{Ab-Ag}$$

Methods for determining dissociation constants (K_D) are well known in the art. For example, a Biacore® (Sweden) assay can be used. Additionally, a KinExA® (Kinetic Exclusion Assay) assay, available from Sapidyne Instruments (Boise, Idaho) can also be used.

As used herein, the term "epitope" or "epitopes" refers to sites or fragments of a polypeptide or protein having antigenic or immunogenic activity in a subject. An epitope having immunogenic activity is a site or fragment of a polypeptide or protein that elicits an antibody response in an animal. An epitope having antigenic activity is a site or fragment of a polypeptide or protein to which an antibody immunospecifically binds as determined by any method well-known to those skilled in the art, for example by immunoassays.

As used herein, the term "equilibrium dissociation constant" or "K_D" as used interchangeably, herein, refers to the value obtained by dividing the dissociation rate (k_{off}) by the association rate (k_{on}). The association rate, the dissociation rate and the equilibrium dissociation constant are used to represent the binding affinity of an antibody to an antigen.

As used herein, the term "human brain natriuretic peptide", "human BNP", "hBNP", "hBNP peptide", "B-type natriuretic peptide" or "hBNP polypeptide" refers to a 32 amino acid molecule representing amino acids 77-108 of the 108 amino acid precursor molecule of human brain natriuretic peptide.

As used herein, the term "hBNP fragment" or "hBNP peptide fragment" as used herein refers to a polypeptide that comprises at least about five contiguous amino acids of amino acids 77-108 of the 108 amino acid BNP precursor molecule. In one aspect, a hBNP fragment or hBNP peptide fragment refers to a polypeptide that comprises at least about ten contiguous amino acids residues of amino acids 77-108 of the 108 amino acid BNP precursor molecule; at least about fifteen contiguous amino acids residues of amino acids 77-108 of the 108 amino acid BNP precursor molecule; at least about 20 contiguous amino acids residues of amino acids 77-108 of the 108

amino acid BNP precursor molecule; at least about 25 contiguous amino acids residues of amino acids 77-108 of the 108 amino acid BNP precursor molecule, or at least about 30 contiguous amino acid residues of amino acids 77-108 of the 108 amino acid BNP precursor molecule. Examples of hBNP fragments or hBNP peptide fragments include, but are not limited to, amino acid sequences containing amino acids residues 1-31, 1-30, 1-29, 1-28, 1-27, 1-26, 1-25, 1-24, 1-23, 1-22, 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 2-32, 2-31, 2-30, 2-29, 2-28, 2-27, 2-26, 2-25, 2-24, 2-23, 2-22, 2-21, 2-20, 2-19, 2-18, 2-17, 2-16, 2-15, 2-14, 2-13, 2-12, 2-11, 2-10, 2-9, 2-8, 2-7, 3-32, 3-31, 3-30, 3-29, 3-28, 3-27, 3-26, 3-25, 3-24, 3-23, 3-32, 3-21, 3-20, 3-19, 3-18, 3-17, 3-16, 3-15, 3-14, 3-13, 3-12, 3-11, 3-10, 3-9, 3-8, 4-32, 4-31, 4-30, 4-29, 4-28, 4-27, 4-26, 4-25, 4-24, 4-23, 4-22, 4-21, 4-20, 4-19, 4-18, 4-17, 4-16, 4-15, 4-14, 4-13, 4-12, 4-11, 4-10, 4-9, 5-32, 5-31, 5-30, 5-29, 5-28, 5-27, 5-26, 5-25, 5-24, 5-23, 5-22, 5-21, 5-20, 5-19, 5-18, 5-17, 5-16, 5-15, 5-14, 5-13, 5-12, 5-11, 5-10, 6-32, 6-31, 6-30, 6-29, 6-28, 6-27, 6-26, 6-25, 6-24, 6-23, 6-22, 6-21, 6-20, 6-19, 6-18, 6-17, 6-16, 6-15, 6-14, 6-13, 6-12, 6-11, 7-32, 7-31, 7-30, 7-29, 7-28, 7-27, 7-26, 7-25, 7-24, 7-23, 7-22, 7-21, 7-20, 7-19, 7-18, 7-17, 7-16, 7-15, 7-14, 7-13, 7-12, 8-32, 8-31, 8-30, 8-29, 8-28, 8-27, 8-26, 8-25, 8-24, 8-23, 8-22, 8-21, 8-20, 8-19, 8-18, 8-17, 8-16, 8-15, 8-14, 8-13, 9-32, 9-31, 9-30, 9-29, 9-28, 9-27, 9-26, 9-25, 9-24, 9-23, 9-22, 9-21, 9-20, 9-19, 9-18, 9-17, 9-16, 9-15, 9-14, 10-32, 10-31, 10-30, 10-29, 10-28, 10-27, 10-26, 10-25, 10-24, 10-23, 10-22, 10-21, 10-20, 10-19, 10-18, 10-17, 10-16, 10-15, 11-32, 11-31, 11-30, 11-29, 11-28, 11-27, 11-26, 11-25, 11-24, 11-23, 11-22, 11-21, 11-20, 11-19, 11-18, 11-17 or 11-16 of hBNP.

As used herein, the term "humanized" antibody refers to an immunoglobulin variant or fragment thereof, which is capable of binding to a predetermined antigen and which comprises framework regions having substantially the amino acid sequence of a human immunoglobulin and CDRs having substantially the amino acid sequence of a non-human immunoglobulin. Ordinarily, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. In general, the humanized antibody will include substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or

substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Generally, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG_1, IgG_2, IgG_3 and IgG_4. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within those skilled in the art.

As used herein, the phrase "immunospecifically binds to a human brain natriuretic peptide", "immunospecifically binds to hBNP", "immunospecifically binds to human brain natriuretic peptide fragment" or "immunospecifically binds to hBNP fragment" and analogous terms thereof refer to peptides, polypeptides, proteins, fusion proteins and antibodies that specifically bind to hBNP or hBNP fragment and do not specifically bind to other peptides. A peptide, polypeptide, protein, or antibody that immunospecifically binds to hBNP or hBNP fragment may bind to other peptides, polypeptides, or proteins with lower binding affinity as determined by, for example, immunoassays, BIAcore, or other assays known in the art. Antibodies or antibody fragments that immunospecifically bind to hBNP or hBNP fragment can be identified, for example, by immunoassays, BIAcore, or other techniques known to those of skill in the art. An antibody binds immunospecifically to a hBNP peptide or hBNP fragment when it binds to hBNP or hBNP fragment with a higher binding affinity than to any cross-reactive antigen as determined using experimental techniques, such as, but not limited to, radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISAs) (See, for example, Paul, ed., *Fundamental Immunology*, 2nd ed., Raven Press, New York, pages 332-336 (1989) for a discussion regarding antibody specificity.). In one aspect of the present invention, an antibody binds immunospecifically to hBNP or hBNP fragment when it has an equilibrium dissociation constant (K_D) for the hBNP or hBNP fragment of at least 2.0×10^{-11} M as determined by a BIAcore assay under standard assay conditions, and in particular the BIAcore assay described in Example 1.

As used herein, the term "isolated" in the context of nucleic acid molecules refers to a nucleic acid molecule which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In one aspect, nucleic acid molecules are isolated. In another aspect, a nucleic acid molecule encoding an antibody of the invention is isolated.

As used herein, the term "stringent conditions" refers to hybridization to filter-bound DNA in 6 x sodium chloride/sodium citrate (SSC) at about 45°C followed by one or more washes in 0.2 x SSC/0.1% SDS at about 50-65°C. The term "under highly stringent conditions", refers to hybridization to filter-bound nucleic acid in 6 x SSC at about 45°C followed by one or more washes in 0.1 x SSC/0.2% SDS at about 68°C, or under other stringent hybridization conditions which are known to those skilled in the art (see, for example, Ausubel, F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

As used herein, the terms "subject" and "patient" are used interchangeably. As used herein, the terms "subject" and "subjects" refer to an animal, in one aspect, a bird (for example, a duck or goose), in another aspect, a shark or whale, or in a further aspect, a mammal including, a non-primate (for example, a cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, cat, dog, rat, and mouse) and a primate (for example, a monkey, such as a cynomolgous monkey, chimpanzee, and a human).

As used herein, the term "test sample" refers to a biological sample derived from serum, plasma, whole blood, lymph, CNS fluid, urine or other bodily fluids of a subject. The test sample can be prepared using routine techniques known to those skilled in the art.

As used herein, the term "therapeutically effective amount" or "pharmaceutically effective amount" means an amount of antibody or antibody portion effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. The exact dose will be ascertainable by one skilled in the art. As known in the art, adjustments based on age, body weight, sex, race, diet, time of administration, drug interaction and severity of condition may be necessary and will be ascertainable with routine experimentation by those skilled in the art. A therapeutically effective amount is also one in which the therapeutically beneficial effects outweigh any toxic or detrimental effects of the antibody or antibody fragment. 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 prophylactically effective amount will be less than the therapeutically effective amount.

II. Antibodies of the Present Invention

The present invention provides antibodies that immunospecifically bind to hBNP or hBNP fragment. In particular, the present invention provides for antibodies that have a high binding affinity for hBNP or hBNP fragment. Specifically, in one aspect, the present invention relates to an antibody that immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP or hBNP fragment with at least about a two fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044 (which is also referred to herein as the "wildtype"). More specifically, the antibodies of the present invention immunospecifically bind to an epitope comprising amino acid residues 5 through 13 of hBNP or hBNP fragment thereof with at least about a three fold improvement, at least about a five fold improvement, at least about a ten fold improvement, at least about a fifteen fold improvement, at least about a twenty fold improvement, at least about a twenty-five fold improvement, at least about a thirty fold improvement, at least about a thirty-five fold improvement, at least about a forty fold improvement, at

least about a forty-five fold improvement, at least about a fifty fold improvement, at least about a fifty-five fold improvement, at least about a sixty fold improvement, at least about a seventy fold improvement or at least about a seventy-five fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3 (the wildtype).

In another aspect, the present invention relates to an antibody that immunospecifically binds to hBNP or hBNP fragment and has a k_{on} (or k_a) of at least about 2.4 x 10^4 $M^{-1}s^{-1}$, of at least about 2.5 x 10^4 $M^{-1}s^{-1}$, of at least about 3.3 x 10^4 $M^{-1}s^{-1}$, of at least about 5.0 x 10^4 $M^{-1}s^{-1}$, of at least about 1.25 x 10^7 $M^{-1}s^{-1}$ of at least about 1.35 x 10^7 $M^{-1}s^{-1}$, of at least about 1.0 x 10^8 $M^{-1}s^{-1}$, of at least about 1.0 x 10^9 $M^{-1}s^{-1}$, or has a k_{on} (or k_a) ranging from about 5.0 x 10^4 $M^{-1}s^{-1}$to about 1.0 x 10^8 $M^{-1}s^{-1}$, from about 3.3 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^9 $M^{-1}s^{-1}$, from about 2.5 x 10^4 $M^{-1}s^{-1}$ to about 1.25 x 10^7 $M^{-1}s^{-1}$, from about 2.4 x 10^4 $M^{-1}s^{-1}$ to about 1.35 x 10^7 $M^{-1}s^{-1}$.

In another aspect, an antibody of the present invention immunospecifically binds to the amino acid residues 5 through 13 of human BNP or hBNP fragment at a k_{on} (or k_a) of at least about 2.4 x 10^4 $M^{-1}s^{-1}$, of at least about 2.5 x 10^4 $M^{-1}s^{-1}$, of at least about 3.3 x 10^4 $M^{-1}s^{-1}$, of at least about 5.0 x 10^4 $M^{-1}s^{-1}$, of at least about 1.25 x 10^7 $M^{-1}s^{-1}$ of at least about 1.35 x 10^7 $M^{-1}s^{-1}$, of at least about 1.0 x 10^8 $M^{-1}s^{-1}$, of at least about 1.0 x 10^9 $M^{-1}s^{-1}$, or has a k_{on} (or k_a) ranging from about 5.0 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^8 $M^{-1}s^{-1}$, from about 3.3 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^9 $M^{-1}s^{-1}$, from about 2.5 x 10^4 $M^{-1}s^{-1}$ to about 1.25 x 10^7 $M^{-1}s^{-1}$, from about 2.4 x 10^{-11} $M^{-1}s^{-1}$ to about 1.35 x 10^7 $M^{-1}s^{-1}$.

In another aspect, the present invention provides antibodies produced by Chinese hamster ovary cell line AM1 (also known as 106.3 L1 B24/H2288). Antibodies produced by this cell line bind to amino acid residues 5 thorough 13 of hBNP or hBNP fragment at a k_{on} (or k_a) of at least about 2.4 x 10^4 $M^{-1}s^{-1}$, of at least about 2.5 x 10^4 $M^{-1}s^{-1}$, of at least about 3.3 x 10^4 $M^{-1}s^{-1}$, of at least about 5.0 x 10^4 $M^{-1}s^{-1}$, of at least about 1.25 x 10^7 $M^{-1}s^{-1}$ of at least about 1.35 x 10^7 $M^{-1}s^{-1}$, of at

least about 1.0 x 10^8 $M^{-1}s^{-1}$, of at least about 1.0 x 10^9 $M^{-1}s^{-1}$, or has a k_{on} (or k_a) ranging from about 5.0 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^8 $M^{-1}s^{-1}$, from about 3.3 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^9 $M^{-1}s^{-1}$, from about 2.5 x 10^4 $M^{-1}s^{-1}$ to about 1.25 x 10^7 $M^{-1}s^{-1}$, from about 2.4 x 10^{-11} $M^{-1}s^{-1}$ to about 1.35 x 10^7 $M^{-1}s^{-1}$.

The present invention provides antibodies that immunospecifically bind to hBNP or hBNP fragment. In particular, the present invention provides for antibodies that have a high binding affinity for hBNP or hBNP fragment. More specifically, in one aspect, an antibody that immunospecifically binds to hBNP or hBNP fragment and has a k_{off} (or k_d) of at least 1.0 x 10^{-3} s^{-1}, of at least 1.0 x 10^{-4} s^{-1}, of at least 1.0 x 10^{-5} s^{-1}, of at least 1.0 x 10^{-6} s^{-1} or has a k_{off} (or k_d) ranging from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-6} s^{-1}, from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-5} s^{-1} or from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-4} s^{-1}.

In another aspect, an antibody of the present invention immunospecifically binds to the amino acid residues 5 through 13 of human BNP or hBNP fragment at a k_{off} (or k_{off}) of at least 1.0 x 10^{-3} s^{-1}, of at least 1.0 x 10^{-4} s^{-1}, of at least 1.0 x 10^{-5} s^{-1}, of at least 1.0 x 10^{-6} s^{-1} or has a k_{off} (or k_d) ranging from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-6} s^{-1}, from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-5} s^{-1} or from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-4} s^{-1}.

In another aspect, the present invention provides antibodies produced by Chinese hamster ovary cell line AM1. Antibodies produced by this cell line bind to amino acid residues 5 thorough 13 of hBNP or hBNP fragment at a k_{off} (or k_d) of at least 1.0 x 10^{-3} s^{-1}, of at least 1.0 x 10^{-4} s^{-1}, of at least 1.0 x 10^{-5} s^{-1}, of at least 1.0 x 10^{-6} s^{-1} or has a k_{off} (or k_d) ranging from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-6} s^{-1}, from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-5} s^{-1} or from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-4} s^{-1}

The present invention provides antibodies that immunospecifically bind to hBNP or hBNP fragment. In particular, the present invention provides for antibodies that have a high binding affinity for hBNP or hBNP fragment. More specifically, in one aspect, the present invention relates to an antibody that immunospecifically binds to hBNP or hBNP fragment and has a K_D of at least 2.0 x 10^{-11} M, of at least 3.0 x 10^{-11} M, of at least 4.0 x 10^{-11} M, of at least 4.2 x 10^{-11} M, of at least 1.0 x 10^{-12} M of at

least 1.0 x 10^{-13} M, of at least 7.4 x 10^{-13} M, of at least 8.0 x 10^{-13} M, of at least 1.0 x 10^{-14} M, of at least 1.0 x 10^{-15} M, or has a K_D ranging from 2.0 x 10^{-11} M to 1.0 x 10^{-15} M, from 3.0 x 10^{-11} M to 1 x 10^{-14} M, from 4.0 x 10^{-11} M to 8.0 x 10^{-13} M or from 4.2 x 10^{-11} M to 7.4 x 10^{-13} M.

In another aspect, an antibody of the present invention immunospecifically binds to the amino acid residues 5 through 13 of human BNP at a K_D of at least 2.0 x 10^{-11} M, of at least 3.0 x 10^{-11} M, of at least 4.0 x 10^{-11} M, of at least 4.2 x 10^{-11} M, of at least 1.0 x 10^{-12} M of at least 1.0 x 10^{-13} M, of at least 7.4 x 10^{-13} M, of at least 8.0 x 10^{-13} M, of at least 1.0 x 10^{-14} M, of at least 1.0 x 10^{-15} M, or has a K_D ranging from 2.0 x 10^{-11} M to 1.0 x 10^{-15} M, from 3.0 x 10^{-11} M to 1 x 10^{-14} M, from 4.0 x 10^{-11} M to 8.0 x 10^{-13} M or from 4.2 x 10^{-11} M to 7.4 x 10^{-13} M.

In another aspect, the present invention provides antibodies produced by Chinese hamster ovary (CHO) cell line AM1. Antibodies produced by this cell line bind to amino acid residues 5 thorough 13 of hBNP or hBNP fragment at a K_D of from 4.2 x 10^{-11} M to 7.4 x 10^{-13} M.

In another aspect, the antibodies of the present invention are derivatives or variants of the antibodies produced by hybridoma cell line 106.3 (ATCC Accession No. HB-12044). More specifically, the inventors of the present invention have discovered that antibodies that are derivatives or variants of the antibodies produced by hybridoma cell line 106.3 can be produced which exhibit a high binding affinity to hBNP or hBNP fragment. More specifically, the antibodies of the present invention exhibit a k_{on} (or k_a) of at least about 2.4 x 10^4 $M^{-1}s^{-1}$, of at least about 2.5 x 10^4 $M^{-1}s^{-1}$, of at least about 3.3 x 10^4 $M^{-1}s^{-1}$, of at least about 5.0 x 10^4 $M^{-1}s^{-1}$, of at least about 1.25 x 10^7 $M^{-1}s^{-1}$ of at least about 1.35 x 10^7 $M^{-1}s^{-1}$, of at least about 1.0 x 10^8 $M^{-1}s^{-1}$, of at least about 1.0 x 10^9 $M^{-1}s^{-1}$, or have a k_{on} (or k_a) ranging from about 5.0 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^8 $M^{-1}s^{-1}$, from about 3.3 x 10^4 $M^{-1}s^{-1}$ to about 1.0 x 10^9 $M^{-1}s^{-1}$, from about 2.5 x 10^4 $M^{-1}s^{-1}$ to about 1.25 x 10^7 $M^{-1}s^{-1}$, from about 2.4 x 10^{-11} $M^{-1}s^{-1}$ to about 1.35 x 10^7 $M^{-1}s^{-1}$, a k_{off} (or k_d) of at least 1.0 x 10^{-3} s^{-1}, of at least 1.0 x 10^{-4} s^{-1}, of at least 1.0 x 10^{-5} s^{-1}, of at least 1.0 x 10^{-6} s^{-1}

or have a k_{off} (or k_d) ranging from 1.0×10^{-3} s^{-1} to 1.0×10^{-6} s^{-1}, from 1.0×10^{-3} s^{-1} to 1.0×10^{-5} s^{-1} or from 1.0×10^{-3} s^{-1} to 1.0×10^{-4} s^{-1} and a K_D of at least 2.0×10^{-11} M, of at least 3.0×10^{-11} M, of at least 4.0×10^{-11} M, of at least 4.2×10^{-11} M, of at least 1.0×10^{-12} M of at least 1.0×10^{-13} M, of at least 7.4×10^{-13} M, of at least 8.0×10^{-13} M, of at least 1.0×10^{-14} M, of at least 1.0×10^{-15} M, or have a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from 4.2×10^{-11} M to 7.4×10^{-13} M. The derived or variant antibodies of the present invention comprise at least one mutation (such as deletions, additions and/or substitutions) in at least one of the heavy chain complementary determining ("CDR") regions (for example, the heavy chain CDR 1, heavy chain CDR 2 and/or heavy chain CDR 3), and/or at least one mutation (such as deletions, additions and/or substitutions) in the light chain CDR regions (for example, the light chain CDR 1, light chain CDR 2, and/or light chain CDR 3) when compared to the amino acid sequence of the antibody produced by hybridoma cell line 106.3 (also referred to herein as the "wildtype"). Moreover, the antibodies of the present invention may also contain one or more other mutations (such as deletions, additions and/or substitutions) in a part or portion of the antibody other than the CDR, such as, but not limited to, the framework region of an antibody. Methods for creating such derivatives are well known in the art and include the use of site-directed mutagenesis and PCR-mediated mutagenesis, which will be discussed in more detail *infra*.

More specifically, in another aspect, the antibody of the present invention immunospecifically binds to hBNP or hBNP fragment and comprises a heavy chain CDR 2 having an amino acid sequence of the formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

where Xaa_1 is selected from the group consisting of proline and alanine and Xaa_2 is selected from the group consisting of isoleucine and tyrosine, provided that when Xaa_1 is proline, Xaa_2 is not isoleucine.

In yet a further aspect, the antibody of the present invention immunospecifically binds to hBNP or hBNP fragment and comprises a heavy chain CDR 2 having the amino acid sequence shown in SEQ ID NO:15. In another aspect, the present invention relates to an antibody that immunospecifically binds to hBNP or hBNP fragment that comprises an amino acid sequence that is at least 35%, preferably at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to an amino acid sequence of SEQ ID NO:15.

In yet another aspect, the antibody of the present invention immunospecifically binds to hBNP or hBNP fragment and comprises a light chain CDR 1 that has an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

where Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine; where Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine and where Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid, provided that Xaa_3 is not serine when Xaa_4 is glutamine and Xaa_5 is serine.

In yet a further aspect, the antibody immunospecifically binds to hBNP or hBNP fragment and has a light chain CDR 1 having the amino acid sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO: 21 or SEQ ID NO:22. In another aspect, the present invention relates to an antibody that immunospecifically binds to hBNP or hBNP fragment that comprises an amino acid sequence that is at least 35%, preferably at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to an amino acid

sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO: 21 or SEQ ID NO:22.

In yet another aspect, the antibody of the present invention immunospecifically binds to hBNP or hBNP fragment and comprises a light chain CDR 2 that has an amino acid sequence having a formula of:

Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

where Xaa_6 is selected from the group consisting of: asparagine and cysteine, where Xaa_7 is selected from the group consisting of: leucine, glycine and alanine and where Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline, provided that Xaa_6 is not asparagine when Xaa_7 is leucine and Xaa_8 is glutamic acid.

In yet a further aspect, the antibody immunospecifically binds to hBNP or hBNP fragment and has a light chain CDR 2 having the amino acid sequence of SEQ ID NO:23 or SEQ ID NO: 24. In another aspect, the present invention relates to an antibody that immunospecifically binds to hBNP or hBNP fragment that comprises an amino acid sequence that is at least 35%, preferably at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24.

In yet a further aspect, the antibody of the present invention immunospecifically binds to hBNP or hBNP fragment and has a heavy chain CDR 1, heavy chain CDR 2, heavy chain CDR 3, a light chain CDR 1, a light chain CDR 2 and a light variable CDR 3 comprising the following amino acid sequences:

(a) Heavy Chain CDR 1 having an amino acid sequence of: Gly-Tyr-Thr-Phe-Thr-His-Tyr-Gly-Ile-Asn (SEQ ID NO:6);

(b) Heavy Chain CDR 2 having an amino acid sequence having a formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

where Xaa_1 is selected from the group consisting of proline and alanine;

where Xaa_2 is selected from the group consisting of isoleucine and tyrosine;

(c) Heavy Chain CDR 3 having an amino acid sequence of: Ser-His-Arg-Phe-Gly-Leu-Asp-Tyr (SEQ ID NO:8);

(d) Light Chain CDR 1 having an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

where Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine;

where Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine;

where Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid;

(e) Light Chain CDR 2 has an amino acid sequence having the formula of: Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

where Xaa_6 is selected from the group consisting of: asparagine and cysteine;

where Xaa_7 is selected from the group consisting of: leucine, glycine and alanine;

where Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline; and

(f) Light Chain CDR 3 has an amino acid sequence of: Gln-Gln-Ser-Asn-Glu-Asp-Pro-Phe-Thr (SEQ ID NO:11),

where the heavy chain CDR 2 has an amino acid sequence other than Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) when the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) and the light chain CDR 2 has the amino acid sequence of Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10), the light chain CDR 1 has an amino acid sequence other than Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) when the heavy chain CDR 2 has the amino acid sequence Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 2 has the amino acid sequence Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:19), or the light chain CDR 2 has an amino acid sequence other than Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10) when the heavy chain CDR 2 has the amino acid sequence of Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9).

Preferably, the antibodies having the above-described formulas comprise a heavy chain CDR 1, heavy chain CDR 2, heavy chain CDR 3, light chain CDR 1, light chain CDR 2 and light chain CDR 3 where Xaa_1-Xaa_8 in the above described formulas have the amino acid residues shown below in Table 2:

Table 2

Xaa_1	Xaa_2	Xaa_3	Xaa_4	Xaa_5	Xaa_6	Xaa_7	Xaa_8
alanine	Tyrosine	serine	Glutamine	serine	asparagine	leucine	glutamic acid
proline	Isoleucine	glutamine	phenylalanine	alanine	asparagine	leucine	glutamic acid
proline	Isoleucine	tyrosine	Alanine	serine	asparagine	leucine	glutamic acid
proline	Isoleucine	glutamine	Tryptophan	glycine	asparagine	leucine	glutamic acid
proline	Isoleucine	threonine	Tryptophan	aspartic acid	asparagine	leucine	glutamic acid
proline	Isoleucine	arginine	Tryptophan	proline	asparagine	leucine	glutamic acid
proline	Isoleucine	alanine	Tyrosine	glycine	asparagine	leucine	glutamic acid
proline	Isoleucine	asparagine	Tryptophan	proline	asparagine	leucine	glutamic acid
proline	Isoleucine	serine	Glutamine	serine	cysteine	glycine	tryptophan
proline	Isoleucine	serine	Glutamine	serine	cysteine	alanine	proline

III. Nucleic Acid Molecules

The present invention provides for a nucleic acid molecule, generally isolated, encoding an antibody of the present invention that immunospecifically binds to hBNP or hBNP fragment. In one aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that binds to an epitope comprising amino acid residues 5 through 13 of hBNP or hBNP fragment thereof with at least about a two fold improvement, at least about a three fold improvement, at least about a five fold improvement, at least about a ten fold improvement, at least about a fifteen fold improvement, at least about a twenty fold improvement, at least about a twenty-five fold improvement, at least about a thirty fold improvement, at least about a thirty-five fold improvement, at least about a forty fold improvement, at least about a forty-five fold improvement, at least about a fifty fold improvement, at least about a fifty-five fold improvement, at least about a sixty fold improvement, at least about a seventy fold improvement or at least about a seventy-five fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody that binds to an epitope comprising amino

acid residues 5 through 13 of hBNP or hBNP fragment with at least about a two fold improvement, at least about a three fold improvement, at least about a five fold improvement, at least about a ten fold improvement, at least about a fifteen fold improvement, at least about a twenty fold improvement, at least about a twenty-five fold improvement, at least about a thirty fold improvement, at least about a thirty-five fold improvement, at least about a forty fold improvement, at least about a forty-five fold improvement, at least about a fifty fold improvement, at least about a fifty-five fold improvement, at least about a sixty fold improvement, at least about a seventy fold improvement or at least about a seventy-five fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044.

In another aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment and that has a K_D of at least 2.0×10^{-11} M, of at least 3.0×10^{-11} M, of at least 4.0×10^{-11} M, of at least 4.2×10^{-11} M, of at least 1.0×10^{-12} M of at least 1.0×10^{-13} M, of at least 7.4×10^{-13} M, of at least 8.0×10^{-13} M, of at least 1.0×10^{-14} M, of at least 1.0×10^{-15} M, or has a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1.0×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from 4.2×10^{-11} M to 7.4×10^{-13} M. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody that immunospecifically binds to hBNP or hBNP fragment and that has a K_D of at least 2.0×10^{-11} M, of at least 3.0×10^{-11} M, of at least 4.0×10^{-11} M, of at least 4.2×10^{-11} M, of at least 1.0×10^{-12} M of at least 1.0×10^{-13} M, of at least 7.4×10^{-13} M, of at least 8.0×10^{-13} M, of at least 1.0×10^{-14} M, of at least 1.0×10^{-15} M, or has a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1.0×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from 4.2×10^{-11} M to 7.4×10^{-13} M.

In another aspect, an isolated nucleic acid molecule encodes an antibody that immunospecifically binds to amino acid residues 5 through 13 of human BNP or hBNP fragment at a K_D of at least 2.0×10^{-11} M, of at least 3.0×10^{-11} M, of at least

4.0×10^{-11} M, of at least 4.2×10^{-11} M, of at least 1.0×10^{-12} M of at least 1.0×10^{-13} M, of at least 7.4×10^{-13} M, of at least 8.0×10^{-13} M, of at least 1.0×10^{-14} M, of at least 1.0×10^{-15} M, or has a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1.0×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from 4.2×10^{-11} M to 7.4×10^{-13} M. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody that immunospecifically binds to amino acid residues 5 through 13 of hBNP or hBNP fragment at a K_D of at least 2.0×10^{-11} M, of at least 3.0×10^{-11} M, of at least 4.0×10^{-11} M, of at least 4.2×10^{-11} M, of at least 1.0×10^{-12} M of at least 1.0×10^{-13} M, of at least 7.4×10^{-13} M, of at least 8.0×10^{-13} M, of at least 1.0×10^{-14} M, of at least 1.0×10^{-15} M, or has a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1.0×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from 4.2×10^{-11} M to 7.4×10^{-13} M.

In yet another aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to amino acid residues 5 through 13 of hBNP or hBNP fragment at a K_D of from 4.2×10^{-11} M to 7.4×10^{-13} M, wherein said nucleic acid molecule comprises the nucleotide sequence of antibody produced by CHO cell line AM1. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody that immunospecifically binds to amino acid residues 5 through 13 of hBNP or hBNP fragment at a K_D of from 4.2×10^{-11} M to 7.4×10^{-13} M, wherein said nucleic acid molecule comprises the nucleotide sequence of antibody produced by CHO cell line AM1.

In another aspect, the present invention provides an isolated nucleic acid molecule that encodes antibodies that immunospecifically bind to hBNP or hBNP fragment, wherein said antibodies comprise derivatives or variants of antibodies produced by hybridoma cell line 106.3 (ATCC Accession No. HB-12044). As discussed previously herein, the inventors of the present invention have discovered

that antibodies that are derivatives or variants of the antibodies produced by hybridoma cell line 106.3 can be produced which exhibit a high binding affinity, specifically a k_{on} (or k_a) of at least about 2.4 x 10^4 $M^{-1}s^{-1}$, of at least about 2.5 x 10^4 $M^{-1}s^{-1}$, of at least about 3.3 x 10^4 $M^{-1}s^{-1}$, of at least about 5.0 x 10^4 $M^{-1}s^{-1}$, of at least about 1.25 x 10^7 $M^{-1}s^{-1}$ of at least about 1.35 x 10^7 $M^{-1}s^{-1}$, of at least about 1.0 x 10^8 $M^{-1}s^{-1}$, of at least about 1.0 x 10^9 $M^{-1}s^{-1}$, or have a k_{on} (or k_a) ranging from 5.0 x 10^4 $M^{-1}s^{-1}$ to 1.0 x 10^8 $M^{-1}s^{-1}$, from 3.3 x 10^4 $M^{-1}s^{-1}$ to 1.0 x 10^9 $M^{-1}s^{-1}$, from 2.5 x 10^4 $M^{-1}s^{-1}$ to 1.25 x 10^7 $M^{-1}s^{-1}$, from 2.4 x 10^{-11} $M^{-1}s^{-1}$ to 1.35 x 10^7 $M^{-1}s^{-1}$, a k_{off} (or k_d) of at least about 1.0 x 10^{-3} s^{-1}, of at least about 1.0 x 10^{-4} s^{-1}, of at least about 1.0 x 10^{-5} s^{-1}, of at least about 1.0 x 10^{-6} s^{-1} or have a k_{off} (or k_d) ranging from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-6} s^{-1}, from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-5} s^{-1} or from 1.0 x 10^{-3} s^{-1} to 1.0 x 10^{-4} s^{-1} and a K_D of at least about 2.0 x 10^{-11} M, of at least about 3.0 x 10^{-11} M, of at least about 4.0 x 10^{-11} M, of at least about 4.2 x 10^{-11} M, of at least about 1.0 x 10^{-12} M of at least about 1.0 x 10^{-13} M, of at least about 7.4 x 10^{-13} M, of at least about 8.0 x 10^{-13} M, of at least about 1.0 x 10^{-14} M, of at least about 1.0 x 10^{-15} M of at least about 1.0 x 10^{-16} M, or have a K_D ranging from 2.0 x 10^{-11} M to 1.0 x 10^{-16} M, from 2.0 x 10^{-11} M to 1.0 x 10^{-15} M, from 3.0 x 10^{-11} M to 1 x 10^{-14} M, from 4.0 x 10^{-11} M to 8.0 x 10^{-13} M or from 4.2 x 10^{-11} M to 7.4 x 10^{-13} M. The derived or variant antibodies of the present invention comprises at least one mutation (such as deletions, additions and/or substitutions) in at least one of the heavy chain complementary determining ("CDR") regions (for example, the heavy chain CDR 1, heavy chain CDR 2, or heavy chain CDR 3), at least one mutation (such as deletions, additions and/or substitutions) in the light chain CDR regions (for example, the light chain CDR 1, light chain CDR 2, or light chain CDR 3) when compared to the amino acid sequence the antibody produced by hybridoma cell line 106.3. Standard techniques known to those of skill in the art can be used to introduce mutations (such as deletions, additions, and/or substitutions) in the nucleic acid molecule encoding an antibody of the present invention, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis which results in amino acid substitutions. In one aspect, the derivatives include less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the original antibody

produced by hybridoma cell line 106.3. In one aspect, the derivatives have conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues (i.e., amino acid residues which are not critical for the antibody to immunospecifically bind to hBNP or hBNP fragment). A "conservative amino acid substitution" is one in which the amino acid residue is replaced with the amino acid residue having a side chain with a similar charge. Families of amino acid residues having side chains with similar charges have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Alternatively, mutations can be introduced randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that exhibit enhanced binding affinity to hBNP or hBNP fragment. Following mutagenesis, the encoded antibody can be expressed and the activity of the antibody can be determined.

In another aspect, the present invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody having a heavy chain CDR 2 having an amino acid sequence of the formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

where Xaa_1 is selected from the group consisting of proline and alanine and Xaa_2 is selected from the group consisting of isoleucine and tyrosine, provided that when Xaa_1 is proline, Xaa_2 is not isoleucine. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes,

under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody having a heavy chain CDR 2 having an amino acid sequence of the above-described formula.

In another aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody comprising (alternatively, consisting of) a heavy chain CDR 2 having an amino acid sequence of SEQ ID NO:15. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody comprising a heavy chain CDR 2 having the amino acid sequence of SEQ ID NO:15.

In another aspect, the present invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody having a light chain CDR 1 that has an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

where Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine; where Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine and where Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid, provided that Xaa_3 is not serine when Xaa_4 is glutamine and Xaa_5 is serine. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody having a light chain CDR 1 having an amino acid sequence of the above-described formula.

In another aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody comprising (alternatively, consisting of) a light chain CDR 1 having an amino acid sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody comprising a light chain CDR 1 having the amino acid sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22.

In another aspect, the present invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody having a light chain CDR 2 that has an amino acid sequence having a formula of:

Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

where Xaa_6 is selected from the group consisting of: asparagine and cysteine, where Xaa_7 is selected from the group consisting of: leucine, glycine and alanine and where Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline, provided that Xaa_6 is not asparagine when Xaa_7 is leucine and Xaa_8 is glutamic acid. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody having a light chain CDR 2 having an amino acid sequence of the above-described formula.

In another aspect, the invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody comprising (alternatively, consisting of) a light chain CDR 2 having an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence

that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody comprising a light chain CDR 2 having the amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24.

In another aspect, the invention provides an isolated nucleic acid molecule that encodes an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody comprising (alternatively, consisting) a heavy chain CDR 2 having an amino acid sequence of SEQ ID NO:15, a light chain CDR 1 having an amino acid sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22, a light chain CDR 2 having an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24 or any combinations these amino acid sequences. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody comprising a heavy chain CDR 2 having an amino acid sequence of SEQ ID NO:15, a light chain CDR 1 having an amino acid sequence of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22, a light chain CDR 2 having an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24 or any combinations these amino acid sequences.

In another aspect, the present invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, said antibody having a heavy chain CDR 1, heavy chain CDR 2, heavy chain CDR 3, a light chain CDR 1, a light chain CDR 2 and a light variable CDR 3 comprising the following amino acid sequences:

(a) Heavy Chain CDR 1 having an amino acid sequence of: Gly-Tyr-Thr-Phe-Thr-His-Tyr-Gly-Ile-Asn (SEQ ID NO:6);

(b) Heavy Chain CDR 2 having an amino acid sequence having a formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

where Xaa_1 is selected from the group consisting of proline and alanine;

where Xaa_2 is selected from the group consisting of isoleucine and tyrosine;

(c) Heavy Chain CDR 3 having an amino acid sequence of: Ser-His-Arg-Phe-Gly-Leu-Asp-Tyr (SEQ ID NO:8);

(d) Light Chain CDR 1 having an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

where Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine;

where Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine;

where Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid;

(e) Light Chain CDR 2 has an amino acid sequence having the formula of: Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

where Xaa_6 is selected from the group consisting of: asparagine and cysteine;

where Xaa_7 is selected from the group consisting of: leucine, glycine and alanine;

where Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline; and

(f) Light Chain CDR 3 has an amino acid sequence of: Gln-Gln-Ser-Asn-Glu-Asp-Pro-Phe-Thr (SEQ ID NO:11),

where the heavy chain CDR 2 has an amino acid sequence other than Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) when the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) and the light chain CDR 2 has the amino acid sequence of Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10), the light chain CDR 1 has an amino acid sequence other than Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) when the heavy chain CDR 2 has the amino acid sequence Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 2 has the amino acid sequence Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:19), or the light chain CDR 2 has an amino acid sequence other than Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10) when the heavy chain CDR 2 has the amino acid sequence of Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9). The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide sequence that hybridizes, under stringent conditions, to the nucleic acid molecule described herein that encodes an antibody having a heavy chain CDR 1 region, a heavy chain CDR 2 region, a heavy chain CDR 3 region, a light chain CDR 1 region, a light chain CDR 2 region and a light chain CDR 3 region having the amino acid sequences pursuant to the above-described formula.

In yet another aspect, the present invention provides an isolated nucleic acid molecule encoding an antibody that immunospecifically binds to hBNP or hBNP fragment, wherein said antibody is produced by CHO cell line AM1. The present invention also provides an isolated nucleic acid molecule that comprises a nucleotide

sequence that hybridizes, under stringent conditions, to the nucleic acid molecule that encodes an antibody that immunospecifically binds to hBNP or hBNP fragment, wherein said antibody is produced by CHO cell line AM1.

IV. Methods for Preparing the Antibodies of the Present Invention

The antibodies of the present invention can be prepared using routine techniques known to those skilled in the art.

In one aspect, the antibodies of the present invention can be prepared by recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying nucleic acid molecules encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are cultures, from which medium the antibodies can be recovered. Standard recombinant nucleic acid (DNA) methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expressions vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning: A Laboratory Manual, Second Edition,* Cold Spring Harbor, New Your, (1989), Ausubel, F. M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates (1989) and in U.S. Patent No. 4,816,397 by Boss et al.

To express the antibodies of the invention, nucleic acid molecules encoding the light and heavy chain regions are first obtained. These nucleic acid molecules may be obtained from the hybridoma cell line expressing monoclonal antibody 106.3 and modified by means well known in the art (such as site-directed mutagenesis) to generate antibodies of the present invention, including, for example, the antibodies produced by CHO cell line AM1. A hybridoma cell line expressing monoclonal antibody 106.3 was deposited with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Virginia 20110 and was accorded accession number HB-12044. The nucleic acid sequence of monoclonal antibody 106.3 is

shown in Figures 3A-3E and SEQ ID NO:1.

For example, once the 106.3 variable heavy (VH) and variable (VL) nucleic acid fragments are obtained, these sequences or specific regions within these sequences, such as the complementary determining ("CDR") regions, can be mutated to encode the AM1 or AM1-related amino acid sequences disclosed herein. The amino acid sequences encoded by the 106.3 VH and VL DNA sequences are compared to the AM1 or AM1-related VH and VL amino acid sequences to identify amino acid residues in the AM1 or AM1-related sequence that differ. The appropriate nucleotides of monoclonal antibody 106.3 are mutated such that the mutated sequence encodes the AM1 or AM1-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of antibody 106.3 sequences can be carried out by standard methods, such as PCR-mediated mutageneisis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Alternatively, in another aspect, nucleic acid molecules encoding the VH and VL chains can be synthesized on a chemical synthesizer, using routine techniques known to those in the art. For example, the VH and VL chains from the nucleic acid molecules described in Section III can be chemically synthesized using routine techniques known in the art. Starting at the 3' terminal base which is attached to a support, nucleotides are coupled in a step-wise fashion. Following the addition of the most 5' nucleotide, the nucleotide is cleaved from the solid support and purified by desalting followed by polyacrylamide gel electrophoresis (PAGE) (Midland Certified Reagents, Midland, TX, www.oligos.com).

Once nucleic acid fragments encoding AM1 or AM1-related VH and VL segments are obtained (by amplification and mutagenesis of VH and VL genes, as described above), these nucleic acid fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to an antibody (such as, but not limited to, a full-length antibody chain genes, to Fab fragment genes or to a scFv gene). In these manipulations, a VL- or VH-encoding

nucleic acid fragment is operatively linked to another nucleic acid fragment encoding another protein, such as antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two nucleic acid fragments are joined such that the amino acid sequences encoded by the two nucleic acid fragments remain in-frame.

In an alternative method, an scFv gene may be constructed with wildtype CDR regions (such as those of monoclonal antibody 106.3) and then mutated using techniques known in the art.

The isolated nucleic acid molecule encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding nucleic acid molecule to another nucleic acid molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (See for example, Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242 (1991)). In another aspect, the present invention further encompasses all known human heavy chain constant regions, including but not limited to, all known allotypes of the human heavy chain constant region. Nucleic acid fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region.

The isolated nucleic acid molecule encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding nucleic acid molecule to another nucleic acid molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see e.g., Kabat, E.A., et al., *Sequences of Proteins of Immunological Interest, Fifth Edition,* U.S. Department of Health and Human Services, NIH Publication No. 91-3242 (1991)). The present invention encompasses all known human light chain constant regions, including but not limited to, all known allotypes of the human light chain constant region. Nucleic acid

fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

It is to be understood that the specific designations of framework (FR) and CDR regions within a particular heavy or light chain region may vary depending on the convention or numbering system used to identify such regions (e.g. Chothia, Kabat, Oxford Molecular's *AbM* modeling software, all of which are known to those of ordinary skill in the art). For the purposes of the present invention, the Kabat numbering system is used.

To create a scFv gene, the VH- and VL-encoding nucleic acid fragments are operatively linked to another fragment encoding a flexible linker, such as, a linker that is encoded by the amino acid sequence GPAKELTPLKEAKVS (SEQ ID NO:4). Examples of other linker sequences that can be used in the present invention can be found in Bird et al., *Science* 242:423-426 (1988), Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883 (1988) and McCafferty et al., *Nature,* 348:552-554 (1990).

To express the antibodies, or antibody portions of the invention, nucleic acid molecules encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vectors or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (for example, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present).

Prior to the insertion of the light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one approach to converting the VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH "segment" within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The single peptide can be an immunoglobin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors can carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology. Methods in Enzymology* 185, Academic Press, San Diego, Calif. (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of the expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus ("CMV") (such as the CMV promoter/enhancer), Simian Virus 40 ("SV40") (such as the SV40 promoter/enhancer), adenovirus, (such as the adenovirus major late promoter ("AdMLP")) and polyoma. For further description of viral regulatory elements, and sequences thereof, see for example, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell et al. and U.S. Patent No. 4,968,615 by Schaffner et al.

In addition to the antibody chain genes and regulatory sequences, recombinant expression vectors may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (See, for example, U.S. Patent Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel et al.). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase ("DHFR") gene for use in dhfr-host cells with methotrexate selection/amplification and the neomycin ("neo") gene for G418 selection.

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains are transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (See, Boss, M. A. and Wood, C. R., *Immunology Today* 6:12-13 (1985)).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include the Chinese Hamster Ovary ("CHO") cells (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220 (1980), used with a DHFR selectable marker, for example, as described in R.J. Kaufman and P.A. Sharp, *Mol. Biol.* 159:601-621 (1982)), NSO myeloma cells, COS

cells, HEK-293 cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments, $F(ab')_2$ fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with nucleic acid molecule encoding either the light chain or the heavy chain (but not both) of an antibody of the present invention. Recombinant DNA technology may also be used to remove some or all of the nucleic acid molecules encoding either or both of the light and heavy chains that are not necessary for binding to hBNP or hBNP fragment. The molecules expressed from such truncated nucleic acid molecules also are encompassed by the antibodies of the invention.

In a preferred system for recombinant expression of an antibody, or antigen binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector. Cells were cultured in medium without hypoxanthine and thymidine to obtain those CHO cells that have acquired the DHFR gene from the transfecting vector. Antigen specific screening methods were used to identify those clones that expressed the highest quantity of antibody. Those individual clones were expanded and were routinely re-screened. The highest producing clone was AM1. The selected transformant host cells are culture to allow for expression of the antibody heavy and

light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium.

In view of forgoing, another aspect of the invention pertains to nucleic acid, vector and host cell compositions that can be used for recombinant expression of the antibodies and antibody portions of the invention. The amino acid sequence encoding the heavy chain CDR 2 region of AM1 and variants thereof is shown in SEQ ID NO:15. The amino acid sequence encoding the AM1 light chain CDR 1 region is shown in SEQ ID NO:22. The nucleic acid molecule encoding the heavy chain CDR 2 region of AM1 is shown in SEQ ID NO:81. The nucleic acid molecule encoding the light chain CDR 1 region of AM1 is shown in SEQ ID NO:82.

V. Selection of Recombinant Antibodies

The antibodies of the present invention, including the AM1 or AM1-related antibodies disclosed herein, can be isolated by screening of a combinatorial antibody library. Preferably, the combinatorial antibody library is a recombinant combinatorial library, preferably a scFv yeast display library, prepared using chimeric, humanized or human VL and VH cDNAs. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available vectors for generating yeast display libraries (such as, the pYD1 vector, Invitrogen, Carlsbad, California) examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, Boder E.T. and Wittrup K.D., Yeast surface display for directed evolution of protein expression, affinity, and stability, *Methods Enzymol.*, 328:430-44 (2000) and Boder E.T. and Wittrup K.D., Yeast surface display for screening combinatorial polypeptide libraries, *Nat Biotechnol.* 15(6):553-7 (June 1997).

In a preferred embodiment, to isolate antibodies with high binding affinity, such as any of the antibodies described in Section II herein, an antibody that is known to immunospecifically bind to hBNP or hBNP fragment (such as, for example,

monoclonal antibody 106.3) is first used to generate human heavy and light chain sequences expressed as scFvs on the surface of yeast (preferably, *Saccaromyces cerevisiae*). These antibody (Such as monoclonal antibody106.3) scFvs are analyzed to determine the dissociation rate (namely, the k_{off} or k_d) of these antibodies. Such constructs then are screened, preferably using biotinylated cyclic hBNP (1-32c). The dissociation rate data can then be plotted as mean fluorescence units ("MFU") versus time (in seconds). A first order decay equation can be used to fit the data. An example of such a formula that can be used is:

$$y=m1*\exp(-m2*M0)+m3$$

where m1 is the maximum fluorescence at time zero (*= time and exp = exponential);

where m2 is the off-rate (the formula for determining off-rate is well known to those skilled in the art);

where M0 is time x (x being the time that is being measured); and

where m3 is the background being generated from the system.

The dissociation rate data can be used to identify off-rate improved antibodies of the present invention from mutagenic libraries.

Those scFv constructs having an improved dissociation rate are selected for subsequent mutagenesis of the heavy and light chain variable regions to generate CDR mutagenic libraries.

To further increase the binding affinity, the VH and VL segments of the preferred VH/VL pair(s) can be randomly mutated, preferably within the CDR2 region of VH, the CDR1 region and/or CDR2 region of VL in a process analogous to the *in vivo* somatic mutation process responsible for affinity maturation of antibodies during a natural immune response. This *in vitro* affinity maturation can be accomplished by replacing a portion of each CDR with a degenerate single-stranded oligonucleotide encoding three amino acids within the CDR being targeted. The

replacement of a portion of each CDR with a new randomized sequence (up to 8000 possibilities) can be accomplished by homologous recombination in yeast (see, e.g. Example 1). These randomly mutated VH and VL segments can be analyzed for binding to hBNP or hBNP fragment in the context of an scFv; scFvs exhibiting an improved fluorescence and that (a) bind to an epitope comprising amino acid residues 5 through 13 of hBNP or hBNP fragment thereof with at least about a two fold improvement, at least about a three fold improvement, at least about a five fold improvement, at least about a ten fold improvement, at least about a fifteen fold improvement, at least about a twenty fold improvement, at least about a twenty-five fold improvement, at least about a thirty fold improvement, at least about a thirty-five fold improvement, at least about a forty fold improvement, at least about a forty-five fold improvement, at least about a fifty fold improvement, at least about a fifty-five fold improvement, at least about a sixty fold improvement, at least about a seventy fold improvement or at least about a seventy-five fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044, (b) exhibits a k_{on} (or k_a) of at least about 2.4×10^4 $M^{-1}s^{-1}$, of at least about 2.5×10^4 $M^{-1}s^{-1}$, of at least about 3.3×10^4 $M^{-1}s^{-1}$, of at least about 5.0×10^4 $M^{-1}s^{-1}$, of at least about 1.25×10^7 $M^{-1}s^{-1}$ of at least about 1.35×10^7 $M^{-1}s^{-1}$, of at least about 1.0×10^8 $M^{-1}s^{-1}$, of at least about 1.0×10^9 $M^{-1}s^{-1}$, or have a k_{on} (or k_a) ranging from 5.0×10^4 $M^{-1}s^{-1}$ to 1.0×10^8 $M^{-1}s^{-1}$, from 3.3×10^4 $M^{-1}s^{-1}$ to 1.0×10^9 $M^{-1}s^{-1}$, from 2.5×10^4 $M^{-1}s^{-1}$ to 1.25×10^7 $M^{-1}s^{-1}$, from 2.4×10^{-11} $M^{-1}s^{-1}$ to 1.35×10^7 $M^{-1}s^{-1}$, (c) exhibits a k_{off} (or k_d) of at least about 1.0×10^{-3} s^{-1}, of at least about 1.0×10^{-4} s^{-1}, of at least about 1.0×10^{-5} s^{-1}, of at least about 1.0×10^{-6} s^{-1} or have a k_{off} (or k_d) ranging from 1.0×10^{-3} s^{-1} to 1.0×10^{-6} s^{-1}, from 1.0×10^{-3} s^{-1} to 1.0×10^{-5} s^{-1} or from 1.0×10^{-3} s^{-1} to 1.0×10^{-4} s^{-1}, or (d) exhibit a K_D of at least about 2.0×10^{-11} M, of at least about 3.0×10^{-11} M, of at least about 4.0×10^{-11} M, of at least about 4.2×10^{-11} M, of at least about 1.0×10^{-12} M, of at least about 1.0×10^{-13} M, of at least about 7.4×10^{-13} M, of at least about 8.0×10^{-13} M, of at least about 1.0×10^{-14} M, of at least about 1.0×10^{-15} M, or have a K_D ranging from 2.0×10^{-11} M to 1.0×10^{-15} M, from 3.0×10^{-11} M to 1×10^{-14} M, from 4.0×10^{-11} M to 8.0×10^{-13} M or from $4.2 \times$

10^{-11} M to 7.4 x 10^{-13} M can then be isolated and the CDR mutation identified by sequencing.

Following screening of a recombinant scFv display library, clones having the desired characteristics are selected for conversion. Nucleic acid molecules encoding the selected antibody can be recovered from the display package (e.g., from the yeast expression vector) and subcloned into other expression vectors by standard recombinant DNA techniques. If desired, the nucleic acid can be further manipulated to create other antibody forms of the invention (e.g., linked to nucleic acid encoding additional immunoglobulin domains, such as additional constant regions). To express a recombinant human antibody isolated by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into a mammalian host cells, as described in further detail in Section IV above.

VI. Immunoassays

In another aspect, the present invention relates to immunoassays that can be used for the qualitative and/or quantitative detection of hBNP or hBNP fragment in a test sample. The immunoassays of the present invention can be conducted using any format known in the art, such as, but not limited to, a sandwich format, a competitive inhibition format (including both forward or reverse competitive inhibition assays) or in a fluorescence polarization format.

In immunoassays for the qualitative detection of hBNP or hBNP fragment in a test sample, at least one antibody that binds to certain epitopes of hBNP or hBNP fragment thereof is contacted with at least one test sample suspected of containing or that is known to contain hBNP or hBNP fragment to form an antibody-hBNP immune complex. The antibodies described in Section II herein can be used in such immunoassays to form such antibody-hBNP immune complexes in at least one test sample. These immune complexes can then detected using routine techniques known to those skilled in the art. For example, the antibody of the present invention can be labeled with a detectable label to detect the presence antibody-hBNP complex.

Alternatively, the hBNP or hBNP fragments in the test sample can be labeled with a detectable label and the resulting antibody-hBNP immune complexes detected using routine techniques known to those skilled in the art. Detectable labels and their attachment to antibodies are discussed in more detail *infra.*

Alternatively, a second antibody that binds to the hBNP or hBNP fragment and that contains a detectable label can be added to the test sample and used to detect the presence of the antibody-hBNP complex. Any detectable label known in the art can be used. Detectable labels and their attachment to antibodies are discussed in more detail *infra.*

In immunoassays for the quantitative detection of BNP, such as a sandwich type format, at least two antibodies are employed to separate and quantify hBNP or hBNP fragment in a test sample. More specifically, the at least two antibodies bind to certain epitopes of hBNP or hBNP fragment forming an immune complex which is referred to as a "sandwich". Generally, one or more antibodies can be used to capture the hBNP or hBNP fragment in the test sample (these antibodies are frequently referred to as a "capture" antibody or "capture" antibodies) and one or more antibodies is used to bind a detectable (namely, quantifiable) label to the sandwich (these antibodies are frequently referred to as the "detection" antibody or "detection" antibodies). In a sandwich assay, it is preferred that both antibodies binding to their epitope are not diminished by the binding of any other antibody in the assay to its respective epitope. In other words, antibodies should be selected so that the one or more first antibodies brought into contact with a test sample suspected of containing hBNP or hBNP fragment do not bind to all or part of an epitope recognized by the second or subsequent antibodies, thereby interfering with the ability of the one or more second detection antibodies to bind to the hBNP or hBNP fragment.

The inventors have discovered that an excellent sandwich immunoassay can be performed using the antibodies of the present invention. More specifically, the antibodies of the present invention can be used as a first antibody in said immunoassay. Preferably, the antibody of the present invention immunospecifically bind to epitopes comprising at least three (3) amino acids of 5-13 of hBNP or hBNP

fragment with a K_D of from 4.2×10^{-11} M to 7.4×10^{-13} M. In addition to the antibodies of the present invention, said immunoassay comprises a second antibody, preferably a monoclonal antibody, that immunospecifically binds to epitopes having an amino acid sequence comprising at least three (3) amino acids of amino acids 27-32 of hBNP. An example of a monoclonal antibody that immunospecifically binds to epitopes having an amino acid sequence containing amino acids 27-32 of hBNP is a monoclonal antibody produced by hybridoma cell line BC203.

In a preferred embodiment, the test sample suspected of containing hBNP or a hBNP fragment can be contacted with at least one first capture antibody (or antibodies) and at least one second detection antibodies either simultaneously or sequentially. In the sandwich assay format, a test sample suspected of containing hBNP or hBNP fragment is first brought into contact with the at least one first capture antibody that specifically binds to a particular epitope under conditions which allow the formation of a first antibody-hBNP complex. If more than one capture antibody is used, a first multiple capture antibody-hBNP complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of hBNP or hBNP fragment expected in the test sample. For example, from about 5 μg/mL to about 1 mg/mL of antibody per mL of microparticle coating buffer can be used.

Optionally, prior to contacting the test sample with the at least one first capture antibody, the at least one first capture antibody can be bound to a solid support which facilitates the separation the first antibody-hBNP complex from the test sample. Any solid support known in the art can be used, including but not limited to, solid supports made out of polymeric materials in the forms of wells, tubes or beads. The antibody (or antibodies) can be bound to the solid support by adsorption, by covalent bonding using a chemical coupling agent or by other means known in the art, provided that such binding does not interfere with the ability of the antibody to bind hBNP or hBNP fragment. Moreover, if necessary, the solid support can be derivatized to allow reactivity with various functional groups on the antibody. Such derivatization requires the use of certain coupling agents such as, but not limited to,

maleic anhydride, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

After the test sample suspected of containing hBNP or an hBNP fragment is brought into contact with the at least one first capture antibody, the test sample is incubated in order to allow for the formation of a first capture antibody (or multiple antibody)-hBNP complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2°C to about 45°C, and for a period from at least about one (1) minute to about eighteen (18) hours, preferably from about 2-6 minutes, most preferably from about 3-4 minutes.

After formation of the first/multiple capture antibody-hBNP complex, the complex is then contacted with at least one second detection antibody (under conditions which allow for the formation of a first/multiple antibody—hBNP-second antibody complex). If the first antibody-hBNP complex is contacted with more than one detection antibody, then a first/multiple capture antibody-hBNP-multiple antibody detection complex is formed. As with first antibody, when the at least second (and subsequent) antibody is brought into contact with the first antibody-hBNP complex, a period of incubation under conditions similar to those described above is required for the formation of the first/multiple antibody-hBNP-second/multiple antibody complex. Preferably, at least one second antibody contains a detectable label. The detectable label can be bound to the at least one second antibody prior to, simultaneously with or after the formation of the first/multiple antibody-hBNP-second/multiple antibody complex. Any detectable label known in the art can be used. For example, the detectable label can be a radioactive label, such as, ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, an enzymatic label, such as horseradish peroxidase, alkaline peroxidase, glucose 6-phosphate dehydrogenase, etc., a chemiluminescent label, such as, acridinium esters, luminal, isoluminol, thioesters, sulfonamides, phenanthridinium esters, etc. a fluorescence label, such as, fluorescein (5-fluorescein, 6-carboxyfluorescein, 3'6-carboxyfluorescein, 5(6)-carboxyfluorescein, 6-hexachloro-fluorescein, 6-tetrachlorofluorescein, fluorescein isothiocyanate, etc.), rhodamine, phycobiliproteins, R-phycoerythrin, quantum dots (zinc sulfide-capped cadmium

selenide), a thermometric label or an immuno-polymerase chain reaction label. An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden, *Introduction to Immunocytochemistry,* 2nd ed., Springer Verlag, N.Y. (1997) and in Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (1996), which is a combined handbook and catalogue published by Molecular Probes, Inc., Eugene, Oregon.

The detectable label can be bound to the antibodies either directly or through a coupling agent. An example of a coupling agent that can be used is EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride) that is commercially available from Sigma-Aldrich, St. Louis, MO. Other coupling agents that can be used are known in the art. Methods for binding a detectable label to an antibody are known in the art. Additionally, many detectable labels can be purchased or synthesized that already contain end groups that facilitate the coupling of the detectable label to the antibody, such as, N10-(3-sulfopropyl)-N-(3-carboxypropyl)-acridinium-9-carboxamide, otherwise known as CPSP-Acridinium Ester or N10-(3-sulfopropyl)-N-(3-sulfopropyl)-acridinium-9-carboxamide, otherwise known as SPSP-Acridinium Ester.

The first antibody/multiple-hBNP-second/multiple antibody complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the label. For example, if the at least first capture antibody is bound to a solid support, such as a well or a bead, separation can be accomplished by removing the fluid (from the test sample) from contact with the solid support. Alternatively, if the at least first capture antibody is bound to a solid support it can be simultaneously contacted with the hBNP-containing sample and the at least one second detection antibody to form a first (multiple) antibody-hBNP-second (multiple) antibody complex, followed by removal of the fluid (test sample) from contact with the solid support. If at least first capture antibody is not bound to a solid support, then the first antibody/multiple-hBNP-second/multiple antibody complex does not have to be removed from the test sample for quantification of the amount of the label.

After formation of the labeled first antibody-hBNP-second antibody complex, the amount of label in the complex is quantified using techniques known in the art. For example, if an enzymatic label is used, the labeled complex is reacted with a substrate for the label that gives a quantifiable reaction such as the development of color. If the label is a radioactive label, the label is quantified using a scintillation counter. If the label is a fluorescent label, the label is quantified by stimulating the label with a light of one color (which is known as the "excitation wavelength") and detecting another color (which is known as the "emission wavelength") that is emitted by the label in response to the stimulation. If the label is a chemiluminescent label, the label is quantified detecting the light emitted either visually or by using luminometers, x-ray film, high speed photographic film, a CCD camera, etc. Once the amount of the label in the complex has been quantified, the concentration of hBNP or hBNP fragment in the test sample is determined by use of a standard curve that has been generated using serial dilutions of hBNP or hBNP fragment of known concentration. Other than using serial dilutions of hBNP or hBNP fragment, the standard curve can be generated gravimetrically, by mass spectroscopy and by other techniques known in the art.

In a forward competitive format, an aliquot of labeled hBNP, hBNP fragment or hBNP analogue thereof of a known concentration is used to compete with hBNP or hBNP fragment in a test sample for binding to hBNP antibody (such as an antibody of the present invention). Peptides of hBNP, hBNP fragments and hBNP analogues thereof and methods of making peptides of hBNP, hBNP fragments and hBNP analogues are known in the art (See, for example, U.S. Patent No. 6,162,902). Moreover, as described in the Examples herein, cyclic hBNP (1-32) can also be used in said competitive formats.

In a forward competition assay, an immobilized antibody (such as an antibody of the present invention) can either be sequentially or simultaneously contacted with the test sample and a labeled hBNP, hBNP fragment or hBNP analogue thereof. The hBNP peptide, hBNP fragment or hBNP analogue can be labeled with any detectable label known to those skilled in the art, including those detectable labels discussed

above in connection with the sandwich assay format. In this assay, the antibody of the present invention can be immobilized on to a solid support using the techniques discussed previously herein. Alternatively, the antibody of the present invention can be coupled to an antibody, such as an antispecies antibody, that has been immobilized on to a solid support, such as a microparticle (See Example 3).

The labeled hBNP peptide, hBNP fragment or hBNP analogue, the test sample and the antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species of antibody-hBNP complexes are then generated. Specifically, one of the antibody-hBNP complexes generated contains a detectable label while the other antibody-hBNP complex does not contain a detectable label. The antibody-hBNP complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the detectable label. Regardless of whether the antibody-hBNP complex is separated from the remainder of the test sample, the amount of detectable label in the antibody-hBNP complex is then quantified. The concentration of hBNP or hBNP fragment in the test sample can then be determined by comparing the quantity of detectable label in the antibody-hBNP complex to a standard curve. The standard curve can be generated using serial dilutions of hBNP or hBNP fragment of known concentration, by mass spectroscopy, gravimetrically and by other techniques known in the art.

The antibody-hBNP complex can be separated from the test sample by binding the antibody to a solid support, such as the solid supports discussed above in connection with the sandwich assay format, and then removing the remainder of the test sample from contact with the solid support.

The labeled hBNP (or hBNP fragment or hBNP analogue thereof) that is used to compete with hBNP or a hBNP fragment in the test sample for binding to the antibody can be intact hBNP 1-32, any hBNP fragment thereof provided that said hBNP fragment comprises at least one amino acid sequence containing (meaning including and between) amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-

11, 10-13, 10-12 or 11-13 of hBNP) or any hBNP analogue provided that said hBNP peptide, hBNP fragment or hBNP analogue contains a sequence of amino acids that corresponds to an epitope that is recognized by the antibody. Preferably, the antibody employed specifically binds to an epitope comprising at least three (3) amino acids of amino acids 5-13 of hBNP (such as the antibody of the present invention, specifically an antibody produced by CHO cell line AM1) or specifically binds to an epitope having an amino acid sequence that contains (meaning that it includes and is between) amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP. Examples of hBNP fragments that can be labeled and used in the present invention, include, but are not limited to, peptide fragments having an amino acid sequence containing amino acids 1-31, 1-30, 1-29, 1-28, 1-27, 1-26, 1-25, 1-24, 1-23, 1-22, 1-21, 1-20, 1-19, 1-18, 1-17, 1-16, 1-15, 2-32, 2-31, 2-30, 2-29, 2-28, 2-27, 2-26, 2-25, 2-24, 2-23, 2-22, 2-21, 2-20, 2-19, 2-18, 2-17, 2-16, 2-15, 2-14, 2-13, 2-12, 2-11, 2-10, 2-9, 2-8, 2-7, 3-32, 3-31, 3-30, 3-29, 3-28, 3-27, 3-26, 3-25, 3-24, 3-23, 3-32, 3-21, 3-20, 3-19, 3-18, 3-17, 3-16, 3-15, 3-14, 3-13, 3-12, 3-11, 3-10, 3-9, 3-8, 4-32, 4-31, 4-30, 4-29, 4-28, 4-27, 4-26, 4-25, 4-24, 4-23, 4-22, 4-21, 4-20, 4-19, 4-18, 4-17, 4-16, 4-15, 4-14, 4-13, 4-12, 4-11, 4-10, 4-9, 5-32, 5-31, 5-30, 5-29, 5-28, 5-27, 5-26, 5-25, 5-24, 5-23, 5-22, 5-21, 5-20, 5-19, 5-18, 5-17, 5-16, 5-15, 5-14, 5-13, 5-12, 5-11, 5-10, 6-32, 6-31, 6-30, 6-29, 6-28, 6-27, 6-26, 6-25, 6-24, 6-23, 6-22, 6-21, 6-20, 6-19, 6-18, 6-17, 6-16, 6-15, 6-14, 6-13, 6-12, 6-11, 7-32, 7-31, 7-30, 7-29, 7-28, 7-27, 7-26, 7-25, 7-24, 7-23, 7-22, 7-21, 7-20, 7-19, 7-18, 7-17, 7-16, 7-15, 7-14, 7-13, 7-12, 8-32, 8-31, 8-30, 8-29, 8-28, 8-27, 8-26, 8-25, 8-24, 8-23, 8-22, 8-21, 8-20, 8-19, 8-18, 8-17, 8-16, 8-15, 8-14, 8-13, 9-32, 9-31, 9-30, 9-29, 9-28, 9-27, 9-26, 9-25, 9-24, 9-23, 9-22, 9-21, 9-20, 9-19, 9-18, 9-17, 9-16, 9-15, 9-14, 10-32, 10-31, 10-30, 10-29, 10-28, 10-27, 10-26, 10-25, 10-24, 10-23, 10-22, 10-21, 10-20, 10-19, 10-18, 10-17, 10-16, 10-15, 11-32, 11-31, 11-30, 11-29, 11-28, 11-27, 11-26, 11-25, 11-24, 11-23, 11-22, 11-21, 11-20, 11-19, 11-18, 11-17 or 11-16 of hBNP.

In a reverse competition assay, an immobilized hBNP peptide, hBNP fragment or hBNP analogue thereof can either be sequentially or simultaneously contacted with a test sample and at least one labeled antibody. Preferably, the antibody specifically

binds to an epitope having an amino acid sequence comprising at least three (3) amino acids of amino acids 5-13 of hBNP or to an epitope having an amino acid sequence containing (meaning including and between) amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13of hBNP. An example of an antibody that specifically binds to epitopes having an amino acid sequence containing at least three (3) amino acids of amino acids 5-13 of hBNP is an antibody produced by CHO cell line AM1. The antibody can be labeled with any detectable label known to those skilled in the art, including those detectable labels discussed above in connection with the sandwich assay format.

The hBNP peptide, hBNP fragment or hBNP analogue can be bound to a solid support, such as the solid supports discussed above in connection with the sandwich assay format. Preferably, the hBNP peptide fragment has an amino acid sequence that contains amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP.

The immobilized hBNP peptide, hBNP peptide fragment or hBNP analogue thereof, test sample and at least one labeled antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species hBNP-antibody complexes are then generated. Specifically, one of the hBNP-antibody complexes generated is immobilized and contains a detectable label while the other hBNP-antibody complex is not immobilized and contains a detectable label. The non-immobilized hBNP-antibody complex and the remainder of the test sample are removed from the presence of the immobilized hBNP-antibody complex through techniques known in the art, such as washing. Once the non-immobilized hBNP antibody complex is removed, the amount of detectable label in the immobilized hBNP-antibody complex is then quantified. The concentration of hBNP or hBNP fragment in the test sample can then be determined by comparing the quantity of detectable label in the hBNP-complex to a standard curve. The standard curve can be generated using serial dilutions of hBNP or hBNP fragment of known

concentration, by mass spectroscopy, gravimetrically and by other techniques known in the art.

In a fluorescence polarization assay, in one embodiment, an antibody or functionally active fragment thereof is first contacted with an unlabeled test sample suspected of containing hBNP or a hBNP fragment thereof to form an unlabeled hBNP-antibody complex. The unlabeled hBNP-antibody complex is then contacted with a fluorescently labeled hBNP, hBNP fragment or hBNP analogue thereof. The labeled hBNP, hBNP fragment or hBNP analogue competes with any unlabeled hBNP or hBNP fragment in the test sample for binding to the antibody or functionally active fragment thereof. The amount of labeled hBNP-antibody complex formed is determined and the amount of hBNP in the test sample determined via use of a standard curve.

Preferably, the antibody used in a fluorescence polarization assay specifically binds to an epitope having an amino acid sequence comprising at least three (3) amino acids of amino acids 5-13 of hBNP or to an epitope having an amino acid sequence containing (meaning including and between) amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP. An example of an antibody that specifically binds to epitopes having an amino acid sequence containing at least three (3) amino acids of amino acids 5-13 of hBNP is a monoclonal antibody produced by CHO cell line AM1.

Preferably, the hBNP peptide fragment has an amino acid sequence that contains amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP. The antibody, labeled hBNP peptide, hBNP peptide fragment or hBNP analogue thereof and test sample and at least one labeled antibody are incubated under conditions similar to those described above in connection with the sandwich assay format.

Alternatively, in another embodiment, an antibody or functionally active fragment thereof is simultaneously contacted with a fluorescently labeled hBNP, hBNP fragment or hBNP analogue thereof and an unlabeled test sample suspected of containing hBNP or hBNP fragment thereof to form both labeled hBNP-antibody complexes and unlabeled hBNP-antibody complexes. The amount of labeled hBNP-antibody complex formed is determined and the amount of hBNP in the test sample determined via use of a standard curve. The antibody used in this immunoassay specifically binds to an epitope having an amino acid sequence comprising at least three (3) amino acids of amino acids 5-13 of hBNP or to an epitope having an amino acid sequence containing (meaning including and between) amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP. An example of an antibody that specifically binds to epitopes having an amino acid sequence containing at least three (3) amino acids of amino acids 5-13 of hBNP is a monoclonal antibody produced by CHO cell line AM1. Additionally, the hBNP peptide fragment has an amino acid sequence that contains amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP.

Alternatively, in yet another embodiment, an antibody (such as antibody of the present invention, such as an antibody produced by CHO cell line AM1) or functionally active fragment thereof is first contacted with a fluorescently labeled hBNP, hBNP fragment or hBNP analogue thereof to form a labeled hBNP-antibody complex. The labeled BNP-antibody complex is then contacted with an unlabeled test sample suspected of containing hBNP or a hBNP fragment thereof. Any unlabeled hBNP or hBNP fragment in the test sample competes with the labeled hBNP, hBNP fragment or hBNP analogue for binding to the antibody or functionally active fragment thereof. The amount of labeled hBNP-antibody complex formed is determined the amount of hBNP in the test sample determined via use of a standard curve. The antibody used in this immunoassay specifically binds to an epitope having an amino acid sequence comprising at least three (3) amino acids of amino acids 5-13 of hBNP or to an epitope having an amino acid sequence containing (meaning

including and between) amino acids 13-20, 13-19, 13-18, 13-17, 13-16, 14-20, 14-19, 14-18, 14-17, 14-16, 15-20, 15-19, 15-18, 16-20, 16-19, 17-24, 17-23, 17-22, 17-21, 17-20, 17-19, 18-24, 18-23, 18-22, 18-21, 18-20, 19-24, 19-23, 19-22 or 19-21 of hBNP. An example of an antibody that specifically binds to epitopes having an amino acid sequence containing at least three (3) amino acids of amino acids 5-13 of hBNP is a monoclonal antibody produced by CHO cell line AM1. Additionally, the hBNP peptide fragment has an amino acid sequence that contains amino acids 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 7-13, 7-13, 7-11, 7-10, 7-9, 8-13, 8-12, 8-11, 8-10, 9-13, 9-12, 9-11, 10-13, 10-12 or 11-13 of hBNP.

VII. Pharmaceutical Compositions and Pharmaceutical Administration

The antibodies of the present invention can be incorporated into pharmaceutical compositions suitable for administration to a subject. Typically, the pharmaceutical composition comprises a therapeutically or pharmaceutically effective amount of an antibody or the present invention invention along with a pharmaceutically acceptable carrier or excipient. As used herein, "pharmaceutically acceptable carrier" or "pharmaceutically acceptable excipient" includes any and all solvents, dispersion media, coating, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers or excipients include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable substances such as wetting or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody or antibody portion also may be included. Optionally, disintegrating agents can be included, such as cross-linked polyvinyl pyrrolidone, agar, alginic acid or a salt thereof, such as sodium alginate and the like. In addition to the excipients, the pharmaceutical composition can include one or more of the following, carrier proteins such as serum albumin, buffers, binding agents, sweeteners and other flavoring agents; coloring agents and polyethylene glycol.

The compositions of this invention may be in a variety of forms. They 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 passive immunization of humans with other antibodies. The preferred mode of administration is parenteral (e.g., intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody is administered by intravenous infusion or injection. In another preferred embodiment, the antibody or antibody fragment is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (i.e. antibody or antibody fragment) 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 of the present invention can be administered by a variety of methods known in the art, although for many therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by those skilled in the art, the route and/or mode of administration will 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, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. (See, e.g. *Sustained and Controlled Release Drug Delivery Systems,* J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978).

In certain embodiments, an antibody of the present invention may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, buccal tablets, troches, capsules, elixiers, suspensions, syrups, wafers, and the like. To administer an antibody or antibody fragment of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Supplementary active compounds also can be incorporated into the compositions. In certain embodiments, the antibody or antibody portion is co-formulated with and/or co-administered with one or more additional therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with monotherapies or alternatively, act synergistically or additively to enhance the therapeutic effect.

Dosage regimens may be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic 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 mammalian subjects to be tested; each unit containing 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 present invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic 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 or antibody portion of the invention is 0.1-20 mg/kg, more preferably 0.5-10 mg/kg. 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 should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Now by way of example, and not of limitation, examples of the present invention shall now be given.

EXAMPLE 1

Identification of immunoglobulin genes

Messenger RNA was isolated from subcloned anti-BNP 106.3 hybridoma cells (hybridoma cell line 106.3 (A.T.C.C. Accession No. HB-12044) is described in U.S. Patent No. 6,162,902). 106.3 mRNA was utilized in a reverse transcriptase–polymerase chain reaction using a mouse Ig primer set kit purchased from Novagen (Novagen (which is an Affiliate of Merck KGaA, Darmstadt, Germany), Cat No. 69831-3) with immunoglobulin gene specific primers contained in the kit. The resulting PCR products were sequenced and thus the immunoglobulin variable heavy and variable light chain genes were identified (See Figures 3A-3E and SEQ ID NO:1).

Cloning 106.3 variable region genes into pYD41 vector

A yeast display system was used to express unmutated anti-BNP proteins (described herein *infra*) and a library of anti-BNP proteins on the yeast surface as a fusion to the yeast protein AGA2. A yeast display vector called pYD (Invitrogen, Carlsbad, California), was used as it allows for cloning of the anti-BNP gene at the C-terminus of the AGA2 gene, a yeast mating factor (See, Boder and Wittrup, *Nature Biotechnology*, 15:553-557 (June 1997). Other critical features of the pYD vector include a galactose inducible promoter and an epitope tag, V5, on the C-terminus of the inserted anti-BNP gene (See, Figure 2 and Figure 6A-6B).

The yeast display platform utilizes an antibody format known as the single-chain variable fragment. In the scFv format, the variable heavy domain is connected to the variable light domain through a flexible linker (variable heavy domain - Linker GPAKELTPLKEAKVS (SEQ ID NO:4) - variable light domain).

PCR single overlap extension (SOE) was used to combine the variable heavy (VH) and the variable light genes (VL) for the 106.3 scFv construct (See, e.g., Figures 4, 6A-6B, and SEQ ID NO:2). The 106.3 scFv DNA was cloned into the yeast display vector pYD41 using vector restriction sites *Sfi*I and *Not*I. The pYD41-106.3scFv vector was transformed into DH5α *E. coli*. Plasmid DNA was then isolated from the *E. coli* and the 106.3 scFv insert was sequenced to ensure the scFv was cloned in frame with the AGA2 protein.

The cloning site for the scFv into the yeast display vector pYD41 is in an ORF that includes the following genes: AGA2-tether linker 41-X press epitope tag-106.3 variable heavy chain-Linker 40-106.3 variable light chain-V5 epitope tag – Six His tag. In addition, the yeast strain EBY100 is a tryptophan auxotroph and the pYD41 vector encodes for tryptophan as the system's selectable marker.

Transformation into *Saccharomyces cerevisiae* strain EBY100

Yeast display plasmid, pYD41-106.3 scFv, was transformed into *S. cerevisiae* EBY100 using Gietz and Schiestl Method (See, Schiestl and Gietz, *Current Genetics*, 16(5-6):339-46 (Dec. 1989)). Dilutions of the transformation reaction were plated on selective glucose plates (2% glucose (0.67% yeast nitrogen base, 0.105% HSM -trp – ura, 1.8% bacterial agar, 18.2% sorbitol, 0.86% NaH_2PO_4 H_2O, 1.02% Na_2HPO_4 $7H_2O$)) and incubated at 30°C for 48-72 hours. Selective glucose media was inoculated with individual colonies and grown shaking at 30°C for 16-20 hours. Protein expression was induced in colonies by transferring 0.5 OD600 of cells/ml (1e7cells/0.5OD/ml) to selective galactose media. Colonies were shaken at 20°C for 16-24 hours and then analyzed by the FACS Aria flow cytometer for binding to cyclic BNP (referred to as "1-32c") (SEQ ID NO:5) and anti-V5. For flow cytometry assays, yeast cells expressing 106.3 scFv were incubated with biotinylated: cyclic BNP (1-32c) (SEQ ID NO:5) or anti-V5 antibody followed by streptavidin: phycoerythrin (SA:PE, BD Pharmingen) or goat anti-mouse immunoglobulin-Alexa Fluora 633 (GAM:633, Molecular Probes (which is an Affiliate of Invitrogen, Carlsbad, California)). The flow cytometry histograms as shown in Figures 7A-7B illustrate full-length surface expression of 106.3 scFv (anti-V5 binding) and binding of 106.3 scFv to cyclic BNP (1-32c) (SEQ ID NO:5).

Off-rate Analysis for 106.3 scFv and 106.3 variants on yeast.

Off-rate measurements of 106.3scFv and 106.3 variants on yeast were measured by incubating 0.05OD yeast ($1x10^6$ cells) with 100-fold molar excess of biotinylated-cyclic BNP 1-32c (~0.3μM) (SEQ ID NO:5) and anti-V5 antibody (2.5 ug/ml) for 30-60 minutes at room temperature. Cells were then washed twice with blocking buffer containing phosphate buffered saline with 1% bovine serum albumin

(PBS/BSA) and incubated at room temperature with 100-fold molar excess unlabelled cyclic BNP 1-32c (SEQ ID NO:5) for varying amounts of time (0, 0.25 hr, 0.5 hr, 1 hr, 2 hr, 4.25 hr, 25.5 hr, 50 hr 75 hr and 144 hr (See Figure 8). At each individual time point, yeast cells were transferred to ice to halt the reaction. Cells were then washed twice with PBS/BSA and suspended in secondary staining reagents, specifically, SA:PE and GAM:633. Cells were incubated on ice for 30 minutes, washed twice and then analyzed on the FACS Aria flow cytometer. Figure 8 shows the off-rate data plotted as mean fluorescence units ("MFU") versus time (in seconds). A first order decay equation was used to fit the data. The off-rate, m2 in the equation shown in Figure 8, was fitted to 8.4^{-5} sec^{-1} with and R value of 0.9993. The 106.3 scFv half-life ($t_{1/2}$) was 137min ($t_{1/2}$= ln2/k_{off}).

An off-rate sorting strategy was used to identify off-rate improved 106.3 variants from mutagenic libraries. Therefore, the 106.3 scFv, unmutated or wildtype ("wt"), half-life was used to determine the appropriate time to sort the mutagenic libraries. 106.3 mutagenic libraries were sorted approximately 3hours after the addition of unlabelled cyclic BNP (1-32c) (SEQ ID NO:5) with the same assay conditions described for wt 106.3 scFv.

Generation of 106.3 CDR directed libraries

Mutagenesis was directed to the three heavy and three light chain complementary determining regions (CDR) of antibody 106.3 (See, e.g., Figures 3-6 and SEQ ID NOS:6-11) since these loops are the major antigen contact sites. CDR loop lengths and numbering were defined using Kabat nomenclature. Individual libraries were composed that randomly mutated three amino acid positions of the CDR in a single library with the mutagenic window shifted by one amino acid per library (See, Figure 9). The library diversity for an individual library totaled 20^3 or 8,000 possible variants with every amino acid sampled at every CDR position. For 106.3scFv, a total of 54 libraries were generated 29 variable heavy and 25 variable light libraries.

Libraries were generated by combining linearized gapped pYD41-106.3 vector and single stranded oligonucleotides with chemically competent EBY100 yeast (See, Figure 10). The gapped pYD41 vector is a vector created by PCR that lacks a specific region of each CDR that is replaced in library construction by the single stranded degenerate oligonucleotide. Degenerate single-stranded oligonucleotides are 90-105 nucleotides long with 39-43 nucleotides of homology to the pYD41-106.3 scFv vector on each side of the nine degenerate nucleotide window. The oligonucleotides for each library, 54 total, were synthesized (See Figures 14A-H and SEQ ID NOS:25-78). Gapped vector (1ug) and the degenerate oligonucleotide (16ug) were combined with EBY100 yeast (3e8 cells) and transformed using the Gietz and Schiestl library transformation protocol (Schiestl and Gietz, *Current Genetics,* 16(5-6):339-46 (Dec 1989)). The degenerate oligonucleotide and the pYD41-106.3scFv gapped vector cyclize during transformation due to homologous recombination facilitated by the nucleotide overlap and the mechanism of yeast endogenous gap repair. Libraries were grown at 30°C for 48-72 hours in selective glucose media and passed again in selective glucose media prior to induction of protein expression for library sorting.

106.3 Mutagenic CDR libraries

106.3 libraries were sorted based on an off-rate sorting strategy. 106.3 CDR mutagenic libraries were induced in galactose expression media at 20°C for 18-24 hours. At room temperature, 106.3 mutagenic libraries were washed with PBS/BSA, incubated with biotinylated cyclic BNP (1-32c) (SEQ ID NO:5) and anti-V5 antibody, washed twice and incubated with unlabelled cyclic BNP (1-32c) (SEQ ID NO:5). After three hours, mutagenic libraries were washed twice and incubated on ice with SA-PE (1:200 dilution) and GAM-633 (1:200 dilution) for 30 minutes. Finally, cells were washed, analyzed and sorted on the FACS Aria. Sort gates were set based on unmutated 106.3 binding at 3 hours with a gate set to sort full-length BNP binding clones. Each sort collected the top 0.1-0.5% of the BNP binding population. Sorted cells were grown in selective glucose media and grown 18-24 hours at 30°C. Sort 1 cells were induced and sorting was repeated for one or two additional rounds.

After the last sort, sorted cells were plated onto selective glucose plates and placed at 30°C for 72 hours. Three libraries showed improvements relative to wt 106.3 scFv: heavy chain library H2 8, light chain library L1 (1-5 pool), and L2 (1-5 pool). Individual yeast colonies from these libraries were inoculated in selective glucose media, cryopreserved and induced in selective galactose media. Individual colonies were then characterized and ranked in an off-rate assay.

Analysis of Selected 106.3 Variants

Selected clones were initially characterized in the off-rate assay described above for wt 106.3 scFv. Figure 11 shows the off-rate values determined from a first order decay curve for each improved 106.3 scFv variant evaluated. Overall, clones exhibited improvements in off-rate better than 2-fold that of the 106.3 scFv wt clone. The clone with the desired slowest off-rate was 106.3 L1 B24 scFv with an off rate of $6.7x10^{-6}sec^{-1}$.

Selected 106.3 scFv variants were sequenced to determine the amino acid mutations being expressed. Initially, plasmid DNA was isolated from yeast suspension cultures using a yeast mini-prep kit (Cat No. D2001, Zymo Research Orange, CA). In order to obtain sequencing grade plasmid DNA, plasmid from the yeast mini-prep kit was transformed into DH5α *E.coli*, and then purified from culture using *E. coli* mini-prep kits (Qiagen). Pure plasmid DNA was then sequenced using pYD41 vector specific primers (pYD41 for –TAGCATGACTGGTGGACAGC (SEQ ID NO:79) and pYD41rev-CGTAGAATCGAGACCGAG (SEQ ID NO:80)). Nucleotide and amino acid sequence data for 106.3 scFv variants is shown in Figures 12A-C. Position numbers refers to amino acid position in the respective CDR (H2 Pos 8 is 8th amino acid of CDR H2).

The sequence data for CDR L1 indicated a strong preference at position 4 for tryptophan or other bulky hydrophobic amino acids such as tyrosine or phenylalanine. A bulky amino acid residue at position 4 may be crucial for the substantial improvements in off-rate for the 106.3 scFv. The cyclic BNP (1-32c) peptide (SEQ

ID NO:5) may become trapped by this bulky amino acid and thus slowing the off-rate. The L2 mutations both contain a cysteine at position 4.

Cloning and Soluble Expression of 106.3 Chimeric Antibodies in a Transient or Stable Expression System

Selected 106.3 variants were converted to chimeric mouse-human IgG_1/human kappa antibodies through cloning of the 106.3 variable domains into the transient expression vector system called pBOS (Abbott Bioresearch Center, Worcester, MA) More specifically, PCR was used to amplify the variable heavy and variable light chain genes with restriction sites for cloning into separate pBOS vectors (Mizushima and Nagata, *Nucleic Acids Research*, 18:5322, (1990)). The variable heavy and variable light genes were ligated in digested/dephosphorylated vector and transformed into DH5α *E. coli*. Plasmid DNA was purified from *E. coli* and transfected into COS-7 cells and 293H cells using lipofectamine (Invitrogen, Carlsbad, California) or electroporation. Transient antibody was expressed for the following 106.3 variants: wt chimeric, L1 B24 chimeric, L1 16 chimeric, L1 B24/H2 288 chimeric, and L1 16/H2 288 chimeric.

Using the pBOS-106.3 heavy and light vectors, a stable CHO cell line plasmid was created in a two step cloning procedure. First, variable heavy chain and variable light genes were ligated in frame to the human constant genes in pBV and pJV plasmids (Abbott Bioresearch Center, Worcester, MA), respectively, using the restriction enzymes SrfI/NotI. Ligation reactions were transformed into DH5α *E. coli* and plasmid DNA was subsequently isolated from individual colonies. The pBV-106.3 mouse variable heavy-human IgG1 and pJV-106.3 mouse variable light-human kappa were sequenced at the cloning sites.

The second cloning step involved combining the heavy chain IgG_1 genes and the light chain kappa genes into a single stable cell line vector. The pBV-106.3 and pJV-106.3 vectors were digested with AscI/PacI. The VL-human kappa constant and the VH-human IgG1 constant DNA fragments were gel purified and ligated to produce the stable cell line vector called pBJ-106.3. The pBJ-106.3 heavy/light

chimeric plasmid was transformed into CHO cells using calcium phosphate protocol. Stable cell lines were subcloned from initial transformation. A stable CHO cell line has been developed for the clone AM1 (also referred to as "BNP106.3sc128am1CHO1162-236" and "106.3 L1 B24/H2 288 chimeric") and deposited with the A.T.C.C. as described in Example 2 herein.

BIAcore Characterization of Engineered Chimeric 106.3 variants

A high density Goat Anti-human Fc (GAHFc) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (an antispecies antibody) surface plasma resonance (SPR) biosensor was prepared by immobilizing GAHFc to a preconditioned BIAcore CM5 chip (Uppsala, Sweden) by amine coupling (amino coupling is well known in the art, for example, see Nordin, H et al., *Analytical Biochemistry,* 340:359-368 (2005)). The carboxymethyl-dextran biosensor is activated with an 8 minute injection of a 1:1 mixture of 0.4 M EDC and 0.1 NHS at 20 μL/minute. GAHFc in 10 mM sodium acetate (pH 5.0) is coupled to the activated surface with a 10 minute injection. The surface is then deactivated with 1 M ethanolamine pH 8.5 for 8 minutes followed by another 10 minute injection of GAHFc. This is followed with a biosensor conditioning of ten 20 second injection of 100 mM H_3PO_4 at a flow rate of 100 μL/min. ~10.5 kRU, resonance units, of GAHFc is coupled to the biosensor in each flow cell.

Purified anti-BNP chimeric antibodies ("cAb"): (1) stable 106.3 AM1 from CHO cells (described above and in Example 2), and (2) transient anti-BNP WT/WT from COS cells are diluted into SPR Running Buffer (BIAcore, Uppsala, Sweden) (degassed/vacuum-filtered HBS-EP (BIAcore, Sweden)) supplemented with 12 mg/mL BSA and 12 mg/mL carboxymethyl dextran sodium salt) to a concentration of 10 μg/mL of purified antibody. A frozen (–80°C) aliquot of BNP in dH_2O at 100 μM is diluted into SPR Running Buffer to a concentration of 100 pM.

At 25°C, 30 μL of each anti-BNP cAbs are injected at 10 μL/min onto individual SPR flow cells with one flow cell left blank as a reference control. After loading each cAb onto the biosensor, all flow cells are allowed to equilibrate for 45

minutes with SPR running buffer at a flow rate of 100 μL/min before the running buffer bottle is substituted (in between syringe fills) for a sample solution of 100 pM BNP for ~16 hours. The sample solution is then switched back to SPR running buffer for another ~7 hours. The surface is then regenerated with three 33 second pulses of 100 mM phosphoric acid at a flow rate of 100 μL/min. A blank run is performed by running SPR running buffer over an anti-BNP cAb loaded sensor for ~23 hours.

The data was double-referenced corrected (the 100 pM BNP sample data was corrected by subtracting the reference data and then subtracting blank buffer data) and fitted to a 1:1 Langmuir Binding model (See, *BIA Evaluation 3 Software Handbook*, edition November 1999 (version AD) Copyright 1997-1999, Biacore AB) with considerations for mass transport and linear drift with BIAevaluation software (version 3.2).

Using BIAcore SPR, the equilibrium dissociation constant (K_D) of the wild-type 106.3 cAb was determined to be 1.9×10^{-11}M with an on-rate of 7.8×10^{6} $M^{-1}sec^{-1}$ and an off-rate of $1.5 \times 10^{-4}sec^{-1}$. The equilibrium dissociation constant (K_D) of the 106.3 AM1 cAb was determined to be 1.9×10^{-12}M with an on-rate of 1.3×10^{7} $M^{-1}sec^{-1}$ and an off-rate of $2.4 \times 10^{-5}sec^{-1}$. Similar K_D values were obtained for both 106.3 and 106.3 AM1, 1.7×10^{-12} M and 9.3×10^{-12} M respectively, using Sapidyne's KinExA instrument that determines K_D values in a solution phase measurement (Sapidyne, Boise, ID).

Specificity of Engineered Chimeric 106.3 variants

Anti-BNP 106.3 AM1 BNP truncated BNP peptide displacment EIA

The 106.3 AM1 mAb's ability to bind to truncated forms of hBNP, namely hBNP 1-26 and hBNP 5-13, was determined in a displacement microtiter CIA (See, Figure 18). Blocked anti-species coated plates were incubated with mAb for 1 hour and washed. Serially diluted free, unconjugated hBNP 1-26 (Abbott, Abbott Park, IL), hBNP 5-13 (AnaSpec, San Jose, CA.), hBNP 1-32 (Peptide Institute, Osaka, Japan) peptides or a 0 peptide control were allowed to react with the AM1 mAb for one hour. The plates were washed and an acridinylated hBNP (1-32 cyclic) conjugate

(Abbott ADD, Abbott Park, IL) was added. The plates were once again incubated and washed. The Relative Luminescence Units (RLUs) were obtained from the chemiluminescence signal generated as the serially-layered pre-trigger/trigger combination (Abbott, Abbott Park, IL) on the Microbeta Jet (Perkin-Elmer, Turku, Finland). Anti-BNP 106.3 AM1 mAb was found to be reactive to the free hBNP fragments amino acids 1-26 and amino acids 5-13 as demonstrated by >85% signal displacement in the microtiter assay.

Fine Epitope Mapping of Engineered Chimeric 106.3 variants

Anti-BNP 106.sc128 L1 B24 H2 288 AM1 Alanine Peptide mapping EIA

The binding site of the 106.3 AM1 mAb was identified using an alanine mutagenesis screening procedure with a cyclic hBNP 1-32 alanine substituted peptide panel. Single amino acids of the hBNP peptide were replaced with an alanine amino acid (except at positions 10 and 26). The 106.3 AM1 mAb was evaluated for its ability to bind the unlabelled alanine substituted peptides versus labeled hBNP 1-32 peptide. The mAb at a constant concentration is incubated on the solid phase coated with an anti-species antibody, then the unbound sample is washed away. The bound antibody is allowed to react with the 2900nM unlabeled peptides. Following incubation, a wash is used to eliminate any unbound free peptide. Next, the biotinylated hBNP 1-32 cyclic peptide (Abbott GPRD, Abbott Park, IL) at 2.9nM is allowed to react with any unbound sites on the anti-BNP 106.3 AM1 mAb. Unbound peptide is washed away prior to the addition of strepavidin-HRPO (Invitrogen, Carlsbad, CA). The OPD substrate system (Abbott, Abbott Park, IL) was used for color development and signals read on a Titertek MAP EIA workstation (Titertek Instruments, Huntsville, Alabama).

This signal displacement EIA assay was used as a tool to determine the fine epitope mapping profile of the 106.3 AM1 mAb. The free peptide concentration was 2-log over that of the labeled peptide to ensure that inhibition occurs. The bar graph in Figure 19 shows the bound over unbound (B/B0) ratio of the AM1 antibody binding signal of free peptide versus labeled peptide. If an amino acid residue is critical for AM1 mAb binding to hBNP, partial to no displacement of signal is

detected. In this example, if a B/Bo ratio of >0.4 is obtained, the specific amino acid is considered critical for mAb binding. The 106.3AM1 mAb functional epitope is identified as V5, Q6, G7, G9, F11, and R13 in bold in the sequence below.

NH2-SPKM**VQG**S**G**C**F**G**R**KMDRISSSSGLGCKVLRRH-COOH (SEQ ID NO:5)

Anti-BNP 106.sc128 L1 B24 H2 288 AM1Alanine Peptide mapping with BIAcore

A high density Goat Anti-human Fc (GAHFc) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (an antispecies antibody) surface plasma resonance (SPR) biosensor was prepared by immobilizing GAHFc to a preconditioned BIAcore CM5 chip (Uppsala, Sweden) by amine coupling as described above.

At 25°C, 60ul of the anti-BNP AM1 cAb are injected at 10 μL/min onto individual SPR flow cells with one flow cell left blank as a reference control. After loading each cAb onto the biosensor, all flow cells are allowed to equilibrate for 10 minutes with SPR running buffer at a flow rate of 100 μL/min. 200 ul of BNP peptide or BNP single alanine substituted peptides (alanine substituted at each position except 10 and 26) at 10 nM was flowed over the AM1 surface at 100uL/min. Dissociation was allowed to take place and monitored for 1800 seconds. The surface is then regenerated as previously described herein.

The data was double-referenced corrected (the sample data was corrected by subtracting the reference data and then subtracting blank buffer data). Off-rates were determined from the dissociation phase of sensograms. Results indicate that amino acids V5, Q6, G7, G9, F11, and R13 are important for stability of the anti-BNP AM1/WT BNP complex. When these residues are individually mutated into alanine, the off-rate increases by at least one order of magnitude. This suggests that the anti-BNP AM1 cAb binding epitope for BNP contains the following BNP residues V5, Q6, G7, G9, F11, and R13A, in accordance with the EIA findings noted above (See Figure 20).

EXAMPLE 2: ATCC Deposit Information

Chinese Hamster Ovary cell line for BNP106.3sc128am1CHO1162-236 was deposited with the American Type Culture Collection (hereinafter referred to as "A.T.C.C."), 10801 University Blvd., Manassas, VA 20110-2209, on September 20, 2005 and assigned A.T.C.C. Accession No. PTA-6987.

EXAMPLE 3: Competitive Immunoassay Using a Single Antibody Format

The antibody produced by CHO cell line AM1 ("antibody AM1") described above in Examples 1 and 2 was purified and tested to determine the antibody's ability to bind human cyclic BNP1-32 in a single antibody format on the ARCHITECT® instrument (Abbott Laboratories, Abbott Park, IL. This instrument is described in U.S. Patent No. 5,468,646). This single antibody format encompasses the use of only one analyte specific antibody in the testing reaction.

Paramagnetic microparticles (hereinafter "microparticles", Polymer Labs, Amherst, MA) were washed and then reacted with serially diluted Goat anti-human antibody (Jackson ImmunoResearch, West Grove, PA). The Goat anti-human antibody was coated onto the paramagnetic microparticles using the techniques described in U.S. Patent No. 6,162,902. Specifically, EDAC coupling was used (EDAC is generally used as a carboxyl activating agent for amide bonding with primary amines. In addition, it reacts with phosphate groups. It is used in peptide synthesis, crosslinking proteins to nucleic acids and in preparing immunoconjugates. The chemical formula for EDAC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride. EDAC is commercially available from Sigma-Aldrich, St. Louis, MO.). After incubating, the microparticles were washed and overcoated with BSA. These Goat anti-human coated microparticles were then reacted with serially diluted antibody AM1, incubated and washed.

These coated microparticles were then tested on the ARCHITECT® instrument (Abbott Laboratories, Abbott Park, Illinois) for reactivity to human cyclic BNP 1-32. An aliquot containing human cyclic BNP 1-32 was delivered to the same

well of the reaction vessel as the microparticles to form a reaction mixture. The reaction mixture was incubated for approximately 18 minutes. After incubation, the microparticles were washed with the ARCHITECT® Line Diluent to remove any of the human cyclic BNP 1-32 that was not captured. The ARCHITECT® Line Diluent is commercially available from Abbott Laboratories, Abbott Park, Illinois. Next, human cyclic BNP 1-32 linked to acridinium (hereinafter "tracer") was dispensed into the reaction vessel and allowed to react with the microparticles for about 4 minutes, after which the microparticles were washed with the ARCHITECT® Line Diluent to remove the unbound materials. The tracer was diluted to about 5-25 ng/mL. A solution of hydrogen peroxide and then sodium hydroxide was added to the reaction vessel and the chemiluminescent signal was measured by the chemiluminescent mircoparticle immunoassay (CMIA) optical assembly of the ARCHITECT® instrument. As shown in Figure 15, in this assay format, the antibody AM1 showed reactivity to the unlabelled human cyclic BNP 1-32 in a concentration dependent manner.

EXAMPLE 4: Sandwich Assays Using Antibodies Produced by CHO Cell Line AM1

For the modified ARCHITECT®-hBNP assay (hereinafter referred to as "Arc-BNP") paramagnetic particles were coated with monoclonal antibody ("mAb") 3-631-436. This mAb binds to an amino acid sequence containing amino acids 13-18 on the hBNP peptide. (Monoclonal antibodies produced by hybridoma cell line 3-631-436 are described in U.S. Patent Application No. 11/135,050, filed on May 25, 2005, the contents of which are herein incorporated by reference. Monoclonal antibodies produced by hybridoma cell line 3-631-436 are also referred interchangeably herein as "monoclonal antibody 3-631-436" and "Fusion 3". Additionally, murine hybridoma cell line 3-631-436 was deposited with the A.T.C.C. on December 21, 2004 and assigned A.T.C.C. Accession No. PTA-6476). Monoclonal antibody 3-631-436 was coated onto a paramagnetic particle (Polymer Laboratories, Amherst, MA) using the techniques described in U.S. Patent No. 6,162,902. Specifically, EDAC coupling was used (EDAC is generally used as a carboxyl activating agent for amide bonding with primary amines. In addition, it reacts with phosphate groups. It is used

in peptide synthesis, crosslinking proteins to nucleic acids and in preparing immunoconjugates. The chemical formula for EDAC is 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride. EDAC is commercially available from Sigma-Aldrich, St. Louis, MO.). Particles were washed and overcoated with BSA. These particles were used to capture BNP peptide in the assay during the first (1^{st}) incubation with specimens.

Alternatively, monoclonal antibody 3-631-436 was biotinylated using NHS-PEO_4-biotin (Pierce Biotechnology, Inc., Rockford, IL) and captured on streptavidin-coated superparamagnetic Dynabeads (Dynal Biotech LLC, Brown Deer, WI). These particles were also used to capture BNP peptide in the assay during the first (1^{st}) incubation with specimens.

Antibody AM1 (See Examples 1 and 2) was conjugated to acridinium (Abbott Laboratories, Abbott Park, IL) and is used in the assay during the second (2^{nd}) incubation to detect the particle-bound hBNP peptide. The conjugation occurred by reaction of antibody AM1 with an activated acridinium-carboxamide ester.

In a complimentary modified Arc-BNP assay to that described above, capture particles were prepared by coating antibody AM1onto paramagnetic particles (Polymer Laboratories, Amherst, MA) utilizing EDAC chemistry or by biotinylation of antibody AM1and capture on streptavidin coated superparamagnetic Dynabeads (Invitrogen, Carlsbad, California). The procedures were identical to those described above for preparation of monoclonal antibody 3-631-436 particles. These particles were also used to capture hBNP peptide in the assay during the first (1^{st}) incubation with specimens. Monoclonal antibody 3-631-436 was conjugated to acridinium the same way antibody AM1was conjugated to acridinium and is used in the assay during the 2nd incubation to detect the particle-bound hBNP peptide.

BNP immunoassays were performed on an ARCHITECT® instrument (this instrument is described in U.S. Patent No. 5,468,646).

An aliquot containing a calibrator solution was delivered to the same well of the reaction vessel as the microparticles to form a reaction mixture. The calibrator solution contained hBNP full-length peptide. The microparticles coated with the capture antibody in a Tris/BSA diluent were pipetted by the sampling probe into the appropriate wells of the reaction vessel in the sampling center. The reaction mixture was incubated for approximately 4 minutes (18 min for streptavidin based particles) at a temperature of about 37°C. After the incubation, the reaction mixture was washed with the ARCHITECT® Line Diluent to remove any of the calibrator that was not captured. The ARCHITECT® Line Diluent is commercially available from Abbott Laboratories, Abbott Park, Illinois.

The mAb-Acridinium-conjugates at about 50-100 ng/mL were dispensed into the reaction vessel and incubated for approximately 4 minutes at a temperature of about 37°C. After the incubation, the reaction vessel was washed with the ARCHITECT® Line Diluent to remove the unbound materials.

A solution of hydrogen peroxide and then sodium hydroxide was added to the reaction vessel and the chemiluminescent signal was measured by the chemiluminescent mircoparticle immunoassay (CMIA) optical assembly of the ARCHITECT® instrument.

The ARCHITECT ® system measures the acridinium signals which are typically measured in relative light units (hereinafter "rlu's"). Measurements were made in triplicate. The results shown in Table 1 below and in Figures 16 and 17 show the mean of the triplicate values. Specifically, the results in Table B and Figures 16 and 17 are shown in pg/mL BNP calibrator.

TABLE 1

uP mAb clone		106.3AM1	3-631-436		106.3AM1	3-631-436
Conj mAb clone		3-631-436	106.3AM1		3-631-436	106.3AM1
BNP (pg/mL)	**Sample**					
0	Cal A	1132	1275		706	528
30	Cal B	1784	2386		671	1291
300	Cal C	19819	35445		3907	12618
1000	Cal D	142648	250363		28612	76400
2000	Cal E	446152	600661		93326	216220
5000	Cal F	1502213	1780437		451856	893368
	Ratio					
	A/A	1.0	1.0		1.0	1.0
	B/A	1.6	1.9		1.0	2.4
	C/A	17.5	27.8		5.5	23.9
	D/A	126.0	196.4		40.5	144.6
	E/A	394.0	471.1		132.2	409.2

In addition, the immunoassays can be used to monitor patients receiving therapeutic doses of hBNP or fragments of hBNP and anti-hBNP treatments.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. An isolated antibody which immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of human brain natriuretic peptide ("hBNP") with at least about a two fold improvement in its equilibrium dissociation constant (K_D) when compared with an antibody produced by hybridoma cell line 106.3, said cell line having A.T.C.C. Accession No. HB-12044.

2. The antibody of claim 1, wherein said isolated antibody exhibits at least about a five fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3.

3. The antibody of claims 1 or 2, wherein said isolated antibody exhibits at least about a ten fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3.

4. The antibody of any of claims 1-3, wherein said isolated antibody exhibits at least about a fifteen fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3.

5. The antibody of any of claims 1-4, wherein said isolated antibody exhibits at least about a twenty fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3.

6. The antibody of any of claims 1-5, wherein said isolated antibody exhibits at least about a twenty-five fold improvement in its K_D when compared with an antibody produced by hybridoma cell line 106.3.

7. The antibody of any of claims 1-6, wherein said isolated antibody is a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single

domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

8. An isolated antibody which immunospecifically binds to hBNP, wherein said antibody has an association rate (k_a) of between about 5.0×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$.

9. The antibody of claim 8, wherein said antibody has an association rate of between about 3.3×10^4 and about 1.0×10^9 $M^{-1}s^{-1}$.

10. The antibody of claims 8 or 9, wherein said antibody has an association rate of between about 2.5×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$.

11. The antibody of any of claims 8-10, wherein said antibody has an association rate of between about 2.4×10^4 and about 1.35×10^7 $M^{-1}s^{-1}$.

12. The antibody of any of claims 8-11, wherein said antibody is a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

13. The antibody of any of claims 8-12, wherein said antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

14. An isolated antibody which immunospecifically binds to hBNP, wherein said antibody has a dissociation rate (k_d) of between about 1.0×10^{-3} and about $1.0 \times 10^{-6} \cdot s^{-1}$.

15. The antibody of claim 14, wherein said antibody has a dissociation rate of between about 1.0×10^{-3} and about $1.0 \times 10^{-5} \cdot s^{-1}$.

16. The antibody of claims 14 or 15, wherein said antibody has a dissociation rate of between about 1.0×10^{-3} and about $1.0 \times 10^{-4} \cdot s^{-1}$.

17. The antibody of any of claims 14-16, wherein said antibody is a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

18. The antibody of any of claims 14-17, wherein said antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

19. An isolated antibody which immunospecifically binds to hBNP wherein said antibody has an equilibrium dissociation constant (K_D) of between about 2×10^{-11} M and about 1×10^{-15} M.

20. The antibody of claim 19, wherein said antibody has an equilibrium dissociation constant of between about 3.0×10^{-11} M and about 1.0×10^{-14} M.

21. The antibody of claims 19 or 20, wherein said antibody has an equilibrium dissociation constant of between about 4.0×10^{-11} M and about 8.0×10^{-13} M.

22. The antibody of any of claims 19-21, wherein said antibody has an equilibrium dissociation constant of between about 4.2×10^{-11} M and about 7.4×10^{-13} M.

23. The antibody of any of claims 19-22, wherein said antibody is a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

24. The antibody of any of claims 19-23, wherein said antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

25. Chinese hamster ovary ("CHO") cell line AM1 having A.T.C.C. Accession No. PTA-6987.

26. An antibody made from DNA extracted from the CHO cell line AM1 having A.T.C.C. Accession No. PTA-6987.

27. A chimeric antibody or a hBNP-epitope binding fragment thereof produced by CHO cell line AM1, wherein said cell line has A.T.C.C. Accession No. PTA-6987.

28. An isolated antibody which immunospecifically binds to hBNP, wherein said antibody has a variable heavy domain and a variable light domain, the variable heavy domain comprising a heavy chain complementarity determining region ("CDR") 1, a heavy chain CDR 2 and a heavy chain CDR 3, the variable light domain comprising a light chain CDR 1, a light chain CDR 2 and a light chain CDR 3, wherein

(a) Heavy Chain CDR 1 has an amino acid sequence of: Gly-Tyr-Thr-Phe-Thr-His-Tyr-Gly-Ile-Asn (SEQ ID NO:6);

(b) Heavy Chain CDR 2 has an amino acid sequence having a formula of:

Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Xaa_1-Xaa_2-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:12)

wherein Xaa_1 is selected from the group consisting of proline and alanine;

wherein Xaa_2 is selected from the group consisting of isoleucine and tyrosine;

(c) Heavy Chain CDR 3 has an amino acid sequence of: Ser-His-Arg-Phe-Gly-Leu-Asp-Tyr (SEQ ID NO:8);

(d) Light Chain CDR 1 has an amino acid sequence having a formula of:

Lys-Ala-Xaa_3-Xaa_4-Xaa_5-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:13)

wherein Xaa_3 is selected from the group consisting of: serine, alanine, asparagine, glutamine, tyrosine, threonine and arginine;

wherein Xaa_4 is selected from the group consisting of: glutamine, tyrosine, tryptophan, alanine and phenylalanine;

wherein Xaa_5 is selected from the group consisting of: serine, glycine, proline, alanine and aspartic acid;

(e) Light Chain CDR 2 has an amino acid sequence having the formula of:
Ala-Ala-Ser-Xaa_6-Xaa_7-Xaa_8-Ser (SEQ ID NO:14)

wherein Xaa_6 is selected from the group consisting of: asparagine and cysteine;

wherein Xaa_7 is selected from the group consisting of: leucine, glycine and alanine;

wherein Xaa_8 is selected from the group consisting of glutamic acid, tryptophan and proline; and

(f) Light Chain CDR 3 has an amino acid sequence of: Gln-Gln-Ser-Asn-Glu-Asp-Pro-Phe-Thr (SEQ ID NO:11),

wherein the heavy chain CDR 2 has an amino acid sequence other than Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) when the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) and the light chain CDR 2 has the amino acid sequence of Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10), the light chain CDR 1 has an amino acid sequence other than Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9) when the heavy chain CDR 2 has the amino acid sequence Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 2 has the amino acid sequence Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:19), or the light chain CDR 2 has an amino acid sequence other than Ala-Ala-Ser-Asn-Leu-Glu-Ser (SEQ ID NO:10) when the heavy chain CDR 2 has the amino acid sequence of Trp-Ile-Asn-Thr-His-Thr-Gly-Glu-Pro-Ile-Tyr-Ala-Asp-Asp-Phe-Lys-Gly (SEQ ID NO:7) and the light chain CDR 1 has the amino acid sequence of Lys-Ala-Ser-Gln-Ser-Val-Asp-Tyr-Asn-Gly-Asp-Ser-Tyr-Leu-Asn (SEQ ID NO:9).

29. The antibody of claim 28, wherein:

Xaa_1 is alanine.

Xaa_2 is tyrosine.

Xaa_3 is serine.

Xaa_4 is glutamine.

Xaa_5 is serine.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

30. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is glutamine.

Xaa_4 is phenylalanine.

Xaa_5 is alanine.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

31. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is tyrosine.

Xaa_4 is alanine.

Xaa_5 is serine.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

32. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is glutamine.

Xaa_4 is tryptophan.

Xaa_5 is glycine.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

33. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is threonine.

Xaa_4 is tryptophan.

Xaa_5 is aspartic acid.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

34. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is arginine.

Xaa_4 is tryptophan.

Xaa_5 is proline.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

35. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is alanine.

Xaa_4 is tyrosine.

Xaa_5 is glycine.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

36. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is asparagine.

Xaa_4 is tryptophan.

Xaa_5 is proline.

Xaa_6 is asparagine.

Xaa_7 is leucine.

Xaa_8 is glutamic acid.

37. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is serine.

Xaa_4 is glutamine.

Xaa_5 is serine.

Xaa_6 is cysteine.

Xaa_7 is glycine.

Xaa_8 is tryptophan.

38. The antibody of claim 28, wherein:

Xaa_1 is proline.

Xaa_2 is isoleucine.

Xaa_3 is serine.

Xaa_4 is glutamine.

Xaa_5 is serine.

Xaa_6 is cysteine.

Xaa_7 is alanine.

Xaa_8 is proline.

39. The antibody of any of claims 26-38, wherein said antibody has an equilibrium dissociation constant of between about 2.0×10^{-11} M and about 1.0×10^{-15} M.

40. The antibody of any of claims 26-39, wherein said antibody has an equilibrium dissociation constant of between about 3.0×10^{-11} M and about 1.0×10^{-14} M.

41. The antibody of any of claims 26-40, wherein said antibody has an equilibrium dissociation constant of between about 4.0×10^{-11} M and about 8.0×10^{-13} M.

42. The antibody of any of claims 26-41, wherein said antibody has an equilibrium dissociation constant of between about 4.2×10^{-11} M and about 7.4×10^{-13} M.

43. The antibody of any of claims 26-42, wherein said antibody further comprises an association rate (k_a) of between about 5.0×10^4 and about 1.0×10^8 $M^{-1}s^{-1}$.

44. The antibody of any of claims 26-43, wherein said antibody further comprises a dissociation rate (k_d) of between about 1.0×10^{-3} and about 1.0×10^{-6} s^{-1}.

45. The antibody of any of claims 26-44, wherein said antibody is a monoclonal antibody, a multispecific antibody, a human antibody, a fully humanized antibody, a partially humanized antibody, an animal antibody, a recombinant antibody, a chimeric antibody, a single-chain Fv, a single chain antibody, a single domain antibody, a Fab fragment, a $F(ab')_2$ fragment, a disulfide-linked Fv, an anti-idiotypic antibody, or a functionally active epitope-binding fragment thereof.

46. The antibody of any of claims 26-45, wherein said antibody immunospecifically binds to an epitope comprising amino acid residues 5 through 13 of hBNP.

47. An immunoassay for hBNP or hBNP fragment, wherein said immunoassay comprises an antibody of any of claims 1-24 and 25-46.

48. The immunoassay of claim 47, wherein said immunoassay comprises a single antibody that immunospecifically binds to hBNP or hBNP fragment.

49. The immunoassay of claims 47 or 48, wherein said immunoassay further comprising an additional specific binding partner for hBNP or hBNP fragment.

50. A pharmaceutical composition comprising a therapeutically effective amount of an antibody of any of claims 1-24 and 25-50.

FIGURE 1

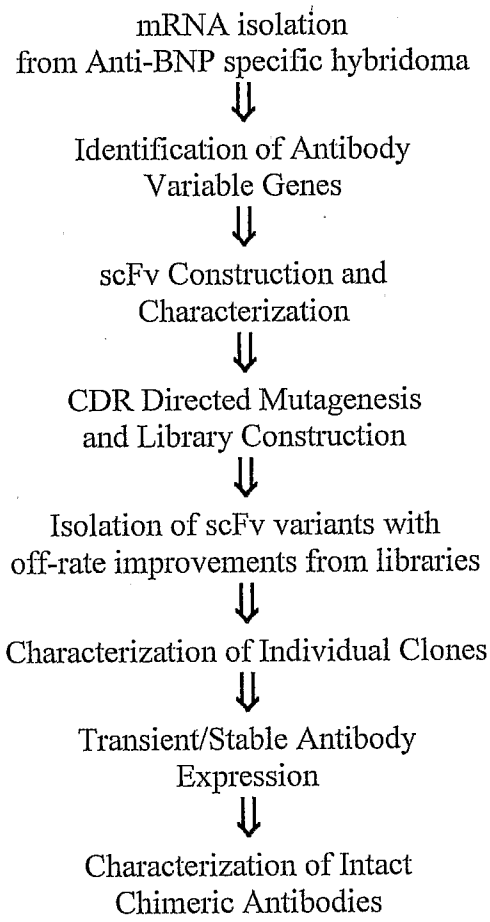

mRNA isolation
from Anti-BNP specific hybridoma
Identification of Antibody
Variable Genes
scFv Construction and
Characterization
CDR Directed Mutagenesis
and Library Construction
Isolation of scFv variants with
off-rate improvements from libraries
Characterization of Individual Clones
Transient/Stable Antibody
Expression
Characterization of Intact
Chimeric Antibodies

FIGURE 2

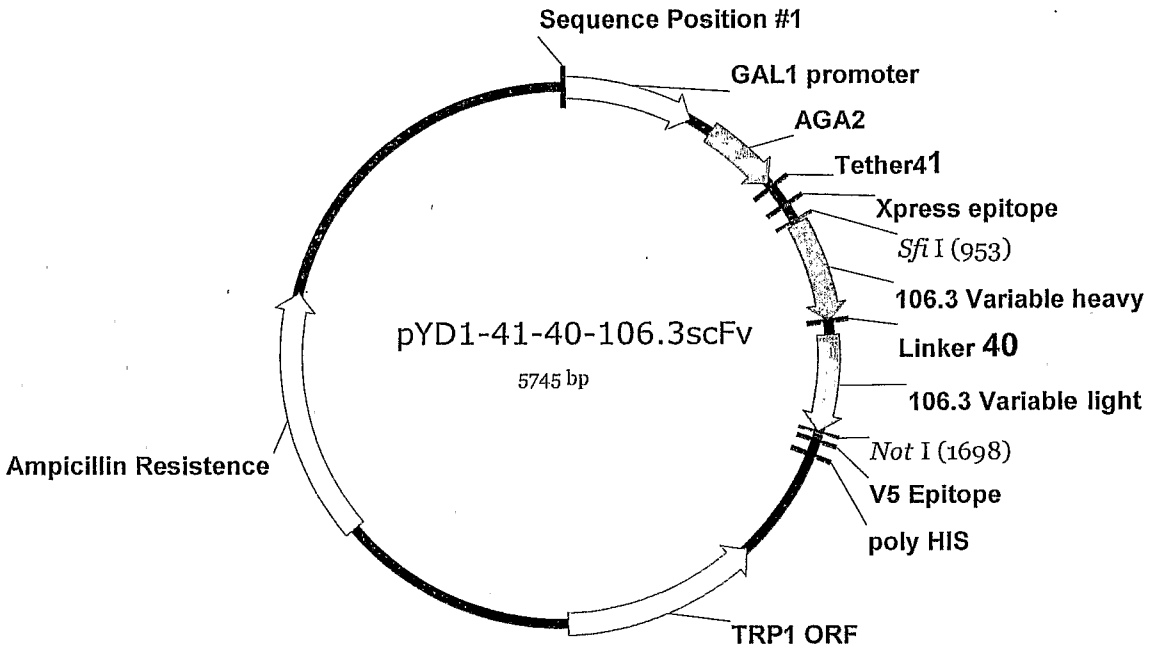

Sequence Position #1
GAL1 promoter
AGA2
Tether41
Xpress epitope
Sfi I (953)
106.3 Variable heavy
Linker 40
106.3 Variable light
Not I (1698)
V5 Epitope
poly HIS
TRP1 ORF
Ampicillin Resistence
pYD1-41-40-106.3scFv
5745 bp

FIGURE 3A

```
1       ACGGATTAGAAG CCGCCGAGCGGG TGACAGCCCTCC
37      GAAGGAAGACTC TCCTCCGTGCGT CCTCGTCCTCAC
73      CGGTCGCGTTCC TGAAACGCAGAT GTGCCTCGCGCC
109     GCACTGCTCCGA ACAATAAAGATT CTACAATACTAG
145     CTTTTATGGTTA TGAAGAGGAAAA ATTGGCAGTAAC
181     CTGGCCCCACAA ACCTTCAAATGA ACGAATCAAATT
217     AACAACCATAGG ATGATAATGCGA TTAGTTTTTTAG
253     CCTTATTTCTGG GGTAATTAATCA GCGAAGCGATGA
289     TTTTTGATCTAT TAACAGATATAT AAATGCAAAAAC
325     TGCATTAACCAC TTTAACTAATAC TTTCAACATTTT
361     CGGTTTGTATTA CTTCTTATTCAA ATGTAATAAAAG
397     TATCAACAAAAA ATTGTTAATATA CCTCTATACTTT
433     AACGTCAAGGAG AAAAAACCCCGG ATCGGACTACTA
469     GCAGCTGTAATA CGACTCACTATA GGGAATATTAAG
                                          AGA2
505     CTAATTCTACTT CATACATTTTCA ATTAAGATGCAG
        ----------------AGA2----------------
541     TTACTTCGCTGT TTTTCAATATTT TCTGTTATTGCT
        ----------------AGA2----------------
577     TCAGTTTTAGCA CAGGAACTGACA ACTATATGCGAG
        ----------------AGA2----------------
613     CAAATCCCCTCA CCAACTTTAGAA TCGACGCCGTAC
        ----------------AGA2----------------
649     TCTTTGTCAACG ACTACTATTTTG GCCAACGGGAAG
        ----------------AGA2----------------
685     GCAATGCAAGGA GTTTTTGAATAT TACAAATCAGTA
        ----------------AGA2----------------
721     ACGTTTGTCAGT AATTGCGGTTCT CACCCCTCAACA
        ----------------AGA2----------------
757     ACTAGCAAAGGC AGCCCCATAAAC ACACAGTATGTT
        ---                   Tether 41-------
793     TTTAAGCTTCTG CAGGCTAGTGGT GAGAACAAGGTG
        ------------------------Tether41
829     GAGTACGCGCCG GCGTTGATGGCC TTGTCTGCTAGC
865     ATGACTGGTGGA CAGCAAATGGGT CGGGATCTGTAC
901     GACGATGACGAT AAGGTACCAGGA TCCAGTGTGGTG
                                          106.3
937     GAATTCGCGGCC CAGCCGGCCATG GCCCAGATCCAG
        variable heavy----------------------
973     TTGGTGCAGTCT GGACCTGAGCTG AGGAAGCCTGGA
        ----------------------------------------
1009    GAGACAGTCAAG ATCTCCTGCAAG GGTTCTGGATAT
        ----------------------------------------
```

FIGURE 3B

```
1045  ACCTTCACACAC TATGGAATAAAC TGGGTGAAGCAG
      ------------------------------------
1081  ACTCCAAGAAAG GATTTAAAGTGG ATGGGCTGGATA
      ------------------------------------
1117  AACACCCATACT GGAGAGCCAATA TATGCTGATGAC
      ------------------------------------
1153  TTCAAGGGACGG TTTGCCTTCTCT TTGGAAACCTCT
      ------------------------------------
1189  GCCAACACTGCC TATTTGCAAATC AACAACCTCAAC
      ------------------------------------
1225  AATGGAGACATG GGTACATATTTC TGTACAAGAAGT
      ------------------------------------
1261  CACCGGTTTGGT TTGGACTACTGG GGTCAAGGTACC
      ----------VH end-Linker 40---------
1297  TCAGTCACCGTC TCGTCAGGTCCC GCCAAGGAGTTG
      ------------------------106.3 ---
1333  ACGCCCCTGAAG GAGGCGAAGGTC TCTGACAATGTG
      Variable light----------------------
1369  CTGACCCAATCT CCACCTTCTTTG GCTGTGTCTCTA
      ------------------------------------
1405  GGGCAGAGGGCC ACCATCTCCTGC AAGGCCAGCCAA
      ------------------------------------
1441  AGTGTTGATTAT AATGGTGATAGT TATCTGAACTGG
      ------------------------------------
1477  TACCAACAGAAG CCAGGACAGCCA CCCAAATTCCTC
      ------------------------------------
1513  ATCTATGCTGCA TCCAATCTAGAA TCTGGGATCCCA
      ------------------------------------
1549  GCCAGGTTTAGT GGCAGTGGGTCT GGGACAGACTTC
      ------------------------------------
1585  AACCTCAACATC CATCCTGTGGAG GAGGAGGATGCT
      ------------------------------------
1621  GCAACCTATTAC TGTCAGCAAAGT AATGAGGATCCA
      ------------------------------------
1657  TTCACGTTCGGC TCGGGGACAAAG TTGGAAATAAAA
      end
1693  CGGGCGGCCGCC CTCGAGTCTAGA GGGCCCTTCGAA
      V5 Epitope-------------------------
1729  GGTAAGCCTATC CCTAACCCTCTC CTCGGTCTCGAT
      ------          Six HIS tag-------STOP
1765  TCTACGCGTACC GGTCATCATCAC CATCACCATTGA
1801  GTTTAAACCCGC TGATCTGATAAC AACAGTGTAGAT
1837  GTAACAAAATCG ACTTTGTTCCCA CTGTACTTTTAG
```

FIGURE 3C

```
1873   CTCGTACAAAAT ACAATATACTTT TCATTTCTCCGT
1909   AAACAACATGTT TTCCCATGTAAT ATCCTTTTCTAT
1945   TTTTCGTTCCGT TACCAACTTTAC ACATACTTTATA
1981   TAGCTATTCACT TCTATACACTAA AAAACTAAGACA
2017   ATTTTAATTTTG CTGCCTGCCATA TTTCAATTTGTT
2053   ATAAATTCCTAT AATTTATCCTAT TAGTAGCTAAAA
2089   AAAGATGAATGT GAATCGAATCCT AAGAGAATTGGG
2125   CAAGTGCACAAA CAATACTTAAAT AAATACTACTCA
                  end TRP1 ORF
2161   GTAATAACCTAT TTCTTAGCATTT TTGACGAAATT
2197   GCTATTTTGTTA GAGTCTTTTACA CCATTTGTCTCC
2233   ACACCTCCGCTT ACATCAACACCA ATAACGCCATTT
2269   AATCTAAGCGCA TCACCAACATTT TCTGGCGTCAGT
2305   CCACCAGCTAAC ATAAAATGTAAG CTCTCGGGGCTC
2341   TCTTGCCTTCCA ACCCAGTCAGAA ATCGAGTTCCAA
2377   TCCAAAAGTTCA CCTGTCCCACCT GCTTCTGAATCA
2413   AACAAGGGAATA AACGAATGAGGT TTCTGTGAAGCT
2449   GCACTGAGTAGT ATGTTGCAGTCT TTTGGAAATACG
2485   AGTCTTTTAATA ACTGGCAAACCG AGGAACTCTTGG
2521   TATTCTTGCCAC GACTCATCTCCG TGCAGTTGGACG
2557   ATATCAATGCCG TAATCATTGACC AGAGCCAAAACA
2593   TCCTCCTTAGGT TGATTACGAAAC ACGCCAACCAAG
2629   TATTTCGGAGTG CCTGAACTATTT TTATATGCTTTT
2665   ACAAGACTTGAA ATTTTCCTTGCA ATAACCGGGTCA
2701   ATTGTTCTCTTT CTATTGGGCACA CATATAATACCC
2737   AGCAAGTCAGCA TCGGAATCTAGA GCACATTCTGCG
2773   GCCTCTGTGCTC TGCAAGCCGCAA ACTTTCACCAAT
2809   GGACCAGAACTA CCTGTGAAATTA ATAACAGACATA
                                  TRP1 ORF
2845   CTCCAAGCTGCC TTTGTGTGCTTA ATCACGTATACT
2881   CACGTGCTCAAT AGTCACCAATGC CCTCCCTCTTGG
2917   CCCTCTCCTTTT CTTTTTTCGACC GAATTTCTTGAA
2953   GACGAAAGGGCC TCGTGATACGCC TATTTTTATAGG
2989   TTAATGTCATGA TAATAATGGTTT CTTAGGACGGAT
3025   CGCTTGCCTGTA ACTTACACGCGC CTCGTATCTTTT
3061   AATGATGGAATA ATTTGGGAATTT ACTCTGTGTTTA
3097   TTTATTTTTATG TTTTGTATTTGG ATTTTAGAAAGT
3133   AAATAAAGAAGG TAGAAGAGTTAC GGAATGAAGAAA
3169   AAAAAATAAACA AAGGTTTAAAAA ATTTCAACAAAA
3205   AGCGTACTTTAC ATATATATTTAT TAGACAAGAAAA
3241   GCAGATTAAATA GATATACATTCG ATTAACGATAAG
3277   TAAAATGTAAAA TCACAGGATTTT CGTGTGTGGTCT
3313   TCTACACAGACA AGATGAAACAAT TCGGCATTAATA
3349   CCTGAGAGCAGG AAGAGCAAGATA AAAGGTAGTATT
3385   TGTTGGCGATCC CCCTAGAGTCTT TTACATCTTCGG
3421   AAAACAAAAACT ATTTTTTCTTTA ATTTCTTTTTTT
3457   ACTTTCTATTTT TAATTTATATAT TTATATTAAAAA
3493   ATTTAAATTATA ATTATTTTTATA GCACGTGATGAA
```

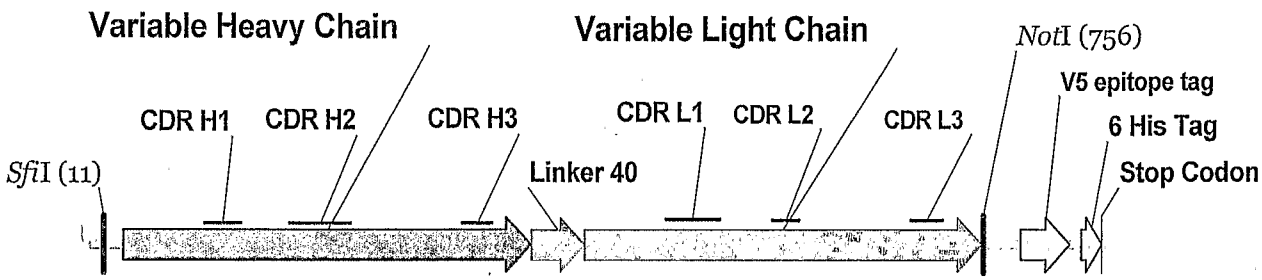
FIGURE 3D

```
3529   AAGGACCCAGGT GGCACTTTTCGG GGAAATGTGCGC
3565   GGAACCCCTATT TGTTTATTTTTC TAAATACATTCA
3601   AATATGTATCCG CTCATGAGACAA TAACCCTGATAA
                                    Amp Res---
3637   ATGCTTCAATAA TATTGAAAAAGG AAGAGTATGAGT
3673   ATTCAACATTTC CGTGTCGCCCTT ATTCCCTTTTTT
3709   GCGGCATTTTGC CTTCCTGTTTTT GCTCACCCAGAA
3745   ACGCTGGTGAAA GTAAAAGATGCT GAAGATCAGTTG
3781   GGTGCACGAGTG GGTTACATCGAA CTGGATCTCAAC
3817   AGCGGTAAGATC CTTGAGAGTTTT CGCCCCGAAGAA
3853   CGTTTTCCAATG ATGAGCACTTTT AAAGTTCTGCTA
3889   TGTGGCGCGGTA TTATCCCGTGTT GACGCCGGGCAA
3925   GAGCAACTCGGT CGCCGCATACAC TATTCTCAGAAT
3961   GACTTGGTTGAG TACTCACCAGTC ACAGAAAAGCAT
3997   CTTACGGATGGC ATGACAGTAAGA GAATTATGCAGT
4033   GCTGCCATAACC ATGAGTGATAAC ACTGCGGCCAAC
4069   TTACTTCTGACA ACGATCGGAGGA CCGAAGGAGCTA
4105   ACCGCTTTTTTG CACAACATGGGG GATCATGTAACT
4141   CGCCTTGATCGT TGGGAACCGGAG CTGAATGAAGCC
4177   ATACCAAACGAC GAGCGTGACACC ACGATGCCTGTA
4213   GCAATGGCAACA ACGTTGCGCAAA CTATTAACTGGC
4249   GAACTACTTACT CTAGCTTCCCGG CAACAATTAATA
4285   GACTGGATGGAG GCGGATAAAGTT GCAGGACCACTT
4321   CTGCGCTCGGCC CTTCCGGCTGGC TGGTTTATTGCT
4357   GATAAATCTGGA GCCGGTGAGCGT GGGTCTCGCGGT
4393   ATCATTGCAGCA CTGGGGCCAGAT GGTAAGCCCTCC
4429   CGTATCGTAGTT ATCTACACGACG GGCAGTCAGGCA
4465   ACTATGGATGAA CGAAATAGACAG ATCGCTGAGATA
             Amp Res end
4501   GGTGCCTCACTG ATTAAGCATTGG TAACTGTCAGAC
4537   CAAGTTTACTCA TATATACTTTAG ATTGATTTAAAA
4573   CTTCATTTTTAA TTTAAAAGGATC TAGGTGAAGATC
4609   CTTTTTGATAAT CTCATGACCAAA ATCCCTTAACGT
4645   GAGTTTTCGTTC CACTGAGCGTCA GACCCCGTAGAA
4681   AAGATCAAAGGA TCTTCTTGAGAT CCTTTTTTTCTG
4717   CGCGTAATCTGC TGCTTGCAAACA AAAAAACCACCG
4753   CTACCAGCGGTG GTTTGTTTGCCG GATCAAGAGCTA
4789   CCAACTCTTTTT CCGAAGGTAACT GGCTTCAGCAGA
4825   GCGCAGATACCA AATACTGTCCTT CTAGTGTAGCCG
4861   TAGTTAGGCCAC CACTTCAAGAAC TCTGTAGCACCG
4897   CCTACATACCTC GCTCTGCTAATC CTGTTACCAGTG
4933   GCTGCTGCCAGT GGCGATAAGTCG TGTCTTACCGGG
4969   TTGGACTCAAGA CGATAGTTACCG GATAAGGCGCAG
5005   CGGTCGGGCTGA ACGGGGGGTTCG TGCACACAGCCC
5041   AGCTTGGAGCGA ACGACCTACACC GAACTGAGATAC
5077   CTACAGCGTGAG CATTGAGAAAGC GCCACGCTTCCC
5113   GAAGGGAGAAAG GCGGACAGGTAT CCGGTAAGCGGC
```

FIGURE 3E

```
5149   AGGGTCGGAACA GGAGAGCGCACG AGGGAGCTTCCA
5185   GGGGGGAACGCC TGGTATCTTTAT AGTCCTGTCGGG
5221   TTTCGCCACCTC TGACTTGAGCGT CGATTTTTGTGA
5257   TGCTCGTCAGGG GGGCCGAGCCTA TGGAAAAACGCC
5293   AGCAACGCGGCC TTTTTACGGTTC CTGGCCTTTTGC
5329   TGGCCTTTTGCT CACATGTTCTTT CCTGCGTTATCC
5365   CCTGATTCTGTG GATAACCGTATT ACCGCCTTTGAG
5401   TGAGCTGATACC GCTCGCCGCAGC CGAACGACCGAG
5437   CGCAGCGAGTCA GTGAGCGAGGAA GCGGAAGAGCGC
5473   CCAATACGCAAA CCGCCTCTCCCC GCGCGTTGGCCG
5509   ATTCATTAATGC AGCTGGCACGAC AGGTTTCCCGAC
5545   TGGAAAGCGGGC AGTGAGCGCAAC GCAATTAATGTG
5581   AGTTACCTCACT CATTAGGCACCC CAGGCTTTACAC
5617   TTTATGCTTCCG GCTCCTATGTTG TGTGGAATTGTG
5653   AGCGGATAACAA TTTCACACAGGA AACAGCTATGAC
5689   CATGATTACGCC AAGCTCGGAATT AACCCTCACTAA
5725   AGGGAACAAAAG CTGGCTAGT
```

FIGURE 4



106.3 scFv

FIGURE 5

QLVQSGPELR KPGETVKISC KGS***GYTFTHY*** ***GIN***WVKQTPR KDLKWMG***WIN***

THTGEPIYAD ***DFKG***RFAFSL ETSANTAYLQ INNLNNGDMG TYFCTR***SHRF***

GLDYWGQGTS VTVSSGPAKE LTPLKEAKVS DNVLTQSPPS LAVSLGQRAT

ISC***KASQSVD*** ***YNGDSYLN***WY QQKPGQPPKF LIY***AASNLES*** GIPARFSGSG

SGTDFNLNIH PVEEEDAATY YC***QQSNEDPF*** ***T***FGSGTKLEI KRAAALESRG

PFEGKPIPNP LLGLDSTRTG HHHHHH*

FIGURE 6A

106.3 Variable heavy
946 CAGATCCAG TTGGTGCAG TCTGGACCT
GTCTAGGTC AACCACGTC AGACCTGGA

106.3 Variable heavy
991 GAGCTGAGG AAGCCTGGA GAGACAGTC AAGATCTCC TGCAAGGGT
CTCGACTCC TTCGGACCT CTCTGTCAG TTCTAGAGG ACGTTCCCA

CDR H1----------(10)
106.3 Variable heavy
1036 TCTGGATAT ACCTTCACA CACTATGGA ATAAACTGG GTGAAGCAG
AGACCTATA TGGAAGTGT GTGATACCT TATTTGACC CACTTCGTC

CDR H2----------
106.3 Variable heavy
1081 ACTCCAAGA AAGGATTTA AAGTGGATG GGCTGGATA AACACCCAT
TGAGGTTCT TTCCTAAAT TTCACCTAC CCGACCTAT TTGTGGGTA

H2----------(17)
106.3 Variable heavy
1126 ACTGGAGAG CCAATATAT GCTGATGAC TTCAAGGGA CGGTTTGCC
TGACCTCTC GGTTATATA CGACTACTG AAGTTCCCT GCCAAACGG

106.3 Variable heavy
1171 TTCTCTTTG GAAACCTCT GCCAACACT GCCTATTTG CAAATCAAC
AAGAGAAAC CTTTGGAGA CGGTTGTGA CGGATAAAC GTTTAGTTG

CDR H3
106.3 Variable heavy
1216 AACCTCAAC AATGGAGAC ATGGGTACA TATTTCTGT ACAAGAAGT
TTGGAGTTG TTACCTCTG TACCCATGT ATAAAGACA TGTTCTTCA

H3----------(8)
106.3 Variable heavy
1261 CACCGGTTT GGTTTGGAC TACTGGGGT CAAGGTACC TCAGTCACC
GTGGCCAAA CCAAACCTG ATGACCCCA GTTCCATGG AGTCAGTGG

106.3 Vh
Linker 40----------
1306 GTCTCGTCA GGTCCCGCC AAGGAGTTG ACGCCCCTG AAGGAGGCG
CAGAGCAGT CCAGGGCGG TTCCTCAAC TGCGGGGAC TTCCTCCGC

106.3 Variable light
Linker 40
1351 AAGGTCTCT GACAATGTG CTGACCCAA TCTCCACCT TCTTTGGCT
TTCCAGAGA CTGTTACAC GACTGGGTT AGAGGTGGA AGAAACCGA

CDR L1-------
106.3 Variable light
1396 GTGTCTCTA GGGCAGAGG GCCACCATC TCCTGCAAG GCCAGCCAA
CACAGAGAT CCCGTCTCC CGGTGGTAG AGGACGTTC CGGTCGGTT

CDR L1----------(15)
106.3 Variable light
1441 AGTGTTGAT TATAATGGT GATAGTTAT CTGAACTGG TACCAACAG
TCACAACTA ATATTACCA CTATCAATA GACTTGACC ATGGTTGTC

FIGURE 6B

```
                                                    CDR L2-------
                    106.3 Variable light
1486    AAGCCAGGA CAGCCACCC AAATTCCTC ATCTATGCT GCATCCAAT
        TTCGGTCCT GTCGGTGGG TTTAAGGAG TAGATACGA CGTAGGTTA

        ------(7)
                    106.3 Variable light
1531    CTAGAATCT GGGATCCCA GCCAGGTTT AGTGGCAGT GGGTCTGGG
        GATCTTAGA CCCTAGGGT CGGTCCAAA TCACCGTCA CCCAGACCC

                    106.3 Variable light
1576    ACAGACTTC AACCTCAAC ATCCATCCT GTGGAGGAG GAGGATGCT
        TGTCTGAAG TTGGAGTTG TAGGTAGGA CACCTCCTC CTCCTACGA

                         CDR L3----------------------(9)
                    106.3 Variable light
1621    GCAACCTAT TACTGTCAG CAAAGTAAT GAGGATCCA TTCACGTTC
        CGTTGGATA ATGACAGTC GTTTCATTA CTCCTAGGT AAGTGCAAG

             106.3 Variable light
                                        end   NotI
1666    GGCTCGGGG ACAAAGTTG GAAATAAAA CGGGCGGCC GCCCTCGAG
        CCGAGCCCC TGTTTCAAC CTTTATTTT GCCCGCCGG CGGGAGCTC

                                       V5 Epitope
1711    TCTAGAGGG CCCTTCGAA GGTAAGCCT ATCCCTAAC CCTCTCCTC
        AGATCTCCC GGGAAGCTT CCATTCGGA TAGGGATTG GGAGAGGAG

          V5 Epitope                        poly HIS
1756    GGTCTCGAT TCTACGCGT ACCGGTCAT CATCACCAT CACCATTGA
        CCAGAGCTA AGATGCGCA TGGCCAGTA GTAGTGGTA GTGGTAACT
```

FIGURE 7A

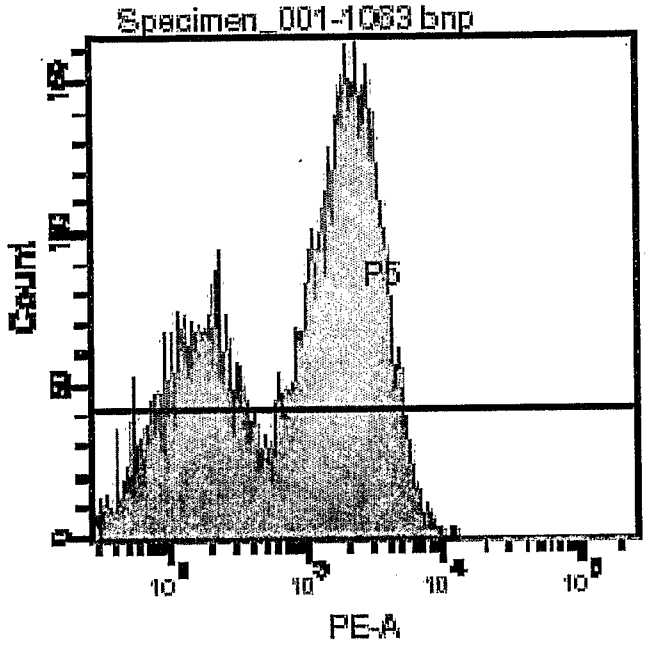


FIGURE 7B

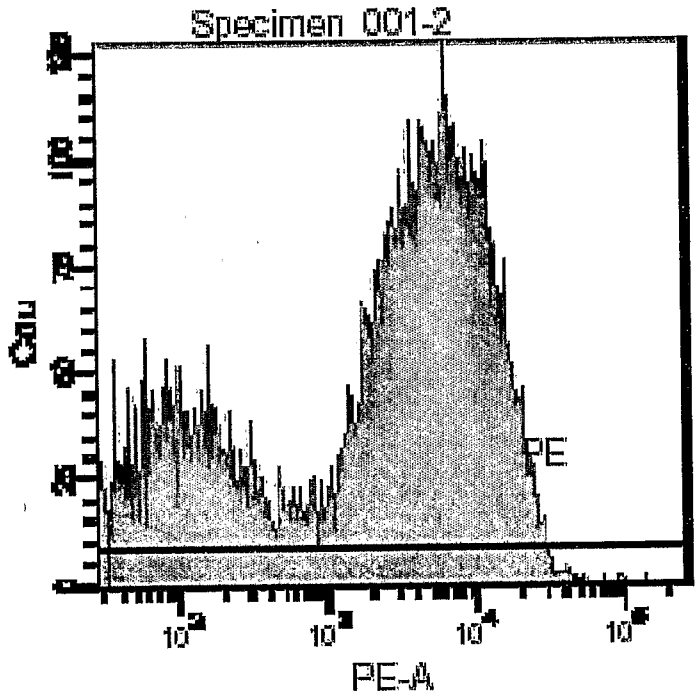


FIGURE 8

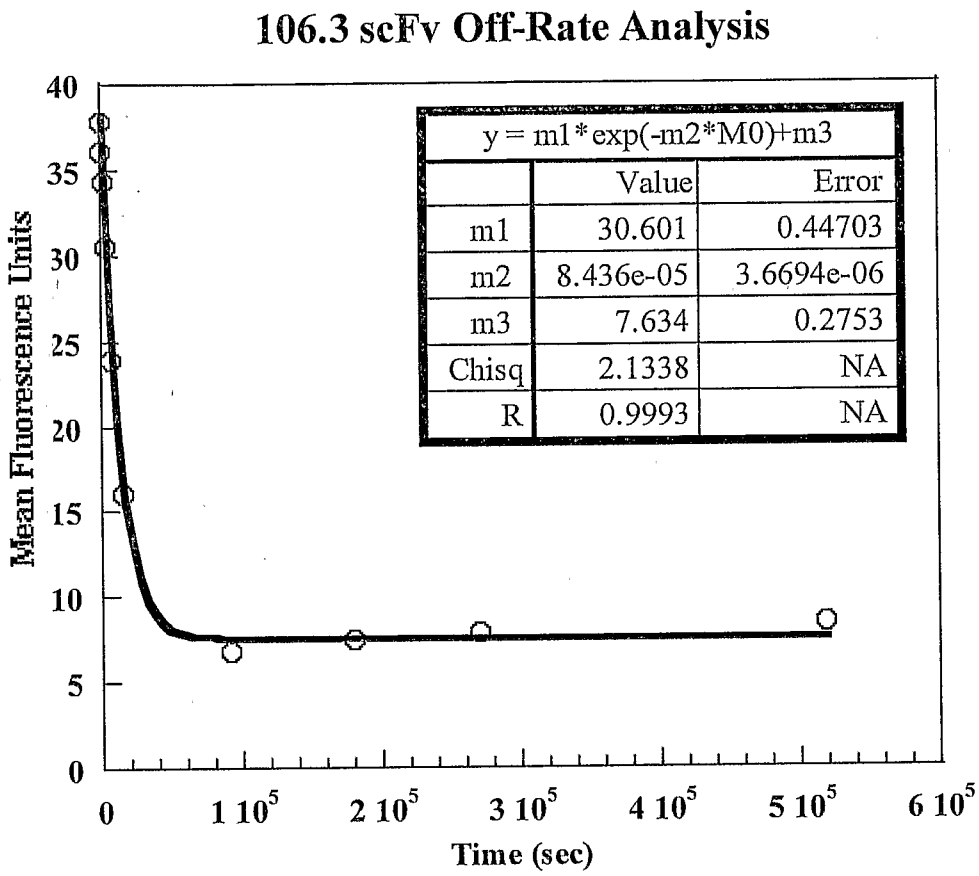
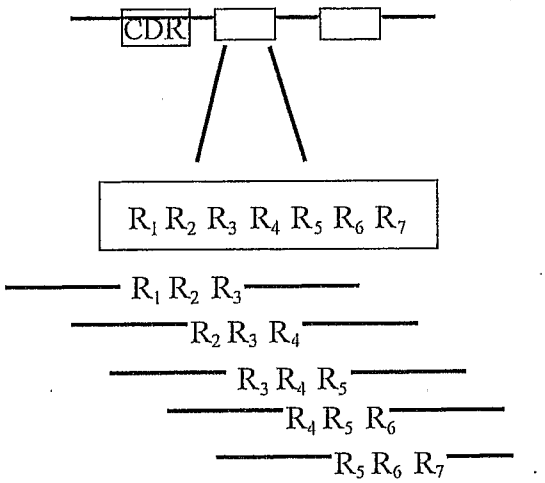
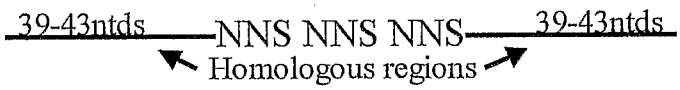

106.3 scFv Off-Rate Analysis
y = m1*exp(-m2*M0)+m3
Value
Error
m1
30.601
0.44703
m2
8.436e-05
3.6694e-06
m3
7.634
0.2753
Chisq
2.1338
NA
R
0.9993
NA
Mean Fluorescence Units
0
5
10
15
20
25
30
35
40
0
1 10^5
2 10^5
3 10^5
4 10^5
5 10^5
6 10^5
Time (sec)

FIGURE 9





N = A,G,C,T

S = C, G

FIGURE 10

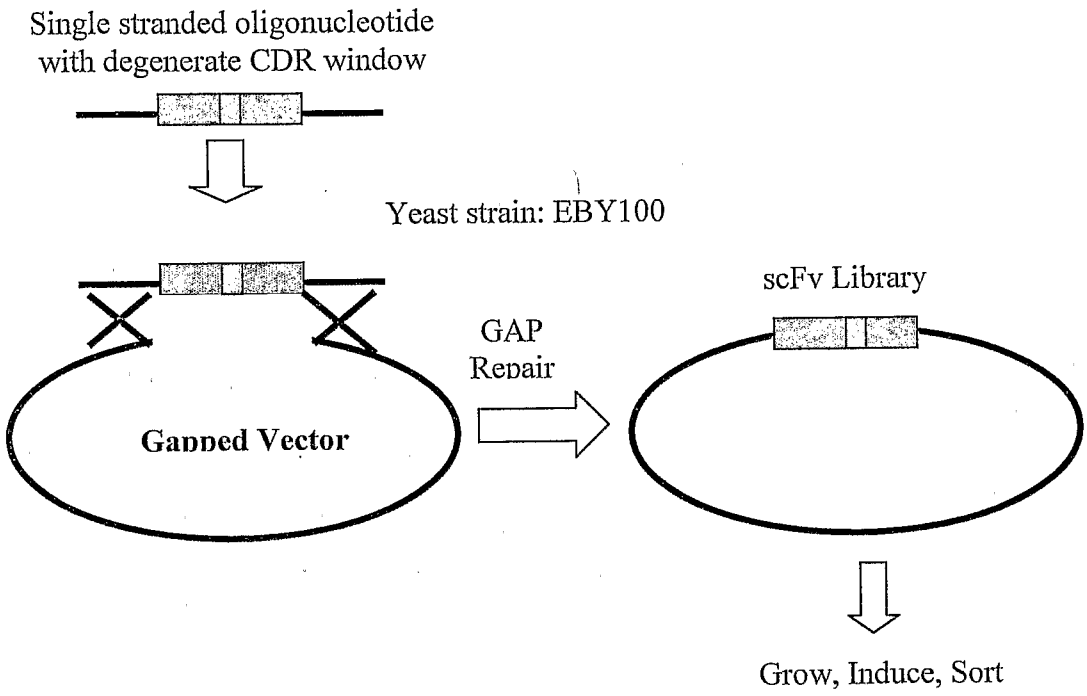

Single stranded oligonucleotide
with degenerate CDR window
Yeast strain: EBY100
scFv Library
GAP
Repair
Gapped Vector
Grow, Induce, Sort

FIGURE 11

Name	k_{off} (sec^{-1})	Fold Improvement
106.3 wt	8.4E-05	-----
H2 288	3.7E-05	2.3
L1 B4	7.3E-06	11.5
L1 B9	3.1E-05	2.7
L1 9b	1.3E-05	6.4
L1 B12	7.6E-06	11.0
L1 B15	8.6E-06	9.7
L1 16	8.7E-06	9.6
L1 B24	6.7E-06	12.5
L2 6	3.2E-05	2.7
L2 21	4.0E-05	2.1

FIGURE 12A

Name	CDR H2 Pos 56	CDR H2 Pos 57	CDR H2 Pos 58
106.3 wt	Glu (GAG)	Pro (CCA)	Ile (ATA)
H2 288	Glu (GAG)	Ala (GCG)	Tyr (TAC)

FIGURE 12B

Name	CDR L1 Pos 26	CDR L1 Pos 27	CDR L1 Pos 27A	Other Mutations
106.3 wt	Ser (AGC)	Gln (CAA)	Ser (AGT)	
L1 B4	Gln (CAG)	Phe (TTC)	Ala (GCG)	
L1 B9	Tyr (TAC)	Ala (GCG)	Ser (AGT)	
L1 9b	Gln (CAG)	Trp (TGG)	Gly (GGC)	R42S
L1 B12	Thr (ACC)	Trp (TGG)	Asp (GAC)	
L1 B15	Arg (AGG)	Trp (TGG)	Pro (CCG)	
L1 16	Ala (GCG)	Tyr (TAC)	Gly (GGC)	
L1 B24	Asn (AAC)	Trp (TGG)	Pro (CCC)	R42S

FIGURE 12C

Name	CDR L2 Pos 53	CDR L2 Pos 54	CDR L2 Pos 55
106.3 wt	Asn (AAT)	Leu (CTA)	Glu (GAA)
L2 6	Cys (TGC)	Gly (GGG)	Trp (TGG)
L2 21	Cys (TGC)	Ala (GCG)	Pro (CCG)

FIGURE 13

Clone Name	Antibody Type	k_{on} ($M^{-1}sec^{-1}$)	k_{off} (sec^{-1})	K_D (pM)
106.3 wt	Mouse IgG1	1.2E+07	2.2E-04	18
106.3 wt	Mouse-Human IgG1	7.8E+06	1.5E-04	19
106.3 AM1	Mouse-Human IgG1	1.3E+07	2.4E-05	1.9

FIGURE 14A

Degenerate Oligonucleotides:
N= A, G, C, T; S = G, C

CDR H3 Degenerate Oligonucleotides

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo1	H3	H3-1	105
106.3	Oligo 2	H3	H3-2	105
106.3	Oligo 3	H3	H3-3	105
106.3	Oligo 4	H3	H3-4	105
106.3	Oligo 5	H3	H3-5	105
106.3	Oligo 6	H3	H3-6	99

Oligo1
AAC CTC AAC AAT GGA GAC ATG GGT ACA TAT TTC TGT ACA AGA **NNS NNS NNS TTT GGT TTG GAC TAC** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC

Oligo2
AAC CTC AAC AAT GGA GAC ATG GGT ACA TAT TTC TGT ACA AGA **AGT NNS NNS NNS GGT TTG GAC TAC** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC

Oligo3
AAC CTC AAC AAT GGA GAC ATG GGT ACA TAT TTC TGT ACA AGA **AGT CAC NNS NNS NNS TTG GAC TAC** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC

Oligo 4
AAC CTC AAC AAT GGA GAC ATG GGT ACA TAT TTC TGT ACA AGA **AGT CAC CGG NNS NNS NNS GAC TAC** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC

Oligo 5
AAC CTC AAC AAT GGA GAC ATG GGT ACA TAT TTC TGT ACA AGA **AGT CAC CGG TTT NNS NNS NNS TAC** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC

Oligo 6
GAC ATG GGT ACA TAT TTC TGT ACA AGA **AGT CAC CGG TTT GGT NNS NNS NNS** TGG GGT CAA GGT ACC TCA GTC ACC GTC TCG TCA GGT CCC GCC GCC AAG

FIGURE 14B

H2 Degenerate Oligonucleotides

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo 7	H2	H2-1	105
106.3	Oligo 8	H2	H2-2	105
106.3	Oligo 9	H2	H2-3	105
106.3	Oligo10	H2	H2-4	105
106.3	Oligo11	H2	H2-5	105
106.3	Oligo12	H2	H2-6	105
106.3	Oligo 13	H2	H2-7	105
106.3	Oligo 14	H2	H2-8	105
106.3	Oligo 15	H2	H2-9	105
106.3	Oligo 16	H2	H2-10	105
106.3	Oligo 17	H2	H2-11	105
106.3	Oligo 18	H2	H2-12	105
106.3	Oligo 19	H2	H2-13	105
106.3	Oligo 20	H2	H2-14	105
106.3	Oligo 21	H2	H2-15	105

Oligo 7

TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC **NNS NNS NNS ACC CAT ACT GGA GAG CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC

Oligo 8

TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG NNS NNS NNS CAT ACT GGA GAG CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC

Oligo 9

TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA NNS NNS NNS ACT GGA GAG CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC

Oligo 10

TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC NNS NNS NNS GGA GAG CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC

Oligo 11

TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC NNS NNS NNS GAG CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC

Oligo 12

CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC CAT NNS NNS NNS CCA ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT

FIGURE 14C

Oligo 13
CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT NNS NNS NNS ATA TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT

Oligo 14
CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA NNS NNS NNS TAT GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT

Oligo15
CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG NNS NNS NNS GCT GAT GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT

Oligo16
CCA AGA AAG GAT TTA AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA NNS NNS NNS GAT GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT

Oligo17
AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA ATA NNS NNS NNS GAC TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT

Oligo18
AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA ATA TAT NNS NNS NNS TTC AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT

Oligo19
AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA ATA TAT GCT NNS NNS NNS AAG GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT

Oligo20
AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA ATA TAT GCT GAT NNS NNS NNS GGA** CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT

Oligo21
AAG TGG ATG GGC **TGG ATA AAC ACC CAT ACT GGA GAG CCA ATA TAT GCT GAT GAC NNS NNS NNS** CGG TTT GCC TTC TCT TTG GAA ACC TCT GCC AAC ACT GCC TAT

FIGURE 14D

CDR H1 Degenerate Oligonucleotides

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo22	H1	H1-1	105
106.3	Oligo23	H1	H1-2	105
106.3	Oligo24	H1	H1-3	105
106.3	Oligo25	H1	H1-4	105
106.3	Oligo26	H1	H1-5	105
106.3	Oligo27	H1	H1-6	99
106.3	Oligo28	H1	H1-7	99
106.3	Oligo29	H1	H1-8	99

Oligo22
AGG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GGT TCT **NNS NNS NNS TTC ACA CAC TAT GGA ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG

Oligo23
AGG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA NNS NNS NNS ACA CAC TAT GGA ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG

Oligo24
AGG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT NNS NNS NNS CAC TAT GGA ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG

Oligo25
AGG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT ACC NNS NNS NNS TAT GGA ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG

Oligo26
AGG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT ACC TTC NNS NNS NNS GGA ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG

Oligo27
ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT ACC TTC ACA NNS NNS NNS ATA AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC

Oligo28
ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT ACC TTC ACA CAC NNS NNS NNS AAC** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC

Oligo29
ACA GTC AAG ATC TCC TGC AAG GGT TCT **GGA TAT ACC TTC ACA CAC TAT NNS NNS NNS** TGG GTG AAG CAG ACT CCA AGA AAG GAT TTA AAG TGG ATG GGC

FIGURE 14E

CDR L1 Degenerate Oligonucleotides (13)

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo30	L1	L1-1	99
106.3	Oligo31	L1	L1-2	99
106.3	Oligo32	L1	L1-3	99
106.3	Oligo33	L1	L1-4	99
106.3	Oligo34	L1	L1-5	99
106.3	Oligo35	L1	L1-6	99
106.3	Oligo36	L1	L1-7	99
106.3	Oligo37	L1	L1-8	99
106.3	Oligo38	L1	L1-9	99
106.3	Oligo39	L1	L1-10	99
106.3	Oligo40	L1	L1-11	99
106.3	Oligo41	L1	L1-12	93
106.3	Oligo42	L1	L1-13	93

Oligo 30
TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATC TCC TGC **NNS NNS NNS CAA AGT GTT GAT TAT AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG

Oligo 31
TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATC TCC TGC **AAG NNS NNS NNS AGT GTT GAT TAT AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG

Oligo 32
TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC NNS NNS NNS GTT GAT TAT AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG

Oligo 33
TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC NNS NNS NNS GAT TAT AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG

Oligo 34
TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA NNS NNS NNS TAT AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG

Oligo 35
GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA AGT NNS NNS NNS AAT GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC

FIGURE 14F

Oligo 36
GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA AGT GTT NNS NNS NNS GGT GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC

Oligo 37
GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT NNS NNS NNS GAT AGT TAT CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC

Oligo38
GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT TAT NNS NNS NNS AGT TAT CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC

Oligo39
GGG CAG AGG GCC ACC ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT TAT AAT NNS NNS NNS TAT CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC

Oligo40
ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT TAT AAT GGT NNS NNS NNS CTG AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC

Oligo41
ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT TAT AAT GGT GAT NNS NNS NNS AAC** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC

Oligo42
ATC TCC TGC **AAG GCC AGC CAA AGT GTT GAT TAT AAT GGT GAT AGT NNS NNS NNS** TGG TAC CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC

CDR L2 Degenerate Oligonucleotides (5)

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo43	L2	L2-1	99
106.3	Oligo44	L2	L2-2	99
106.3	Oligo45	L2	L2-3	99
106.3	Oligo46	L2	L2-4	99
106.3	Oligo47	L2	L2-5	99

Oligo43
CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC ATC TAT **NNS NNS NNS AAT CTA GAA TCT** GGG ATC CCA GCC AGG TTT AGT GGC AGT GGG TCT GGG ACA

Oligo44
CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC ATC TAT **GCT NNS NNS NNS CTA GAA TCT** GGG ATC CCA GCC AGG TTT AGT GGC AGT GGG TCT GGG ACA

FIGURE 14G

Oligo45
CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC ATC TAT **GCT GCA NNS NNS NNS GAA TCT** GGG ATC CCA GCC AGG TTT AGT GGC AGT GGG TCT GGG ACA

Oligo46
CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC ATC TAT **GCT GCA TCC NNS NNS NNS TCT** GGG ATC CCA GCC AGG TTT AGT GGC AGT GGG TCT GGG ACA

Oligo47
CAA CAG AAG CCA GGA CAG CCA CCC AAA TTC CTC ATC TAT **GCT GCA TCC AAT NNS NNS NNS** GGG ATC CCA GCC AGG TTT AGT GGC AGT GGG TCT GGG ACA

CDR L3 Degenerate Oligonucleotides (7)

System	Code	CDR	Library name	Length(ntd)
106.3	Oligo48	L3	L3-1	99
106.3	Oligo49	L3	L3-2	99
106.3	Oligo50	L3	L3-3	99
106.3	Oligo51	L3	L3-4	99
106.3	Oligo52	L3	L3-5	99
106.3	Oligo53	L3	L3-6	93
106.3	Oligo54	L3	L3-7	93

Oligo48
CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT **NNS NNS NNS AAT GAG GAT CCA TTC ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG

Oligo49
CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT **CAG NNS NNS NNS GAG GAT CCA TTC ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG

Oligo50
CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT **CAG CAA NNS NNS NNS GAT CCA TTC ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG

Oligo51
CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT **CAG CAA AGT NNS NNS NNS CCA TTC ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG

Oligo52
CAT CCT GTG GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT **CAG CAA AGT AAT NNS NNS NNS TTC ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG

FIGURE 14H

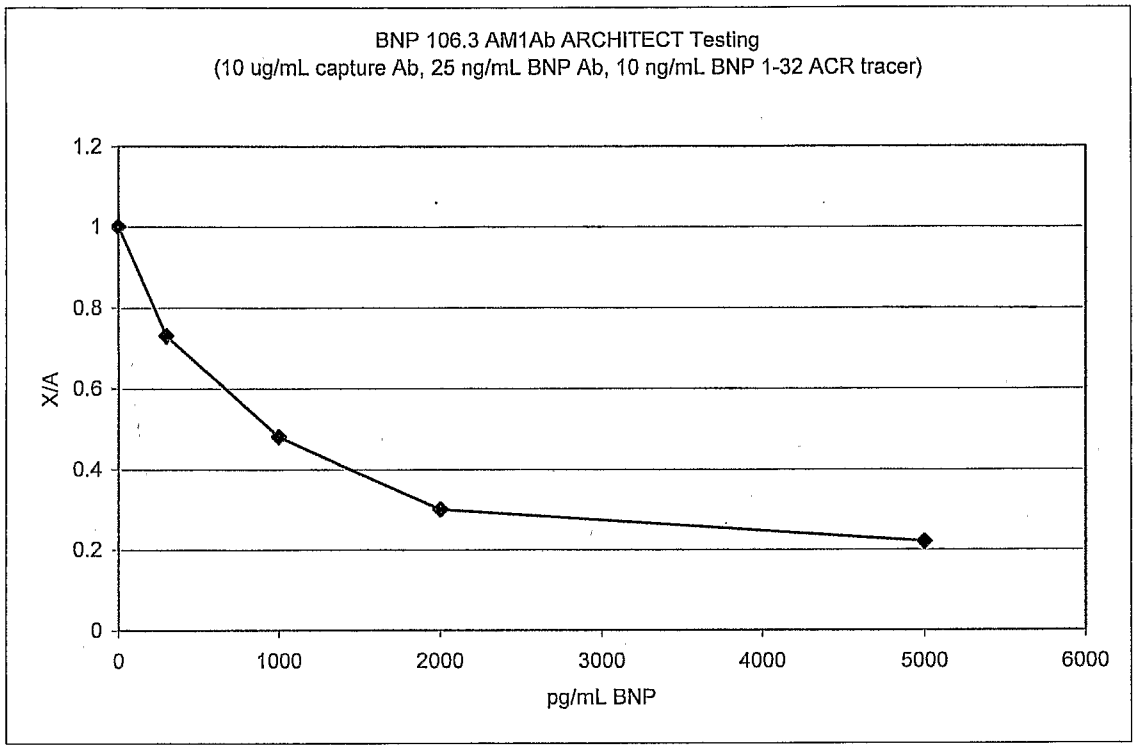
Oligo53

GAG GAT GCT GCA ACC TAT TAC TGT **CAG CAA AGT AAT GAG NNS NNS NNS ACG** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG GCG GCC GCC

Oligo54

GAG GAT GCT GCA ACC TAT TAC TGT **CAG CAA AGT AAT GAG GAT NNS NNS NNS** TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG GCG GCC GCC

FIGURE 15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(10 ug/mL capture Ab, 25 ng/mL BNP Ab, 10 ng/mL BNP 1-32 ACR tracer)
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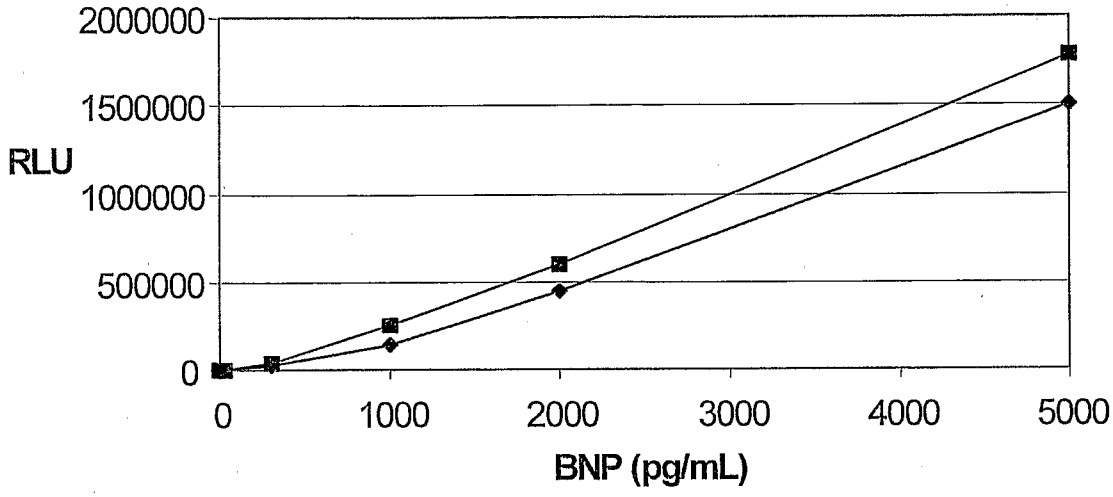
0.6
0.4
0.2
0
0
1000
2000
3000
4000
5000
6000
pg/mL BNP

FIGURE 16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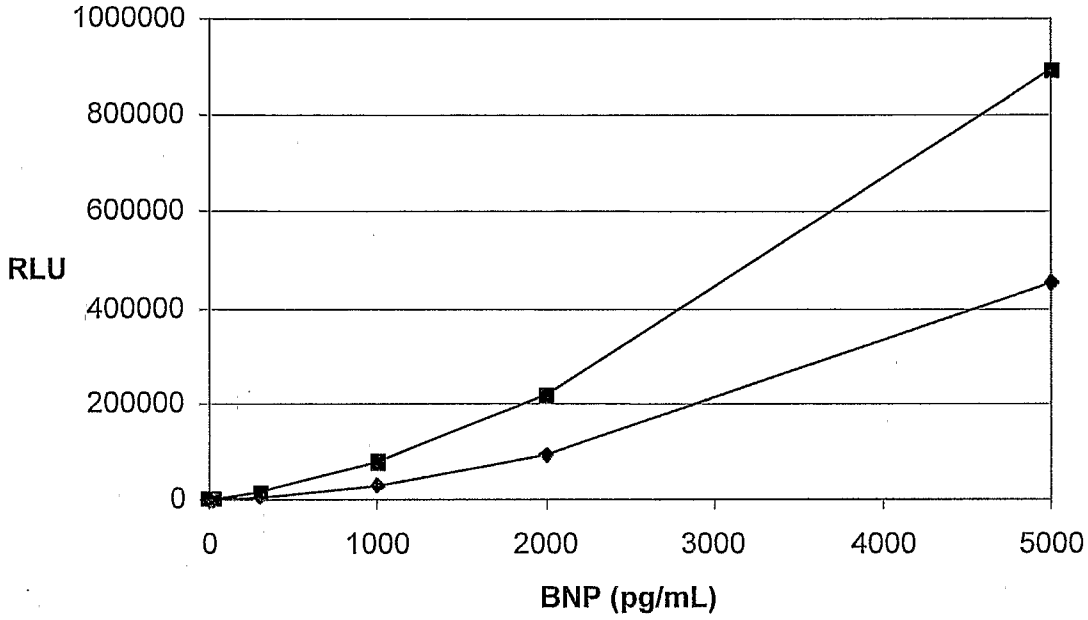
1500000
RLU
1000000
500000
0
0
1000
2000
3000
4000
5000
BNP (pg/mL)

FIGURE 17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RLU
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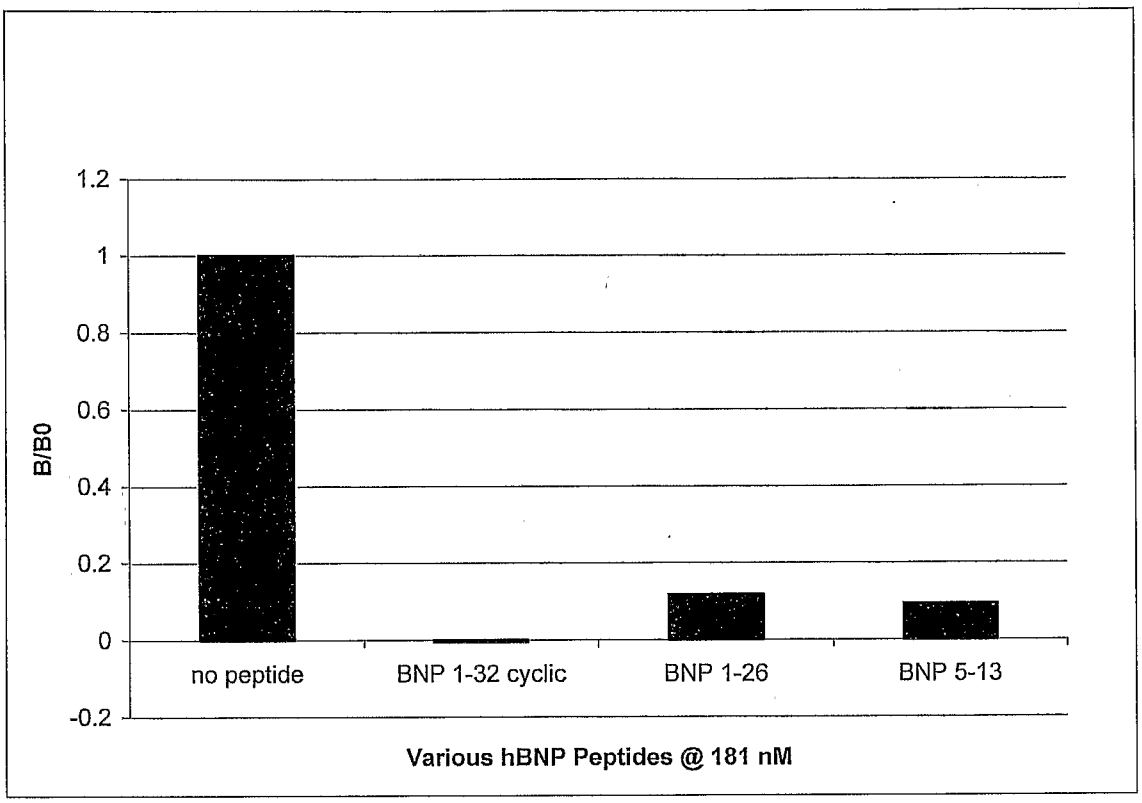
200000
0
0
1000
2000
3000
4000
5000
BNP (pg/mL)

FIGURE 18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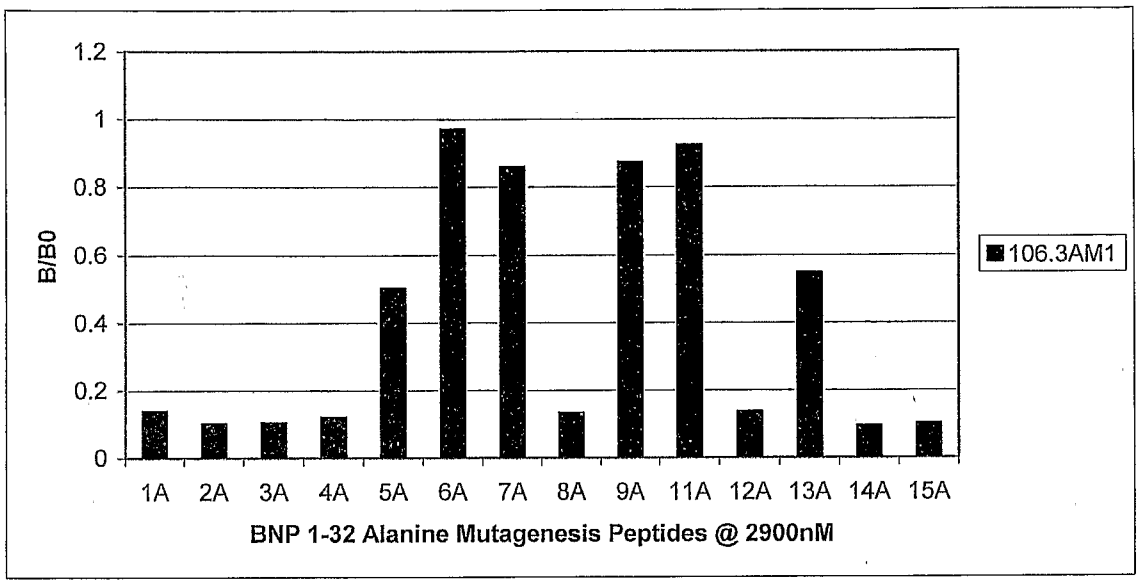
1
0.8
0.6
0.4
0.2
0
-0.2
B/B0
no peptide
BNP 1-32 cyclic
BNP 1-26
BNP 5-13
Various hBNP Peptides @ 181 nM

FIGURE 19


1.2
1
0.8
0.6
0.4
0.2
0
B/B0
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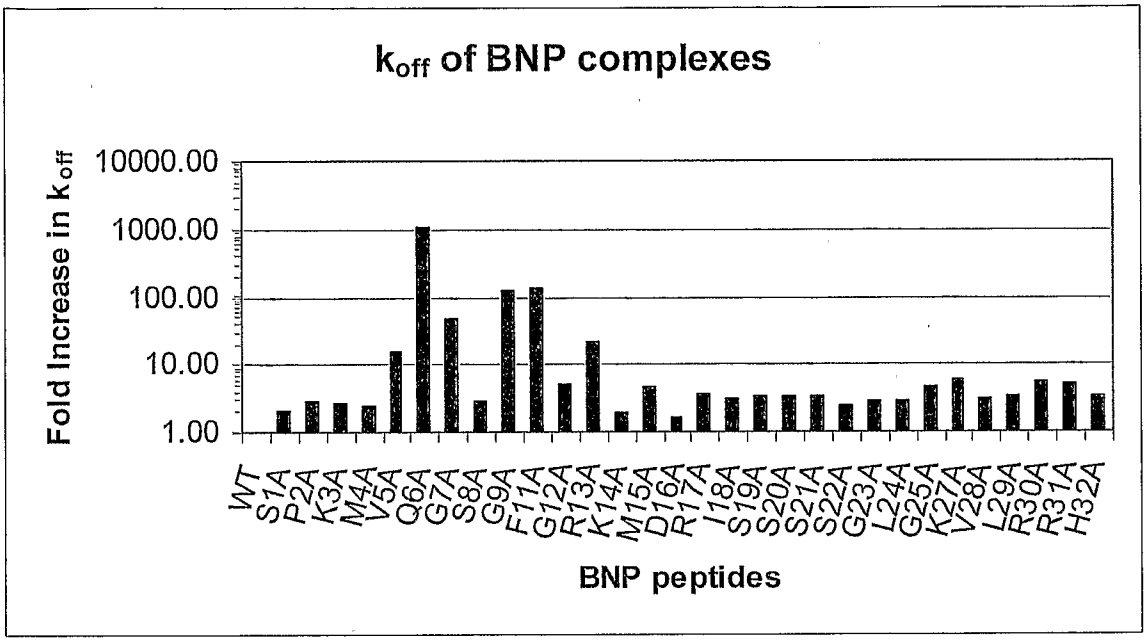
BNP 1-32 Alanine Mutagenesis Peptides @ 2900nM
106.3AM1

FIGURE 20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Fold Increase in koff
10000.00
1000.00
100.00
10.00
1.00
WT
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P2A
K3A
M4A
V5A
Q6A
G7A
S8A
G9A
F11A
G12A
R13A
K14A
M15A
D16A
R17A
I18A
S19A
S20A
S21A
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K27A
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R30A
R31A
H32A
BNP peptides

SEQUENCE LISTING

<110> Abbott Laboratories
Brophy, Susan El
Tyner, Joan D.
Tu, Bailin
Pinkus, Mary S.
Tieman, Brian C.
Shih, Jessie

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Ile Asn Thr His Thr Gly Glu Pro Ile Tyr Ala Asp Asp Phe Lys Gly
    50                  55                  60
Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Asn Thr Ala Tyr Leu Gln
65                  70                  75                  80
Ile Asn Asn Leu Asn Asn Gly Asp Met Gly Thr Tyr Phe Cys Thr Arg
                85                  90                  95
Ser His Arg Phe Gly Leu Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr
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Val Ser Asp Asn Val Leu Thr Gln Ser Pro Pro Ser Leu Ala Val Ser
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225                 230                 235                 240
Lys Arg Ala Ala Ala Leu Glu Ser Arg Gly Pro Phe Glu Gly Lys Pro
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<222> (3)...(5)

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1 5 10 15

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1 5 10 15
Gly

<210> 16
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<223> CDR L1 Mutation 26 S with A and Mutation 27 Q with Y and 27A with Ser to G

<400> 21
Lys Ala Ala Tyr Gly Val Asp Tyr Asn Gly Asp Ser Tyr Leu Asn
1 5 10 15

<210> 22
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> CDR L1 Mutation 26 S with N and Mutation 27 Q with W and 27A with Ser to P

<400> 22
Lys Ala Asn Trp Pro Val Asp Tyr Asn Gly Asp Ser Tyr Leu Asn
1 5 10 15

<210> 23
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> CDR L2 Mutation 53 N with C, Mutation 54 L with G and Mutation 55 E with W

<400> 23

Ala Ala Ser Cys Gly Trp Ser
1 5

<210> 24
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> CDR L2 Mutation 53 N with C, Mutation 54 L with A
and Mutation 55 E with P

<400> 24
Ala Ala Ser Cys Ala Pro Ser
1 5

<210> 25
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N= A, G, C, T; S = G, C ; Degenerate
Oligonucleotides

<400> 25
aacctcaaca atggagacat gggtacatat ttctgtacaa gannsnnsnn stttggtttg 60
gactactggg gtcaaggtac ctcagtcacc gtctcgtcag gtccc 105

<210> 26
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 26
aacctcaaca atggagacat gggtacatat ttctgtacaa gaagtnnsnn snnsggtttg 60
gactactggg gtcaaggtac ctcagtcacc gtctcgtcag gtccc 105

<210> 27
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 27
aacctcaaca atggagacat gggtacatat ttctgtacaa gaagtcacnn snnsnnsttg 60
gactactggg gtcaaggtac ctcagtcacc gtctcgtcag gtccc 105

<210> 28
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 28
aacctcaaca atggagacat gggtacatat ttctgtacaa gaagtcaccg gnnsnnsnns 60
gactactggg gtcaaggtac ctcagtcacc gtctcgtcag gtccc 105

<210> 29
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (55)...(63)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 29
aacctcaaca atggagacat gggtacatat ttctgtacaa gaagtcaccg gtttnnsnns 60
nnstactggg gtcaaggtac ctcagtcacc gtctcgtcag gtccc 105

<210> 30
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N= A, G, C, T; S = G, C; Degenerate

Oligonucleotides

<400> 30
gacatgggta catatttctg tacaagaagt caccggtttg gtnnsnnsnn stggggtcaa 60
ggtacctcag tcaccgtctc gtcaggtccc gccgccaag 99

<210> 31
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 31
tgggtgaagc agactccaag aaaggattta aagtggatgg gcnnsnnsnn sacccatact 60
ggagagccaa tatatgctga tgacttcaag ggacggtttg ccttc 105

<210> 32
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 32
tgggtgaagc agactccaag aaaggattta aagtggatgg gctggnnsnn snnscatact 60
ggagagccaa tatatgctga tgacttcaag ggacggtttg ccttc 105

<210> 33
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N= A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 33
tgggtgaagc agactccaag aaaggattta aagtggatgg gctggatann snnsnnsact 60
ggagagccaa tatatgctga tgacttcaag ggacggtttg ccttc 105

<210> 34
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 34
tgggtgaagc agactccaag aaaggattta aagtggatgg gctggataaa cnnsnnsnns 60
ggagagccaa tatatgctga tgacttcaag ggacggtttg ccttc 105

<210> 35
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (55)...(63)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 35
tgggtgaagc agactccaag aaaggattta aagtggatgg gctggataaa caccnnsnns 60
nnsgagccaa tatatgctga tgacttcaag ggacggtttg ccttc 105

<210> 36
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 36
ccaagaaagg atttaaagtg gatgggctgg ataaacaccc atnnsnnsnn sccaatatat 60
gctgatgact tcaagggacg gtttgccttc tctttggaaa cctct 105

<210> 37
<211> 105
<212> DNA
<213> Artificial Sequence

<220>

<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 37
ccaagaaagg atttaaagtg gatgggctgg ataaacaccc atactnnsnn snnsatatat 60
gctgatgact tcaagggacg gtttgccttc tctttggaaa cctct 105

<210> 38
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 38
ccaagaaagg atttaaagtg gatgggctgg ataaacaccc atactggann snnsnnstat 60
gctgatgact tcaagggacg gtttgccttc tctttggaaa cctct 105

<210> 39
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 39
ccaagaaagg atttaaagtg gatgggctgg ataaacaccc atactggaga gnnsnnsnns 60
gctgatgact tcaagggacg gtttgccttc tctttggaaa cctct 105

<210> 40
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (55)...(63)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 40
ccaagaaagg atttaaagtg gatgggctgg ataaacaccc atactggaga gccannsnns 60
nnsgatgact tcaagggacg gtttgccttc tctttggaaa cctct 105

<210> 41
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 41
aagtggatgg gctggataaa cacccatact ggagagccaa tannsnnsnn sgacttcaag 60
ggacggtttg ccttctcttt ggaaacctct gccaacactg cctat 105

<210> 42
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 42
aagtggatgg gctggataaa cacccatact ggagagccaa tatatnnsnn snnsttcaag 60
ggacggtttg ccttctcttt ggaaacctct gccaacactg cctat 105

<210> 43
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 43
aagtggatgg gctggataaa cacccatact ggagagccaa tatatgctnn snnsnnsaag 60
ggacggtttg ccttctcttt ggaaacctct gccaacactg cctat 105

<210> 44
<211> 105

<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 44
aagtggatgg gctggataaa cacccatact ggagagccaa tatatgctga tnnsnnsnns 60
ggacggtttg ccttctcttt ggaaacctct gccaacactg cctat 105

<210> 45
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (55)...(63)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 45
aagtggatgg gctggataaa cacccatact ggagagccaa tatatgctga tgacnnsnns 60
nnscggtttg ccttctcttt ggaaacctct gccaacactg cctat 105

<210> 46
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 46
aggaagcctg gagagacagt caagatctcc tgcaagggtt ctnnsnnsnn sttcacacac 60
tatggaataa actgggtgaa gcagactcca agaaaggatt taaag 105

<210> 47
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 47
aggaagcctg gagagacagt caagatctcc tgcaagggtt ctggannsnn snnsacacac 60
tatggaataa actgggtgaa gcagactcca agaaaggatt taaag 105

<210> 48
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 48
aggaagcctg gagagacagt caagatctcc tgcaagggtt ctggatatnn snnsnnscac 60
tatggaataa actgggtgaa gcagactcca agaaaggatt taaag 105

<210> 49
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 49
aggaagcctg gagagacagt caagatctcc tgcaagggtt ctggatatac cnnsnnsnns 60
tatggaataa actgggtgaa gcagactcca agaaaggatt taaag 105

<210> 50
<211> 105
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (55)...(63)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 50
aggaagcctg gagagacagt caagatctcc tgcaagggtt ctggatatac cttcnnsnns 60

nnsggaataa actgggtgaa gcagactcca agaaaggatt taaag 105

<210> 51
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 51
acagtcaaga tctcctgcaa gggttctgga taccttca cannsnnsnn sataaactgg 60
gtgaagcaga ctccaagaaa ggatttaaag tggatgggc 99

<210> 52
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 52
acagtcaaga tctcctgcaa gggttctgga taccttca cacacnnsnn snnsaactgg 60
gtgaagcaga ctccaagaaa ggatttaaag tggatgggc 99

<210> 53
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 53
acagtcaaga tctcctgcaa gggttctgga taccttca cacactatnn snnsnnstgg 60
gtgaagcaga ctccaagaaa ggatttaaag tggatgggc 99

<210> 54
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 54
ttggctgtgt ctctagggca gagggccacc atctcctgcn nsnnsnnsca aagtgttgat 60
tataatggtg atagttatct gaactggtac caacagaag 99

<210> 55
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 55
ttggctgtgt ctctagggca gagggccacc atctcctgca agnnsnnsnn sagtgttgat 60
tataatggtg atagttatct gaactggtac caacagaag 99

<210> 56
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 56
ttggctgtgt ctctagggca gagggccacc atctcctgca aggccnnsnn snnsgttgat 60
tataatggtg atagttatct gaactggtac caacagaag 99

<210> 57
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate

Oligonucleotides

<400> 57
ttggctgtgt ctctagggca gagggccacc atctcctgca aggccagcnn snnsnnsgat 60
tataatggtg atagttatct gaactggtac caacagaag 99

<210> 58
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 58
ttggctgtgt ctctagggca gagggccacc atctcctgca aggccagcca annsnnsnns 60
tataatggtg atagttatct gaactggtac caacagaag 99

<210> 59
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 59
gggcagaggg ccaccatctc ctgcaaggcc agccaaagtn nsnnsnnsaa tggtgatagt 60
tatctgaact ggtaccaaca gaagccagga cagccaccc 99

<210> 60
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 60
gggcagaggg ccaccatctc ctgcaaggcc agccaaagtg ttnnsnnsnn sggtgatagt 60
tatctgaact ggtaccaaca gaagccagga cagccaccc 99

<210> 61
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 61
gggcagaggg ccaccatctc ctgcaaggcc agccaaagtg ttgatnnsnn snnsgatagt 60
tatctgaact ggtaccaaca gaagccagga cagccaccc 99

<210> 62
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 62
gggcagaggg ccaccatctc ctgcaaggcc agccaaagtg ttgattatnn snnsnnsagt 60
tatctgaact ggtaccaaca gaagccagga cagccaccc 99

<210> 63
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 63
gggcagaggg ccaccatctc ctgcaaggcc agccaaagtg ttgattataa tnnsnnsnns 60
tatctgaact ggtaccaaca gaagccagga cagccaccc 99

<210> 64
<211> 93
<212> DNA
<213> Artificial Sequence

<220>

<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 64
atctcctgca aggccagcca aagtgttgat tataatggtn nsnnsnnsct gaactggtac 60
caacagaagc caggacagcc acccaaattc ctc 93

<210> 65
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 65
atctcctgca aggccagcca aagtgttgat tataatggtg atnnsnnsnn saactggtac 60
caacagaagc caggacagcc acccaaattc ctc 93

<210> 66
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 66
atctcctgca aggccagcca aagtgttgat tataatggtg atagtnnsnn snnstggtac 60
caacagaagc caggacagcc acccaaattc ctc 93

<210> 67
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 67
caacagaagc caggacagcc acccaaattc ctcatctatn nsnnsnnsaa tctagaatct 60
gggatcccag ccaggtttag tggcagtggg tctgggaca 99

<210> 68
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 68
caacagaagc caggacagcc acccaaattc ctcatctatg ctnnsnnsnn sctagaatct 60
gggatcccag ccaggtttag tggcagtggg tctgggaca 99

<210> 69
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 69
caacagaagc caggacagcc acccaaattc ctcatctatg ctgcannsnn snnsgaatct 60
gggatcccag ccaggtttag tggcagtggg tctgggaca 99

<210> 70
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 70
caacagaagc caggacagcc acccaaattc ctcatctatg ctgcatccnn snnsnnstct 60
gggatcccag ccaggtttag tggcagtggg tctgggaca 99

<210> 71
<211> 99

<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 71
caacagaagc caggacagcc acccaaattc ctcatctatg ctgcatccaa tnnsnnsnns 60
gggatcccag ccaggtttag tggcagtggg tctgggaca 99

<210> 72
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 72
catcctgtgg aggaggagga tgctgcaacc tattactgtn nsnnsnnsaa tgaggatcca 60
ttcacgttcg gctcggggac aaagttggaa ataaaacgg 99

<210> 73
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 73
catcctgtgg aggaggagga tgctgcaacc tattactgtc agnnsnnsnn sgaggatcca 60
ttcacgttcg gctcggggac aaagttggaa ataaaacgg 99

<210> 74
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (46)...(54)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 74
catcctgtgg aggaggagga tgctgcaacc tattactgtc agcaannsnn snnsgatcca 60
ttcacgttcg gctcggggac aaagttggaa ataaaacgg 99

<210> 75
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (49)...(57)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 75
catcctgtgg aggaggagga tgctgcaacc tattactgtc agcaaagtnn snnsnnscca 60
ttcacgttcg gctcggggac aaagttggaa ataaaacgg 99

<210> 76
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (52)...(60)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 76
catcctgtgg aggaggagga tgctgcaacc tattactgtc agcaaagtaa tnnsnnsnns 60
ttcacgttcg gctcggggac aaagttggaa ataaaacgg 99

<210> 77
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (40)...(48)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 77
gaggatgctg caacctatta ctgtcagcaa agtaatgagn nsnnsnnsac gttcggctcg 60

gggacaaagt tggaaataaa acgggcggcc gcc 93

<210> 78
<211> 93
<212> DNA
<213> Artificial Sequence

<220>
<221> variation
<222> (43)...(51)

<223> N = A, G, C, T; S = G, C; Degenerate
Oligonucleotides

<400> 78
gaggatgctg caacctatta ctgtcagcaa agtaatgagg atnnsnnsnn sttcggctcg 60
gggacaaagt tggaaataaa acgggcggcc gcc 93

<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> pYD41 vector specific primers

<400> 79
tagcatgact ggtggacagc 20

<210> 80
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> pYD41 rev vector specific primers

<400> 80
cgtagaatcg agaccgag 18

<210> 81
<211> 51
<212> DNA
<213> Artificial Sequence

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<223> Heavy chain CDR 2 region of AM1

<400> 81
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<210> 82
<211> 45
<212> DNA

<213> Artificial Sequence

<220>
<223> Light chain CDR 1 region of AM1

<400> 82
aaggccaact ggcccgttga ttataatggt gatagttatc tgaac 45

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/043608

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/26 C12N5/10 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 162 902 A1 (MISCHAK RONALD P [US] ET AL) 19 December 2000 (2000-12-19) cited in the application the whole document	1-50
Y	BODER ERIC T ET AL: "Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 97, no. 20, 26 September 2000 (2000-09-26), pages 10701-10705, XP002185398 ISSN: 0027-8424 the whole document	1-50

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2007	27/03/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Domingues, Helena

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/043608

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZAHND CHRISTIAN ET AL: "Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOCHEMICAL BIOLOGISTS, BIRMINGHAM,, US, vol. 279, no. 18, 30 April 2004 (2004-04-30), pages 18870-18877, XP002406477 ISSN: 0021-9258 pages 18871-4; table II	1-50
Y	ITOH H ET AL: "PREPARATION OF MONOCLONAL ANTIBODIES AGAINST BRAIN NATRIURETIC PEPTIDE AND THEIR APPLICATION TO RADIOIMMUNOASSAY AND PASSIVE IMMUNIZATION" ENDOCRINOLOGY, vol. 127, no. 3, 1990, pages 1292-1300, XP009080182 ISSN: 0013-7227 the whole document	1-50
Y	DATABASE WPI Week 199207 Derwent Publications Ltd., London, GB; AN 1992-053618 XP002423680 -& JP 03 297392 A (SHIONOGI & CO LTD) 27 December 1991 (1991-12-27) the whole document	1-50
T	TETIN SERGEY Y ET AL: "Interactions of two monoclonal antibodies with BNP: High resolution epitope mapping using fluorescence correlation spectroscopy" BIOCHEMISTRY, vol. 45, no. 47, 28 November 2006 (2006-11-28), pages 14155-14165, XP002423678 ISSN: 0006-2960	1-50

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/043608

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6162902 A1		NONE	
JP 3297392 A	27-12-1991	JP 2676114 B2	12-11-1997