

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 July 2009 (16.07.2009)

PCT

(10) International Publication Number
WO 2009/089295 A2

- (51) International Patent Classification:
C07K 16/00 (2006.01) C07K 16/10 (2006.01)
 - (21) International Application Number:
PCT/US2009/030351
 - (22) International Filing Date: 7 January 2009 (07.01.2009)
 - (25) Filing Language: English
 - (26) Publication Language: English
 - (30) Priority Data:
61/019,426 7 January 2008 (07.01.2008) US
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 - (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
 - (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished upon receipt of that report

(54) Title: ANTI-HIV DOMAIN ANTIBODIES AND METHOD OF MAKING AND USING SAME

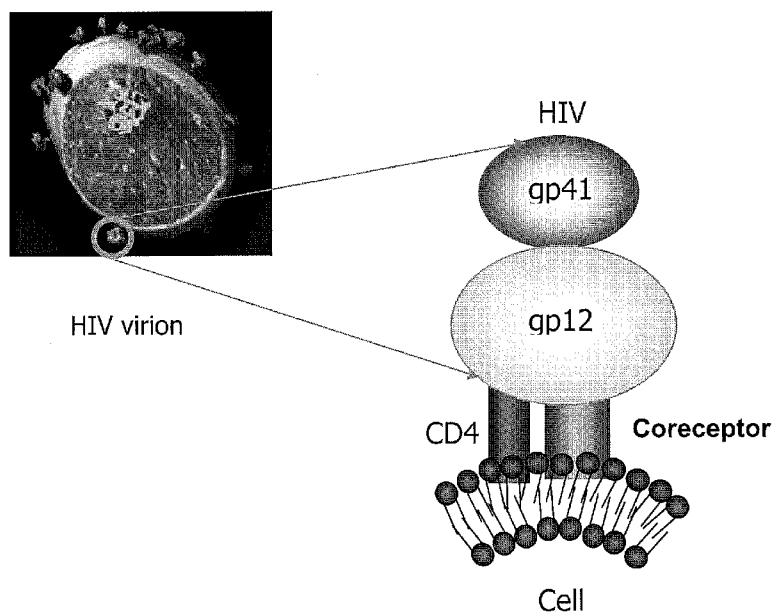


Figure 1

(57) Abstract: The invention provides single domain antibodies and derivatives thereof that bind antigens of interest, which are stable, soluble, and do not tend to aggregate. The invention also provides methods for constructing a dAb library and methods for screening dAb libraries to identify the dAb of the invention. The invention also provide methods of treating or preventing conditions by antigen neutralization by administering the dAbs of the invention.



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TITLE OF THE INVENTION

ANTI-HIV DOMAIN ANTIBODIES AND METHOD OF MAKING AND USING
SAME

INCORPORATION BY REFERENCE

5 This application claims priority to U.S. Provisional Application Serial No.
61/019,426, filed January 7, 2008, the entire contents of which are incorporated herein
by reference.

 All documents cited or referenced herein and all documents cited or referenced
in the herein cited documents, together with any manufacturer's instructions,
10 descriptions, product specifications, and product sheets for any products mentioned
herein or in any document incorporated by reference herein, are hereby incorporated by
reference, and may be employed in the practice of the invention.

BACKGROUND OF THE INVENTION**1. Field of the Invention**

15 The present invention relates to domain antibodies, including derivatives,
fusions and/or fragments thereof, having specificity for HIV and to their identification
and manufacture. The invention further relates to the use of the domain antibodies of
the invention in treating, preventing, and/or diagnosing various conditions, and
particularly, HIV infections.

20 2. Background

 The life-cycle of the human immunodeficiency virus (both HIV-1 and HIV-2) is
well known. HIV primarily infects cells of the human immune system, such as helper T
cells (specifically CD4+ T cells), macrophages and dendritic cells. Entry to cells of the
immune system is mediated through interaction of the virion envelope glycoproteins
25 (gp120 and gp41) with the receptor CD4 on the target cells. In addition, viral entry is
modulated through at least two co-receptors known as CXCR4 and CCR5, which are
members of the chemokine receptor family of proteins, and have been shown to
function with CD4 as coreceptors for HIV-1 isolates that are tropic for T-cell lines or
macrophages, respectively (Feng et al., 1996, Science 272:872-876; Alkhatib et al.,
30 1996, Science 272:1955-1958; Deng et al., 1996, Nature 381:661-666; Dragic et al.,

1996, *Nature* 381:667-673). Other molecules in this family including CCR3 and CCR2b, also appear to function as cofactors for some HIV-1 isolates (Doranz et al., 1996, *Cell* 85:1149-1158; Berson et al., 1997, *J. Virol.* 71: 1692-1696; Choe et al., 1996, *Cell* 85:1135-1148).

35 Current anti-HIV therapy includes the use of compounds which inhibit various aspects of the HIV life-cycle, including entry, fusion and replication in a target cell. While these therapies, particularly when used in combination with one another, are effective, they are frequently short-lived in that the viral strains rapidly develop resistance to one or more of the compounds used—a widespread and major problem in
40 the current approaches in treating HIV infections.

 Antibodies represent yet another promising approach in the treatment of HIV infections. Human monoclonal antibodies (mAbs) currently represent an important and growing technology in the development of inhibitors, vaccines, diagnostic and research tools. In fact, 22 mAbs have been approved by the US Food and Drug Administration
45 against various disease in the past several decades for various disease indications, including rheumatoid arthritis (Centacor's REMICADE and Abbott Laboratories' HUMIRA), non-Hodgkin's lymphoma (Genentech's RITUXAN and IDEC's ZEVALIN) and respiratory syncytial virus infection (Medimmune's SYNAGIS). Many other antibody drug candidates are in the late stages of clinical trials and, as such,
50 antibodies are now well established as both highly potent and well tolerated therapeutics.

 However, no mAbs have yet been approved for clinical use against HIV-1. A fundamental problem in the development of HIV-1-neutralizing antibodies is the virus's innate ability to escape human immune surveillance during the long chronic infection.
55 Several known mAbs, however, have been shown to exhibit potent and broad HIV-1 neutralizing activity in vitro, and can prevent HIV-1 infection in animal models (reviewed in Burton, 2002, Ferrantelli et al., 2002, and Veazey et al., 2003). A recent clinical trial suggested that two of these broadly HIV-1 neutralizing human mAbs, 2F5 and 2G12, lack side effects in humans (Armbruster et al., 2002; Stiegler et al., 2002).
60 However, the potency of 2F5 and 2G12 used in combination in this clinical trial was

significantly lower than currently available treatments and relapses occurred (Stiegler et al., 2002). Further increase in the potency of anti-HIV antibodies and/or new, more effective anti-HIV antibodies would be a significant advancement in the art.

Another fundamental problem in the development of effective therapeutic
65 antibodies against HIV is the problem of epitope accessibility. It has been reported that some epitopes are sterically inaccessible to full size antibodies. For example, a study relating to HIV CD4-inducible (CD4i) epitopes by one of the present inventors has suggested that the size of the CD4i-specific neutralizing antibodies inversely correlates with neutralization efficiency and that perhaps antibody fragments might be more
70 effective than whole antibodies in neutralizing the virus at such epitopes. See Labrijn et al., *J. Virol.*, 2003. The study suggests that HIV's ability to evade the host's immune system may be linked in part to its having found a way to sterically hinder full-sized antibodies from accessing the CD4i epitopes. This study was limited, however, to exploring the effectiveness of scFv and Fab antibody fragments.

75 In the late 1980s, domain antibodies were identified as the smallest known antigen-binding fragments (Holt et al., 2003). Structurally, domain antibodies comprise the single chain variable heavy (VH) or variable light (VL) polypeptides, and due to their single-chain nature, range in size of only 11 kDa to 15 kDa. Domain antibodies, however, have a number of acknowledged problems to overcome to be suitable as
80 potential therapeutics. Domain antibodies, particularly those derived from human antibodies, suffer from poor stability and solubility, and have a tendency to aggregate due to exposed regions of hydrophobicity in the absence of the paired VH or VL.

New and effective domain antibodies which would overcome the problems in the art, and an effective means of identifying and obtaining such domain antibodies,
85 would be a valuable advance in the art. Such antibodies could be the basis of new methods and approaches for treating and/or prophylaxis of a variety of infections and conditions, such as HIV or cancer, in particular, infections and conditions which are capable of evading the immune system or therapeutic compounds and antibodies because certain epitope targets are sterically restricted.

90 **SUMMARY OF THE INVENTION**

The present invention relates to single domain antibodies that overcome the known problems in the art relating to domain antibodies and other anti-HIV antibodies, particularly poor stability, solubility and effectiveness. The domain antibodies of the invention show high affinity for their target epitopes, are highly expressed, are stable, and are capable of potent neutralization of a broad range of HIV isolates. The invention also relates to a novel VH framework identified by the present inventors that can be used as the basis of a highly diverse dAb library from which the inventive domain antibodies having potent neutralization activity against a broad range of HIV isolates can be obtained. The novel VH framework of the invention unexpectedly showed a high degree of compatibility with a wide diversity of CDR sequences, displayed properly folded dAbs, and expressed dAbs at high levels. The present invention also relates to novel fusion proteins containing the domain antibodies of the invention fused to agents (e.g., proteins) which function to enhance the stability of the dAbs of the invention and/or to enhance the effectiveness of the domain antibodies against their targets. In addition, the present invention provides therapeutic methods employing the domain antibodies and fusion proteins comprising the domain antibodies of the invention for treating and/or preventing HIV infections. Methods for preparing and screening domain antibody libraries are also provided herein.

In one embodiment, the present invention provides an isolated domain antibody or fragment thereof according to the amino acid sequence of m36, or an amino acid molecule having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with the amino acid sequence of m36. In one aspect, the domain antibody or fragment can be immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive isotopes, or antiviral agents. In another aspect, the antiviral agent can be zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

In another embodiment, the present invention provides an isolated domain

120 antibody or fragment thereof comprising (a) the m0 framework amino acid sequence or
an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or
99% sequence identity thereto and (b) the CDR3 amino sequence of m36 or an amino
acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%
sequence identity thereto. In another aspect, the domain antibody or fragment thereof
125 can be immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents,
natural or synthetic toxins, radioactive isotopes, or antiviral agents. In a further aspect,
the antiviral agent can be zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine,
trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir,
indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

130 In yet another embodiment, the present invention provides an isolated domain
antibody or fragment thereof comprising (a) the m0 framework amino acid sequence or
an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or
99% sequence identity thereto and (b) at least one of CDR1, CDR2 or CDR3 of m36 or
an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or
135 99% sequence identity thereto. In one aspect, the domain antibody or fragment thereof
can be immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents,
natural or synthetic toxins, radioactive isotopes, or antiviral agents. In another aspect,
the antiviral agent can be zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine,
trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir,
140 indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

In still another embodiment, the present invention relates to a domain antibody
framework for the construction of a domain antibody library, said domain antibody
framework comprising the framework sequence of m0 or an amino acid sequence
having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity
145 thereto.

In yet another embodiment, the present invention provides a fusion protein
comprising a domain antibody or fragment thereof and a fusion partner, wherein the
domain antibody or fragment thereof is the amino acid sequence of m36, or an amino
acid molecule having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%

150 sequence identity with the amino acid sequence of m36. In yet another embodiment,
the present invention relates to a fusion protein comprising a domain antibody or
fragment thereof and a fusion partner, wherein the domain antibody or fragment thereof
comprises (a) the m0 framework amino acid sequence or an amino acid sequence
having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity
155 thereto and (b) at least one of CDR1, CDR2 or CDR3 of m36 or an amino acid
sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence
identity thereto.

In certain aspects, the fusion protein can be immunoconjugated to one or more
cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive
160 isotopes, or antiviral agents. The antiviral agent can be zidovudine, acyclovir,
gancyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet,
amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir,
adefovir, clevadine, entecavir, or pleconaril.

In yet another aspect, the fusion partner of the fusion proteins of the invention
165 can be fused to the m36 or fragment thereof via a linker, such as, for example, a human
IgG hinge region.

In certain other aspects, the fusion partner of the fusion proteins of the invention
increases the stability of m36 or fragment thereof as compared to the m36 or fragment
thereof alone. In certain other aspects, the fusion partner of the fusion proteins of the
170 invention induces a CD4i epitope on Env thereby synergistically increases the
effectiveness of the domain antibodies of the invention.

In one aspect, the fusion partner of the fusion proteins of the invention can be
serum albumin-binding protein.

In certain other aspects, the fusion partner of the fusion proteins of the invention
175 can be CD4 or a fragment or mimic thereof.

The fusion protein of claim 21, wherein the fusion partner is fused to the domain
antibody or fragment thereof via a linker. In a further aspect, the linker can be an IgG
hinge region.

In still another embodiment, the present invention provides a pharmaceutical

180 composition comprising a therapeutically effective amount of a domain antibody or a fusion protein in accordance with the invention and a pharmaceutically acceptable salt.

In another embodiment, the invention relates to a method of treating and/or preventing an HIV infection in a patient in need thereof comprising, administering a therapeutically effective amount of (a) a domain antibody of the invention (b) a fusion
185 protein of the invention, or (c) a pharmaceutical composition comprising a domain antibody or a fusion protein of the invention. In one aspect, the treatment method can further include co-administering a cytokine, anti-angiogenic agent, immunotherapeutic agent, anti-cancer agent, anti-bacterial agent, or anti-viral agent. In still another aspect, the treatment method further comprises co-administering a therapeutically effective
190 amount of soluble CD4 (sCD4) or functional fragment or mimic thereof.

In yet another embodiment, the present invention provides a domain antibody library comprising a plurality of unique clones wherein each unique clone comprises the framework sequence of m0 or an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto. In one aspect, the
195 domain antibody library comprises at least 10^8 or 10^9 or 10^{10} unique clones.

In still another embodiment, the present invention provides a method of constructing a dAb library comprising the steps of (a) obtaining an isolated VH framework nucleotide sequence and (b) introducing a CDR1, CDR2 and CDR3 repertoire into the isolated VH framework nucleotide sequence, thereby forming a dAb
200 library. In one aspect, the step of introducing the CDR1, CDR2 and CDR3 repertoire is achieved by graft replacement of the corresponding CDR sequences of the VH framework with the CDR1, CDR2 and CDR3 repertoire. In another aspect, the CDR1, CDR2 and CDR3 repertoire sequences can be obtained by PCR amplification using the primers of Table 1 and a non-immunized or immunized human antibody library
205 template. In still another aspect, the graft replacement can be achieved by extension reactions between the PCR CDR products and the VH framework. In another aspect one, two or all CDRs could be mutagenized. The dAb library of the invention can be a phage-display library.

In still another embodiment, the present invention provides a method of

210 identifying a dAb that binds to an antigen of interest, comprising panning the dAb
phage-display library of the invention with an antigen of interest. In one aspect, the
antigen of interest is HIV-1 CD4i antigen. In another aspect, the panning can be
sequential panning using at least two antigens. The at least two antigens can be two
HIV-1 CD4i antigens from different HIV-1 isolates.

215 In still another embodiment, the present invention provides a method for treating
or preventing an HIV infection comprising administering in a therapeutically effective
amount a composition comprising a dAb that binds to HIV-1 CD4i with a dissociation
constant (K_d) of about 1 nM to about 500 nM.

These and other embodiments are disclosed or are obvious from and
220 encompassed by, the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description, given by way of example, but not intended
to limit the invention solely to the specific embodiments described, may best be
understood in conjunction with the accompanying drawings.

225 **Figure 1** is a simplified depiction of the general structure of the HIV envelope
glycoprotein (Env) bound to CD4 and a coreceptor at the surface of a target cell. The
HIV Env complex is embedded in the HIV envelope and consists of three (3) molecules
of gp120 and a stem consisting of three (3) molecules gp41 of which only one is shown.
The coreceptor can include chemokine receptors, CCR5 or CXCR4.

230 **Figure 2** depicts a general overview of a phage display antigen panning process
that can be used to identify the antibodies of the invention.

Figure 3 depicts in ribbon form the general structure of a human IgG molecule,
with emphasis on the VH region, which may form a domain antibody of the invention.

235 **Figure 4** is a photograph of an SDS-PAGE gel showing the R3H1 Fab and its
VH domain, m0, of the invention. The VH domain, m0, was identified by panning a
human naïve antibody library, which had been recently constructed according to the
procedure and primers described in (de Haard HJ et al. 1999. J. Biol. Chem. 274:18218-
30) but with one-step overlapping PCR cloning (Zhu Z and Dimitrov DS, in press,
Therapeutic antibodies, A. Dimitrov, Ed., Methods in Molecular Biology, Humana

240 Press), with an HIV-1 antigen, recombinant soluble Env with truncated transmembrane portion and cytoplasmic tail - gp140. The R3H1 Fab was identified with a stop codon in the light chain, after which its reading frame was corrected. The VH domain of R3H1 was sub-cloned into a phagemid vector, expressed and purified.

Figure 5 depicts the m0 VH framework. The m0 gene and the deduced amino acid sequence were compared to human VH germline sequences from IMGT (ImMunoGeneTics) database (http://imgt.cines.fr/textes/IMGTrepertoire/Proteins/protein/human/IGH/IGHV/Hu_IGHVallgenes.html). The alignments to the known human VH germline sequences indicated VH3-23*04 as having the closest sequence identity with m0 although there are still significant differences. The figure displays the m0 amino acid sequence on the top row, followed below by its corresponding nucleotide sequence, and followed below the aligned VH3-23*04 sequences showing only those amino acid residues and nucleotide residues differing from the m0 sequences. The CDR (complementary determining region) and FR (framework) regions are shown according to IMGT numbering system. The italicized regions flanking the CDR2 and CDR3 regions at their 5' and 3' ends correspond to the target sites for the primers used herein to amplify CDR2 and CDR3 segments from other sources in accordance with the invention, e.g. as described in Example 2. Figure 5B provides separate amino acid and nucleotide sequences for m0, indicating the locations of the CDR sequences.

260 **Figure 6** illustrates a process of constructing the VH library, which comprises the m0 framework and a diversity of CDR sequences from different sources. The VH library was constructed in three steps. In the first step, PCRs were performed to amplify CDR2 and CDR3 gene segments from various antibody libraries (plasmid DNA containing human antibody gene fragments). CDR2 amplification products from different libraries were pooled. Similarly, CDR3 amplification products generated from different libraries were pooled together. The CDR1 segment was prepared by PCR amplification from a m0 template using the degenerate primer H1R covering the entire length of CDR1 to generate mutations at four positions to A, D, S or Y. The FR3 segment was also obtained from m0 for assembly of entire VHs. In the second step,

270 overlapping PCRs were performed to join CDR1s to CDR2s and FR3 to CDR3s, respectively. In the third step, entire VHs were assembled by overlapping PCR of the products of the second step. The products were cloned into a phagemid vector and a library was obtained by performing electroporations as described in the Examples.

Figure 7 is a photograph of an SDS-PAGE gel showing soluble VHs selected against HIV-1 antigens. Twelve positive clones (c3, c6, d1, d7, b4, c11, d10, b3, b5 (m36), b7, g6 and e11) were selected after three rounds of sequential panning of library with HIV-1 antigens gp120-CD4 and gp140, expressed and purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA resin.

Figure 8 illustrates the sequence diversity analysis of the VH domain library of the invention, and as described in Example 2. To evaluate the sequence diversity of the library, clones were randomly selected and sequenced. Regarding the diversity of CDR1 sequences, Figure 8A shows the frequency of A/D/S/Y usage in each mutated position. Regarding the diversity of CDR2, Figure 8B provides two bar graphs that show the number of VH germline subgroups of 1-7 as compared to the frequency of the appearance of the corresponding CDR2 in the library. Figure 8C is a bar graph showing the frequency of mutations in CDR2. Regarding CDR3 sequence diversity, Figure 8D provides bar graphs comparing the frequency of CDR3 lengths in vivo against the frequency of library clones having the same lengths.

Figure 9 shows the diversity in the lengths of the CDR3s of the different human VH germlines genes.

Figure 10 shows the frequency of A/D/S/Y residues at positions 27, 28, 29, 30, 31 and 32 in CDR1 before selection against Protein A versus after selection against Protein A in accordance with Example 2. Selection against Protein A was carried out, as explained in Example 2, to evaluate the extent to which correct folding occurred in the VH library of phagemid clones.

Figure 11 shows SDS-PAGE gels of three sets of clones grouped in A, B and C gels. For each gel, the clone number, the origin of the CDR2, the length in amino acids of CDR3, and the yield in milligrams are indicated for each set of clones. See Example

2 for further description. The figure shows the expression profile before (11A and B)
300 and after (11C) panning with antigens.

Figure 12 shows the results of size exclusion chromatography analysis to measure the oligomerization of select isolated VHs, c6, d7, b5 (m36), b7 and d10. See Example 2 for further description.

Figure 13 shows the results of size exclusion chromatography analysis to
305 measure the oligomerization of m36. Purified m36 in PBS was subjected to size exclusion chromatography with Superdex75 column calibrated with protein molecular mass standard shown by the arrows.

Figure 14 shows the binding specificity of m36. Binding of m36 was tested by ELISA using Corning high-binding 96-well plates coated with 1 $\mu\text{g/ml}$ of antigens.
310 Bound m36 were detected by adding 1:5000 diluted HRP-conjugated anti-FLAG antibody. The assay was developed at 37°C with ABST substrate and monitored at 405 nm as described.

Figure 15 shows that the binding of m36 to gp120 is induced by the gp120 interaction with CD4. gp120_{Bal}-CD4 was coated on Corning high-binding 96-well
315 plates. M36 with consensus concentration was mixed with either a known CD4i antibody (m16) or CD4bs antibody (m14) (negative control) in IgG format serially diluted, and added to 96-well plates coated with antigen. Bound m36 were detected by 1:5000 diluted HRP-conjugated anti-FLAG antibody. The assay was developed at 37°C with ABST substrate and monitored at 405 nm. See Figure 27 for complementary data
320 showing the specific binding of m36 to gp120_{Bal}-CD4 but not to gp120_{Bal} alone.

Figure 16 shows the cross-reactivity of m36. GXC gp140 from an isolate of clade C was coated in the presence or absence of CD4. Serially diluted m36 was added and detected by HRP-conjugated anti-FLAG antibody. The assay was developed at 37°C with ABST substrate and monitored at 405 nm.

Figure 17 shows the results of testing the IC₅₀ (the minimum inhibitory concentration needed to inhibit or neutralize 50% of the total virus), IC₉₀ (the minimum inhibitory concentration needed to inhibit or neutralize 90% of the total virus) and %In (percentage inhibition for the highest concentration of antibody – m36: 10 $\mu\text{g/ml}$; scFv
325

m9: 20µg/ml; c34: 4 µg/ml) of m36 antibody, scFv m9 antibody, and c34 antibody
330 against 11 different HIV-1 isolates from a total of 5 clades, A, B, C, D and E.

Figure 18 shows the amino acid and nucleotide sequences for m36. The CDR1, CDR2 and CDR3 sequences are indicated. Also presented are the amino acid and nucleotide sequences of the human germline gene VH3-48*03. Only those amino acid and nucleotide residues that differ from the m36 sequences are indicated in the figure.

335 **Figure 19** provides the nucleotide and amino acid sequences of m36.

Figure 20 provides a schematic representations of the m0 master framework and the cloning region of phagemid vector. **(A)** m0 master framework is analyzed using IMGT/V-QUEST tool provided by IMGT immunoglobulin database (http://imgt.cines.fr/IMGT_vquest/vquest?livret=0&Option=humanIg). The FRs and CDRs regions of the master gene are indicated according to the database. **(B)** Brief
340 description of the cloning region of the phagemid vector phagemid vector. SfiI restriction sites are frequently used for cloning of genes. Hexahistidine tag and HA tag are included for purification and detection of protein products.

Figure 21 shows the preparation of cDNA by reverse transcription of total RNA
345 from human peripheral blood mononuclear cells. Total RNA was extracted from human mononuclear cells with RNeasy Mini Kit (Qiagen, Cat. #74104) as described below in Example 2. Using a SuperScript III First-Strand Synthesis System (Invitrogen, Cat. #18080-051) containing oligo (dT)₂₀ primers and random hexamers, total RNA was then reverse transcribed into cDNA. The cDNA products were separated on a 0.8%
350 (w/v) agarose gel. The cDNA 1 and cDNA 2 are from reactions using oligo (dT)₂₀ primers and random hexamers, respectively. Two molecular weight DNA mass markers, Marker 1 (Invitrogen Cat. #15628-019) and Marker 2 (Invitrogen Cat. #10787-018) were included.

Figure 22 demonstrates the results obtained from PCR amplification of CDR2
355 and CDR3 repertoires from cDNA. **(A)** Eight recombinations of primers were used for CDR2s amplification as described below in Example 2. The products of the first five recombinations (H2-F1/H2-R1, H2-F1/H2-R2, H2-F1/H2-R5, H2-F2/H2-R3, H2-F3/H2-R1, and H2-F3/H2-R2) were shown on lane 1 to lane 5, respectively. **(B)** Three

recombinations of primers were used for CDR3 amplification. The products of the first
360 two recombinations (H3-F1/H3R and H3-F2/H3R) were shown on lane 1 and 2,
respectively. The correct-sized bands were indicated by arrows.

Figure 23 depicts the construction of a human antibody VH library. A stable
VH (m0) was used as a scaffold for grafting CDR2s and CDR3s from five human
antibody Fab libraries. CDR1 residues #27, 29, 31 and 32 (IMGT numbering system)
365 were randomized to A, D, S or Y. The numbers denote the positions of the amino acid
residues corresponding to the respective regions of the antibody VH gene where the
CDRs were grafted; the # denotes the positions of the CDR1 randomization. The SfiI
denotes the restriction enzyme sites used for cloning.

Figure 24 shows an alignment of CD4i antibody VH sequences. The amino acid
370 sequences of 12 known CD4i antibody VHs were obtained from a previous report
(Huang et al. (2004), Proc. Natl. Acad. Sci. USA, 101:2706-2711, the contents of which
are incorporated herein by reference). A multiple sequence alignment of these
antibodies in addition to m36 is shown with CDRs indicated. Sequences are ordered
based on the CDR3 lengths; IMGT numbering system is used. The acidic CDR
375 residues are underlined with red. The gene usage with the FR and CDR regions and
their CDR3 lengths are shown on the right.

Figure 25 provides a table showing pseudotyped virus neutralization by m36
and its fusion proteins, as described below in Example 3. The table provides the
antibody concentration (nM) resulting in 50% inhibition of virus infection (IC_{50}).

380 **Figure 26** shows potent m36-mediated neutralization of viruses pseudotyped
with Envs of HIV-1 primary isolates. (A) Dose-dependent inhibition of Bal by m36,
scFv and C34, respectively. (B) Percentage inhibition of a panel of viruses by m36,
scFv m9 and C34 at 667 nM, respectively.

Figure 27 show that the binding of m36 to gp120 is induced by the gp120
385 interaction with CD4. The graph shows the specific binding of m36 to gp120_{Bal}-CD4
but not to gp120_{Bal} alone. See Figure 15 for complementary data showing the
competition of m36 with a CD4i antibody (IgG m16) for binding to gp120_{Bal}-CD4.

Figure 28 depicts the design of m36 fusion proteins described in Example 3. Schematic representation of m36 fused with SAAb, human IgG1 CH3 domain or Fc. The names of the constructs and their molecular weights are also shown. The sequences of the SAAb and the linkers used to join m36 with Fc by human IgG1 and IgG3 hinge, and camel IgG2 hinge are QRHPEDICLPRWGCLWGDDD (SEQ ID NO: 1), DKTHTCPPCP (SEQ ID NO: 2), EPKIPQPQPKPQPQPQPQPKPQKPEPECTCPKCP (SEQ ID NO: 3), and ELKTPLGDTTHTCPRCPEPKSCDTPPPCPRCP (SEQ ID NO: 4), respectively.

Figure 29 depicts the cloning of m36 and its fusion proteins of Figure 28. Schematic representation of the m36 gene cassettes, and its fusion proteins (A) and plasmids used for cloning (B).

Figure 30 shows SDS-PAGE of m36 and its fusion proteins under reducing (-) and non-reducing (+) conditions.

Figure 31 demonstrates that m36 and its fusion proteins have similar binding activities. (A) Binding of m36, m36SAAb, m36CH3 and m36b0Fc to gp120_{Bal}-CD4 and gp120_{Bal}, respectively. (B) Binding of m36h1Fc, m36c2Fc and m36h3Fc to gp120_{Bal}-CD4 and gp120_{Bal}, respectively.

Figure 32 provides a table showing pseudotyped virus neutralization by m36 and its fusion proteins, as described below in Example 3. The table provides the antibody concentration (nM) resulting in 90% inhibition of virus infection (IC₉₀).

Figure 33 demonstrates that pre-triggering (sensitization) of virus by sCD4 dramatically increases neutralization by large molecules fused with m36. Viruses were pre-inoculated with different concentrations of antibodies and/or sCD4 at 8 nM for 1 h at 37°C, and then the mixture was added to 1.5×10^4 HOS-CD4-CCR5 cells grown in each well of 96-well plates. Luminescence was measured 48 h post infection and percentage inhibition was calculated as described in Example 3.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have developed new and useful single domain antibodies that overcome the known problems in the art relating to domain antibodies, particularly those problems associated with domain antibodies derived from human antibodies.

Unlike the domain antibodies of the art, the antibodies of the invention are stable, highly soluble, and do not tend to form aggregates or polymerization products in solution. In addition, the domain antibodies of the invention have high affinity for their target epitopes and are highly expressed. The advantageous features of the inventive antibodies of the invention stem at least in part to the novel VH framework identified by the present inventors that is used as the basis of a highly diverse dAb library from which the inventive antibodies can be obtained. The novel VH framework of the invention unexpectedly and surprisingly showed a high degree of compatibility with a wide diversity of CDR sequences, maintains proper folding, expresses dAbs at high levels and which are highly soluble.

It is to be understood that present invention as described herein is not to be limited to the particular details set forth herein regarding any aspect of the present invention, including, the anti-HIV domain antibodies and variants and/or fragments thereof, VH framework, method of preparing diverse dAb library, methods of treatment, protocols, cell lines, animal species or genera, constructs, immunoconjugates and reagents described and, as such, may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, the term “**antibody**” refers to immunoglobulin molecules (*e.g.*, any type, including IgG, IgE, IgM, IgD, IgA and IgY, and/or any class, including, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) isolated from nature or prepared by recombinant means or chemically synthesized. The terms “**antibody**” and “**immunoglobulin**” can be used synonymously throughout the specification, unless indicated otherwise.

As used herein, the term “**antibody fragment**” refers to a portion of a whole antibody which retains the ability to exhibit antigen binding activity. Examples include, but are not limited to, Fv, disulphide-linked Fv, single-chain Fv, Fab, variable heavy

region (V_H), variable light region (V_L), and fragments of any of the above antibody fragments which retain the ability to exhibit antigen binding activity, e.g., a fragment of
450 the variable heavy region V_H retains its ability to bind its antigen.

As used herein, the term “**antibody framework**” is intended to mean the portion of an antibody variable domain which serves as a scaffold for the antigen binding loops of the variable domain, i.e., the CDR sequences, which is the same definition proffered by Kabat et al. 1991, which is incorporated herein by reference).

455 As used herein, the term “**complementarity determining regions**” (CDRs), or synonymously, antibody CDR, refers to the amino acid segments of an antibody that function as the antigen binding loops, as defined by Kabat et al. (1991). Each of the two variable domains of an antibody Fv fragment each contain three CDRs. The CDRs for the antigen binding loops, or synonymously, the “**antigenic binding site**”.

460 As used herein, the term “**domain antibody**” (dAb) refers to an antibody whose complementary determining regions (CDRs) are part of a single domain polypeptide. Examples include, but are not limited to, variable heavy region (V_H) or fragment thereof, variable light region (V_L) or fragment thereof, heavy chain antibody (i.e. antibody devoid of light chain), single domain antibodies derived or engineered from
465 conventional 4-chain antibodies, engineered antibodies and single domain scaffolds, wherein the scaffolds can be derived from any natural or synthetic source of antibody. The CDRs can be from any natural or synthetic source, and can be modified by any known or suitable means, e.g. site-directed mutagenesis.

As used herein, the term “**framework region**” refers to the nucleic acid
470 sequence regions of an antibody gene that encode the structural elements of the molecule. In a domain antibody, e.g. the V_H or V_L of a human IgG, the framework region represents the sequences surrounding each of the three CDR sequences of the fragment polypeptides.

As used herein, the term “**library**” refers to a collection of nucleic acid
475 sequences, wherein the individual nucleic acid molecules are carried or contained in a suitable vector, e.g. a DNA vector, expression vector, phagemid vector.

As used herein, the term “**naive library**” refers to a collection of nucleic acid sequences encoding the naturally occurring V_H or V_L repertoire from a non-immunized source.

480 As used herein, the term “**repertoire**” refers to the genetic diversity of a collection of molecules, e.g. a collection of CDR sequences.

As used herein, the terms “**biological sample**” or “**patient sample**” as used herein, refers to a sample obtained from an organism or from components (e.g., cells) of an organism. The sample may be of any biological tissue or fluid. The sample may be
485 a clinical sample which is a sample derived from a patient. Such samples include, but are not limited to, sputum, blood, serum, plasma, blood cells (e.g., white cells), tissue samples, biopsy samples, urine, peritoneal fluid, and pleural fluid, saliva, semen, breast exudate, cerebrospinal fluid, tears, mucous, lymph, cytosols, ascites, amniotic fluid, bladder washes, and bronchioalveolar lavages or cells therefrom, among other body
490 fluid samples. The patient samples may be fresh or frozen, and may be treated, e.g. with heparin, citrate, or EDTA. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes.

As used herein and in the appended claims, the singular forms “a,” “and,” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for
495 example, reference to “a gene” is a reference to one or more genes and includes equivalents thereof known to those skilled in the art, and so forth.

As used in this invention, the term “**epitope**” means any antigenic determinant on an antigen, e.g. a gp120 protein, to which an antibody binds through an antigenic binding site. Determinants or antigenic determinants on an antigen usually consist of
500 chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. In one embodiment of the invention, the HIV-1 epitope is the CD4-inducible epitope (“CD4i”), which becomes exposed on gp120 only after gp120 binds to the CD4 receptor.

505 As used herein, the term antibody that “**specifically binds to**” or is “**specific for**” a particular polypeptide or an epitope on a particular polypeptide is one that binds

to that particular polypeptide or epitope on a particular polypeptide without substantially binding to any other polypeptide or polypeptide epitope. Alternatively, an antibody that specifically binds to an antigen, in accordance with this invention, refers to the binding of an antigen by an antibody or fragment thereof with a dissociation constant (K_d) of $1\mu\text{M}$ or lower, as measured by surface plasmon resonance analysis using, for example, a BIACORE® surface plasmon resonance system and BIACORE® kinetic evaluation software (eg. version 2.1). The affinity or dissociation constant (K_d) for a specific binding interaction is preferably about 500 nM or lower, more preferably about 300 nM or lower and preferably at least 300 nM to 50 pM, 200 nM to 50 pM, and more preferably at least 100 nM to 50 pM, 75 nM to 50 pM, 10 nM to 50 pM.

As used herein, the term “**fusion protein**” refers to two or more polypeptides coupled together which are not naturally found in a coupled arrangement. This can include translational fusions of two polypeptides, e.g., an antibody of the invention translationally fused via recombinant means to a fusion partner, such as an adduct molecule to enhance the stability of the antibody. Fusion proteins can also include proteins, e.g., an antibody of the invention, which have been physically coupled to another polypeptide. The two polypeptides in either case can be joined via a linker molecule.

As use herein, the term “**fusion partner**” refers to each of the polypeptides of a fusion protein.

As used herein, the term “**linker**” refers to a flexible molecular connection between two or more proteins, e.g., the linker between two m36 molecules. The molecular connection can be obtained from any suitable natural or synthetic source. The linker can be obtained, for example, from an antibody hinge region, e.g., IgG hinge region or from another natural or synthetic polypeptide source. The linker can be encoded, as in translational fusions. The linker can also be used to join or couple two already existing proteins.

As used herein, the term “**mimic**” refers to a second molecule, compound or substance which has the same or similar function or characteristics as a first molecule, compound or substance, while at the same time having a different structure.

VH framework

In one embodiment, the present invention provides a novel and advantageous VH framework, which the present inventors have used as the starting point for
540 generating a highly diversified phage-displayed domain antibody library which, in turn, can be used as convenient source of domain antibodies which are unexpectedly highly soluble and stable and possess good folding fidelity (despite a wide diversity of CDR molecules) and high affinity for their cognate antigenic targets.

In a preferred embodiment of the invention, the present invention provides a VH
545 framework having the amino acid and nucleotide sequence of Figure 5 (indicated as m0, herein as “the m0 framework”). The construction of the m0 framework is described in Example 2 herein. Essentially, the m0 framework is the VH region of the Fab antibody, R3H1, which was identified by screening a large non-immune human Fab library (containing $\sim 1.5 \times 10^{10}$ members) derived from the lymph nodes, spleen and peripheral
550 blood lymphocytes of 50 human donors. As shown in Example 2, the m0 framework was found to have high levels of expression and high solubility. This completely natural VH domain antibody, belonging to the VH3 germline family, was then used as a framework to construct a large human VH domain library (with $\sim 2.5 \times 10^{10}$ members) by grafting in a diverse repertoire of CDRs and/or mutating existing framework CDRs.

555 In another embodiment, the present invention provides a VH framework derived from m0, wherein the m0 contains certain advantageous amino acid modifications to enhance the properties of the framework antibody, including its solubility, stability, and lack of tendency to aggregate. Recombinant DNA techniques for modifying and/or changing the nucleic acid sequence and/or amino acid sequence of antibodies, including
560 m0, are well known to those having ordinary skill in the art. For example, techniques for introducing genetic changes include site-directed mutagenesis, random mutagenesis, insertions, deletions, and PCR mutagenesis methods. All of these techniques are well known to those skilled in the art. See Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000, incorporated herein by reference.

565 Accordingly, the present invention relates to VH framework polypeptides that are at least 60%, preferably at least 80% identical, more preferably at least 90%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence of the m0 framework.

The invention also relates to the nucleic acid encoding the VH framework m0 as shown in Figure 5, or a VH framework nucleic acid sequence that is at least 90%, 95%,
570 96%, 97%, 98% or 99% identical to the nucleotide sequence of the m0 framework.

As used herein, the terms percent (%) sequence identity or percent (%) homology are used synonymously as a measure of the similarity of two or more amino acid sequences, or alternatively, between two or more nucleotide sequences. Methods for determining percent (%) sequence identity or percent (%) homology are well known
575 in the art.

For the purposes of the present invention, percent (%) sequence identity or homology can be determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical
580 algorithms. A nonlimiting example of a mathematical algorithm used for comparison of two sequences is the algorithm of Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1990;87: 2264-2268, modified as in Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1993;90: 5873-5877.

Another example of a mathematical algorithm used for comparison of sequences
585 is the algorithm of Myers & Miller, CABIOS 1988;4: pp11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local
590 sequence similarity and alignment is the FASTA algorithm as described in Pearson & Lipman, Proc. Natl. Acad. Sci. USA 1988;85: 2444-2448. Advantageous for use according to the present invention is the WU-BLAST (Washington University BLAST) version 2.0 software. This program is based on WU-BLAST version 1.4, which in turn is based on the public domain NCBI-BLAST version 1.4 (Altschul & Gish, 1996, Local

595 alignment statistics, Doolittle ed., Methods in Enzymology 266: 460-480; Altschul et al., Journal of Molecular Biology 1990;215: 403-410; Gish & States, 1993; Nature Genetics 3: 266-272; Karlin & Altschul, 1993; Proc. Natl. Acad. Sci. USA 90: 5873-5877; all of which are incorporated by reference herein).

In general, comparison of amino acid sequences is accomplished by aligning an amino acid sequence of a polypeptide of a known structure with the amino acid
600 amino acid sequence of a the polypeptide of unknown structure. Amino acids in the sequences are then compared and groups of amino acids that are homologous are grouped together. This method detects conserved regions of the polypeptides and accounts for amino acid insertions and deletions. Homology between amino acid sequences can be determined
605 by using commercially available algorithms (see also the description of homology above). In addition to those otherwise mentioned herein, mention is made too of the programs BLAST, gapped BLAST, BLASTN, BLASTP, and PSI-BLAST, provided by the National Center for Biotechnology Information. These programs are widely used in the art for this purpose and can align homologous regions of two amino acid sequences.

In all search programs in the suite the gapped alignment routines are integral to
610 the database search itself. Gapping can be turned off if desired. The default penalty (Q) for a gap of length one is Q=9 for proteins and BLASTP, and Q=10 for BLASTN, but may be changed to any integer. The default per-residue penalty for extending a gap (R) is R=2 for proteins and BLASTP, and R=10 for BLASTN, but may be changed to any
615 integer. Any combination of values for Q and R can be used in order to align sequences so as to maximize overlap and identity while minimizing sequence gaps. The default amino acid comparison matrix is BLOSUM62, but other amino acid comparison matrices such as PAM can be utilized.

Alternatively or additionally, the term "homology" or "identity", for instance,
620 with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as $(N_{ref} - N_{dif}) * 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence

625 identity of 75% with the sequence AATCAATC ($N_{\text{ref}}=8$; $N_{\text{dif}}=2$).

Alternatively or additionally, "homology" or "identity" with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the
630 Wilbur and Lipman algorithm (Wilbur & Lipman, Proc Natl Acad Sci USA 1983;80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™
635 Suite, Intelligenetics Inc. CA). When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus, RNA sequences are within the scope of the invention and can be derived from DNA sequences, by thymidine (T) in the DNA sequence being-considered equal to uracil (U)
640 in RNA sequences.

And, without undue experimentation, the skilled artisan can consult with many other programs or references for determining percent homology.

The modifications to the amino acid sequence of the m0 framework to form variants that are at least 60%, preferably at least 80% identical, more preferably at least
645 90%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence of the m0 framework, can be made using conservative amino acid substitutions. A conservative substitution is one in which the substituted amino acid has similar structural or chemical properties with the corresponding amino acid in the reference sequence. By way of example, conservative amino acid substitutions involve substitution of one aliphatic or
650 hydrophobic amino acid, e.g. alanine, valine, leucine and isoleucine, with another; substitution of one hydroxyl-containing amino acid, e.g. serine and threonine, with another; substitution of one acidic residue, e.g. glutamic acid or aspartic acid, with another; replacement of one amide-containing residue, e.g. asparagine and glutamine, with another; replacement of one aromatic residue, e.g. phenylalanine and tyrosine, with

655 another; replacement of one basic residue, e.g. lysine, arginine and histidine, with another; and replacement of one small amino acid, e.g., alanine, serine, threonine, methionine, and glycine, with another.

Domain antibody library construction and screening methods

In yet another embodiment, the present invention provides a domain antibody
660 library prepared by introducing CDR diversity into the m0 framework (or derivative thereof having various amino acid substitutions as described above). Any suitable method for introducing CDR diversity is contemplated, including mutagenesis of one or more of the existing m0 CDR1, CDR2 or CDR3 sequences and the grafting in or replacement of one or more of the existing m0 CDR1, CDR2 or CDR3 sequences with a
665 diverse repertoire of synthetically-prepared or naturally-obtained CDR sequences. The naturally-obtained CDR sequences can comprise a naive repertoire (not exposed to antigen) or an immunized repertoire (exposed to antigen).

The CDR sequences may be obtained from several sources, for example, databases such as the National Centre for Biotechnology Information protein and
670 nucleotide databases www.ncbi.nlm.nih.gov, The Kabat Database of Sequences of Proteins of Immunological Interest www.kabatdatabase.com, or the IMGT database www.imgt.cines.fr. Alternatively, the CDR regions can be predicted from the VH and VL domain repertoire (see for example Kabat E A and Wu T T Attempts to locate complementarity determining residues in the variable positions of light and heavy
675 chains. Ann. NY Acad. Sci. 190:382-393 (1971)). The CDR sequence may be a genomic DNA or a cDNA.

PCR amplification using primers that amplify the CDR1, CDR2 and CDR3 sequences, which may include framework sequence at the 5' and 3' ends of the CDR sequences, can be used to obtain the CDR repertoire for grafting into the framework
680 sequence of the invention. Such PCR methods will be known to those of ordinary skill in the art. CDR sequences can also be obtained by any known nucleotide sequence synthesis methods, or by obtaining DNA fragments (e.g. restriction fragments) carrying the CDR sequences of interest. Any suitable method for obtaining the CDR sequences of the invention for use in grafting into the framework sequence is contemplated.

685 In a particular embodiment, diversity can be introduced into the m0 framework
by grafting in a natural repertoire of one or more human CDR1, CDR2 or CDR3
sequences, obtained from known and available human antibody libraries, while
synthetically modifying at least one existing CDR sequence of the m0 framework.
Alternatively, diversity can be introduced by either only grafting in a CDR1, CDR2 and
690 CDR3 repertoire, or by synthetically modifying each existing CDR sequence of the
framework, without simultaneous grafting. Human antibody libraries can be obtained
from any suitable source (e.g. commercial, university laboratories, public repositories of
antibody libraries) and can include, for example, both immunized and non- immunized
type libraries. As used herein, a non- immunized antibody library is one in which the
695 patient(s) from which the antibodies were derived has not been exposed to the antigen
against which the domain antibody library will ultimately be screened. An immunized
antibody library is one in which the patient(s) from which the antibodies were derived
have been exposed to the antigen against which the domain antibody library will
ultimately be screened. As an example, where the goal is to pan or screen the domain
700 antibody library for domain antibodies that neutralize an HIV antigen, an immune
antibody library would be one whereby the patient from which the antibodies were
sampled was infected with HIV.

Alternatively, the source libraries can comprise a synthetically prepared CDR
repertoire.

705 Conveniently, the mutagenesis and/or grafting of the CDRs may be achieved by
the method of overlap extension using primers which contain at each end sequences that
are complementary or homologous to the anchor regions that form the basis of the
framework region of m0 (or a variant thereof) and, in between, the CDR sequences. In
one aspect, the primers of Table 1 can be used to obtain CDR products from any human
710 antibody library and/or introduce mutations in CDR1 (with the H1R primer, which
contains four triplet regions of degenerate sequences (underlined regions). Similarly
mutations can be introduced in CDR2 and CDR3.

Regarding CDR mutagenesis, it is important when designing the CDR primers
also to take into account sequence homology within the CDR regions which was

715 observed in the sequence data from the naive clones, as the amino acids concerned are thought to play a structural role in the VH. It is desirable that highly conserved sequences within the CDRs, that is, residues that are conserved amongst a substantial proportion of the VH domains in the naive repertoire, should be retained in the synthetically modified primers, and excluded as targets for mutagenesis.

720 Splicing by overlap extension is a modification of the polymerase chain reaction, which has been used to generate gene fusions at very specific positions. It is based on the ability to fuse and amplify two DNA fragments containing homologous sequences i.e. `anchors` around the fusion point.

For example, for the preparation of a `synthetic` expression library, CDR
725 primers incubated with framework region fragments will anneal at their complementary ends and fuse to generate randomised framework-CDR encoding fragments. This process yields CDR-1/FR-2, CDR-2/FR-3 and CDR-3/FR-4 fusion fragments. Two of these fragments are then fused, and so forth.

There then follows a denaturation step after which the fragments can be further
730 annealed at the `anchor-regions` and extended yielding the fused, double stranded gene product. If required this reaction can be followed by the PCR reaction amplifying the quantity of fused gene material. This method can easily be extended to fuse three or more fragments. Such or similar methods are disclosed in U.S. Patent No. 7,196,187, and U.S. published application No. 2007/0202105, each of which are incorporated
735 herein by reference.

Splicing by overlap extension allows the linking of the fusion fragments at specific positions to produce a fully assembled VH gene which can be cloned into a suitable phage display vector, such as the vector pHEN.5, using restriction enzymes such as SfiI/NotI.

740 It will be appreciated that other methods of introducing mutations, preferably including random or partially random sequences, into the CDRs would also be applicable. Such methods include, for example, cassette mutagenesis or the use of error-prone `mutator` strains as bacterial hosts.

Any suitable methods can then be used to evaluate the size and sequence
745 diversity of the domain antibody library. It will be appreciated that size and sequence
diversity are key features of high-quality libraries. A finding consistent with theoretical
considerations is that the affinity of antibodies selected is proportional to the size of the
library, with K_{d} s ranging from 10^{6-7} for the smaller libraries to 10^9 for the larger ones,
and antibodies with affinity comparable to those obtained from immune libraries can be
750 selected from naïve libraries that are large enough (Andrew et al., J. Immunol. Methods,
2004). For example, random nucleotide sequencing can be performed on a random
selection of clones of the library to evaluate the source and extent of sequence diversity
of the CDRs of the library. Diverse antibody libraries contain a high number of
uniquely arranged VH sequences, i.e. a diverse assemblage of CDR1, CDR2 and CDR3
755 with unique combinations and sequences. Preferably, the domain antibody library of
the invention comprises preferably at least about 10^8 , preferably at least about 10^9 , 10^{10} ,
more preferably about 10^{11} or 10^{12} unique whole VH sequences.

In addition, the expressed VH products of the library clones can be evaluated for
physical properties, such as solubility, stability and tendency to aggregate. Such
760 methods would require expression of individual clones in a suitable vector in a host cell,
e.g. *E. coli*, followed by the isolation of the expressed product, followed then by an
analysis of the solubility, stability and tendency to aggregate, or any other desire trait.

Once a domain antibody library has been constructed, the present invention
contemplates the screening of the library for antibodies that bind to or which are
765 specifically immunoreactive against an antigen of interest, e.g. CD4i antigen.

The host cell used to express the VH products of the domain antibody library
(e.g. phagemid library) may be prokaryotic or eukaryotic but is preferably bacterial,
particularly *E. coli*.

The domain antibody library (e.g. phagemid library) according to the invention
770 may be screened for antigen binding activity using conventional techniques well known
in the art as described, for example, in Hoogenboom, Tibtech, 1997 (15), 62 70. By
way of illustration, bacteriophage displaying a repertoire of nucleic acid sequences
according to the invention on the surface of the phage may be screened against different

antigens by a 'panning' process (see McCafferty, J., Griffiths, AD, Winter, G. and
775 Chiswell, DJ, Nature, 348 (1990) 552-554, which is incorporated herein by reference in
its entirety) whereby the VH domains are screened for binding to an immobilized
antigen (e.g. antigen on magnetic bead or bottom of microtiter well). Non-binding
phage are removed. Binding phage are retained, eluted and amplified in bacterial or
other suitable host (depending on the vector used). The panning cycle is repeated until
780 enrichment of phage or antigen is observed and individual phage clones are then
assayed for binding to the panning antigen and to uncoated polystyrene by phage
ELISA.

As an indication of the binding affinities of antibodies that result from the
screening described in the invention, dissociation constants for the VHs recognizing a
785 protein antigen will typically be less than 100 nM, preferably less than 75 nM, more
preferred less than 50 nM, still more preferred at less than 40 nM, most preferred less
than 25 nM.

In one particular embodiment, a sequential panning technique is employed to
obtain antibodies that bind to antigens from the same, but genetically distinct viruses or
790 organisms. This approach is advantageous in identifying antibodies that might be more
cross-reactive between different genetically distinct isolates of the same virus or other
organisms, e.g. different isolates of HIV-1 or HIV-1 viruses from different clades.
Antibodies with broader cross reactivity against different isolates of the same target
would be advantageous. It is commonly known that in certain situations, e.g. HIV-1, it is
795 difficult to obtain antibodies that will be effective against HIV-1 isolates too genetically
distinct from the isolate against which the antibody was prepared. That is, with some
anti-HIV antibodies, the antibodies can suffer from having too narrow of an
effectiveness.

In the context of phage display technology, this can be done by sequentially
800 changing the antigen during the panning of a phage display library of the invention. By
sequentially changing the antigen during panning of phage display libraries and
screening the panned libraries using different antigens, the selected phage will display
dAbs against conserved epitopes shared among all antigens used during the entire

selection process. In one embodiment, gp120 antigens or gp120/CD4 complex antigens
805 from two or more different HIV-1 isolates can be sequentially changed during the
panning of phage display libraries of the invention, which will result in the enrichment
of phages that display dAbs having affinity to shared epitopes of both or each of the
gp120 antigens used during panning. Sequential panning of HIV antigens is discussed
further in Zhang et al., 2004 and Zhang et al., 2003, each of which are incorporated
810 herein by reference. The herein Example 2 employs a sequential panning approach.

As an example, antibodies which bind to HIV CD4i can be identified using a
phagemid panning process. The HIV CD4i antigen may be coated on a microtiter plate
or a magnetic bead and incubated with the domain antibody phagemid library of
interest. Phage-linked VHs that do not bind to the CD4i antigen may be washed from
815 the plate, leaving only bound phage. The bound phage may be eluted by addition of a
thiol reducing agent such as dithiotreitol (DTT) resulting in cleavage of the disulfide
bond linking the antibody to the phage. The recovered population of phage may be
amplified by infection of *E. coli* hosts. This panning process may be repeated using the
enriched population of phage to further enrich for a population of phage-linked
820 antibodies that bind to the HIV CD4i antigen. The gene sequence encoding the dAbs
may then be excised using standard cloning techniques and transferred to an *E. coli*
expression vector which is used to transform an *E. coli* expression cell line. dAbs from
the enriched pool may then be expressed and purified and characterized.

Domain antibodies

825 In another embodiment, the present invention provides novel domain antibodies.
The domain antibodies of the invention are advantageous over prior art antibodies,
particularly domain antibodies, because the inventive antibodies are more stable, more
soluble and do not tend to form aggregates or polymerization products in solution. In
addition, the domain antibodies of the invention have high affinity for their target
830 epitopes, are highly expressed, possess strong antigen neutralization action, and
optionally can be broadly cross-reactive, e.g. cross-reactive antibody against HIV
isolates from different clades. Accordingly, in one aspect, the domain antibodies of the
invention can be used therapeutically to treat or prevent a number of conditions (e.g.

cancer) or infections (e.g. HIV), or diagnostically to diagnose or detect condition- or
835 infection-related antigens. As noted above, the advantageous features of the inventive
antibodies of the invention stem at least in part to the novel VH framework, m0, and its
derivatives, of the invention that forms the basis of the dAb library from which the
inventive antibodies can be obtained.

In one aspect, the present invention embodies a number of different antibodies
840 having gp120/CD4 CD4i binding characteristics identified by screening the m0 based
domain antibody library of the invention using a sequential panning approach. In one
aspect, the present invention provides a novel dAb, designated as m36, which possesses
good solubility, has no detectable oligomerization tendencies, is highly specific for the
CD4i antigen, and has broad affinity for different HIV isolates from different clades.
845 (See Example 2). The amino acid and nucleotide sequence of m36 are given in Figures
18 and 19. The CDR sequences and framework sequences of m36 are shown in Figure
18.

The present invention also contemplates, in accordance with the methods
previously described or any other suitable known methods, derivatives of m36, which
850 have been derivatived in any advantageous manner, such as, introducing changes in the
at the nucleotide sequence level of the framework and/or the CDR1, CDR2 or CDR3 of
m36. Methods for introducing genetic change to the nucleotide sequence of m36 are
well known in the art, and can include PCR-based random or site-directed mutagenesis,
insertions, deletions, gene shuffling, CDR grafting, etc. The present invention
855 particular contemplates dAbs that have framework and/or CDR amino acid sequences
having at least 60%, preferably at least 80%, or 85%, preferably at least 90%, 95%,
96%, 97%, 98%, or 99% or greater sequence identity with the framework and/or CDR
sequences of m36.

Domain antibodies and/or fragments or derivatives thereof of the invention may
860 be purified from any cell that expresses the antibodies, including host cells that have
been transfected with antibody-encoding expression constructs. The host cells may be
cultured under conditions whereby the antibodies are expressed. Purified dAbs
antibodies may be separated from other cellular components that may associate with the

antibodies in the cell, such as certain proteins, carbohydrates, or lipids using methods
865 well known in the art. Such methods include, but are not limited to, size exclusion
chromatography, ammonium sulfate fractionation, ion exchange chromatography,
affinity chromatography, and preparative gel electrophoresis. Purity of the preparations
may be assessed by any means known in the art, such as SDS-polyacrylamide gel
electrophoresis.

870 Alternatively, antibodies may be produced using chemical methods to synthesize
its amino acid sequence or portions of the antibody sequence (e.g. CDR sequences),
such as by direct peptide synthesis using solid-phase techniques (e.g., Merrifield, J. Am.
Chem. Soc. 85:2149-2154, 1963; Roberge, et al., Science 269:202-204, 1995, each of
which are incorporated herein by reference). Protein synthesis may be performed using
875 manual techniques or by automation. Automated synthesis may be achieved, for
example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer).
Optionally, fragments of antibodies may be separately synthesized and combined using
chemical methods to produce a full-length molecule.

In embodiments where the VH framework is obtained from a non-human animal
880 and used to construct a dAb library, methods for humanization are contemplated. It will
be generally appreciated by those skilled in the art that the most critical determinants of
antibody selectivity and binding affinity are the sequences and resulting conformations
of the complementarity regions (CDRs). Whole antibodies (e.g. human IgG) contain
six CDRs, three within the heavy chain variable region (VH) and three within the light
885 chain variable region (VL), for each Fab arm of the complete molecule. The
intervening sequences between the CDRs within VH and VL are the framework regions
which spacially orient the CDRs. The CDRs together form the antigenic binding sites
within antibodies. The critical role of these CDRs in determining the functional
properties of antibodies has long been exploited in the processes of antibody
890 humanization and antibody optimization. In the former process, the CDRs from a
monoclonal antibody, for example, a mouse antibody, are transferred to a human
antibody of similar framework design thereby resulting in an antibody with the same
functional properties and reduced immunogenicity in man.

The success of this process is evident from the number of humanized antibodies
895 that have been successfully commercialized as human therapeutics and include
HERCEPTIN® (trastuzumab, Genentech, Inc., South San Francisco, CA), SYNAGIS®
(palivizumab, Medimmune, Inc., Gaithersburg, MD), CAMPATH® (alemtuzumab,
Genzyme Oncology, Cambridge, MA), ZENAPAX® (daclizumab, Roche
Pharmaceuticas, Nutley, NJ), XOLAIR® (omalizumab, Genentech, Inc., South San
900 Francisco, CA), RAPTIVA® (efalizumab, Genentech, Inc., South San Francisco, CA),
AVASTIN® (bevacizumab, Genentech, Inc., South San Francisco, CA), and
MYLOTARG® (gemtuzumab ozogamicin, Wyeth-Ayerst, Madison, NJ). Other
examples have been described in Singer, et al., (J. Immunol, 150:2844-2857, 1993);
Luo, et al., (J. Immunol Meth., 275:31-40, 2002); and Kostelny, et al., (Int. J. Cancer
905 93;556-565, 2001).

In principal, a framework sequence from any human antibody may serve as the
template for CDR grafting. However, it has been demonstrated that straight CDR
replacement onto such a framework often leads to significant loss of binding affinity to
the antigen (Glaser, et al., J. Immunol. 149:2606, 1992); Tempest, et al., Biotechnology
910 9:266, 1992; Shalaby, et al., J. Exp. Med. 17: 217, 1992). The more homologous a
human antibody is to the original antibody, the less likely combining the CDRs with the
human framework will be to introducing distortions into the CDRs that could reduce
affinity. In view of this general principle, it is quite unexpected then that the VH
framework of the dAb library of the invention is compatible in terms of domain folding
915 and solubility with such a highly diverse repertoire of CDRs. (See Example 2).

Domain antibody modifications

In yet another embodiment, the present invention relates to modified dAbs, and
provides methods for modifying domain antibodies identified in accordance with the
invention. The modifications may be genetic modifications to the nucleic acid encoding
920 a domain antibody polypeptide of the invention or they may be chemical, structural, or
physical modifications made directly to an isolated domain antibody of the invention to
impart additional advantageous properties to a domain antibody of the invention

regarding, for example, the level of expression, stability, solubility, epitope affinity, antigen neutralization activity, or penetration characteristics, etc.

925 In one aspect, the present invention contemplates introducing genetic modifications into one or more CDRs or to the framework sequence of the domain antibodies of the invention. Such genetic modifications can confer additional advantageous characteristics, i.e. genetic optimization, of the domain antibodies identified from library screening, including, for example, enhanced solubility, enhanced affinity, 930 and enhanced stability. Any type of genetic modification is contemplated by the present invention, including, for example, site-directed mutagenesis, random mutagenesis, insertions, deletions, CDR grafting (i.e. genetic replacement of one CDR for another CDR), and the construction and/or preparation of fusion proteins between domain antibodies of interest and desired fusion partners, e.g., serum albumin-binding peptide 935 (SaAb), which was unexpectedly found to increase the stability of the dAbs of the invention, or soluble CD4, which was unexpectedly found to synergistically impact the neutralization capacity of some of the domain antibodies of the invention. All of these techniques are well known to those skilled in the art. See Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 2000, incorporated 940 herein by reference. Reference to CDR grafting can be made to Nicaise, et al., *Protein Science* 13:1882-1891, 2004. The effect of any genetic modification can be tested or screened without undue experimentation using any of the methods described herein or other methods already known to one of ordinary skill in the art. For example, affinity of a domain antibody to a target antigen can be assessed using the herein described BIA 945 procedure.

 In another aspect, other modifications contemplated by the present invention relate to chemical modifications of the domain antibodies of the invention to confer additional advantageous features, such as enhanced stability and/or solubility and/or half-life.

950 In one particular aspect, the domain antibodies of the present invention can be PEGylated, or coupled to polymers of similar structure, function and purpose (“PEG or PEG-like polymers”), to confer enhanced stability and half-life. PEGylation can

provide increased half-life and resistance to degradation without a loss in activity (e.g. binding affinity) relative to non-PEGylated antibody polypeptides. The skilled artisan
955 will appreciate, however, that PEGylation may not be advantageous with respect to
some targets, in particular, those epitopes which are sterically-obstructed, e.g. CD4i
epitope. Thus, in cases where the inventive domain antibody targets a size-restricted
epitope, the domain antibody should minimally PEGylated so as not to negatively
impact the accessibility of the antibody to the size-restricted antigen. The skilled artisan
960 will appreciate that this general principle should be applied to any modifications made
to the dAbs of the invention.

Any method known in the art to couple the domain antibodies of the invention to
PEG or PEG-like polymers is contemplated by the present invention. PEG or PEG-like
moieties which can be utilized in the invention can be synthetic or naturally occurring
965 and include, but are not limited to, straight or branched chain polyalkylene,
polyalkenylene or polyoxyalkylene polymers, or a branched or unbranched
polysaccharide, such as a homo- or heteropolysaccharide. Preferred examples of
synthetic polymers which can be used in the invention include straight or branched
chain poly(ethylene glycol) (PEG), poly(propylene glycol), or poly(vinyl alcohol) and
970 derivatives or substituted forms thereof. Substituted polymers for linkage to the domain
antibodies of the invention can also particularly include substituted PEG, including
methoxy(polyethylene glycol). Naturally occurring polymer moieties which can be
used in addition to or in place of PEG include, for example, lactose, amylose, dextran,
or glycogen, as well as derivatives thereof which would be recognized by persons
975 skilled in the art.

PEGylation of the domain antibodies of the invention may be accomplished by
any number of means (see for example Kozlowski-A & Harris-J M (2001) *Journal of
Controlled Release* 72:217). PEG may be attached to the domain antibody construct
either directly or by an intervening linker. Linkerless systems for attaching
980 polyethylene glycol to proteins is described in Delgado et al., (1992), *Crit. Rev. Thera.
Drug Carrier Sys.* 9:249-304 Francis et al., (1998), *Intern. J. Hematol.* 68:1-18; U.S.
Pat. No. 4,002,531; U.S. Pat. No. 5,349,052; WO 95/06058; and WO 98/32466, the

disclosures each of which are incorporated herein by reference. The first step in the attachment of PEG or other polymer moieties to the domain antibody construct of the invention typically is the substitution of the hydroxyl end-groups of the PEG polymer by electrophile-containing functional groups. Particularly, PEG polymers are attached to either cysteine or lysine residues present in the domain antibody construct monomers or multimers. The cysteine and lysine residues can be naturally occurring, or can be engineered into the domain antibody molecule.

990 One system for attaching polyethylene glycol directly to amino acid residues of proteins without an intervening linker employs tresylated MPEG, which is produced by the modification of monomethoxy polyethylene glycol (MPEG) using tresylchloride. Following reaction of amino acid residues with tresylated MPEG, polyethylene glycol is directly attached to the amine groups. Thus, the invention includes protein-
995 polyethyleneglycol conjugates produced by reacting proteins of the invention with a polyethylene glycol molecule having a 2,2,2-trifluoroethane sulphonyl group.

Polyethylene glycol can also be attached to proteins using a number of different intervening linkers. For example, U.S. Pat. No. 5,612,460 discloses urethane linkers for connecting polyethylene glycol to proteins. Protein-polyethylene glycol conjugates
1000 wherein the polyethylene glycol is attached to the protein by a linker can also be produced by reaction of proteins with compounds such as MPEG-succinimidylsuccinate, MPEG activated with 1,1'-carbonyldiimidazole, MPEG-2,4,5-trichloropenylcarbonate, MPEG-p-nitrophenolcarbonate, and various MPEG-succinate derivatives. A number of additional polyethylene glycol derivatives and reaction
1005 chemistries for attaching polyethylene glycol to proteins are described in WO 98/32466, the entire disclosure of which is incorporated herein by reference.

Other derivatized forms of polymer molecules include, for example, derivatives which have additional moieties or reactive groups present therein to permit interaction with amino acid residues of the domain antibodies described herein. Such derivatives
1010 include N-hydroxysuccinimide (NHS) active esters, succinimidyl propionate polymers, and sulfhydryl-selective reactive agents such as maleimide, vinyl sulfone, and thiol.

The reactive group (e.g., MAL, NHS, SPA, VS, or Thiol) may be attached directly to the PEG polymer or may be attached to PEG via a linker molecule.

The size of polymers useful in the invention can be in the range of 500 Da to 60
1015 kDa, for example, between 1000 Da and 60 kDa, 10 kDa and 60 kDa, 20 kDa and 60
kDa, 30 kDa and 60 kDa, 40 kDa and 60 kDa, and up to between 50 kDa and 60 kDa.
The polymers used in the invention, particularly PEG, can be straight chain polymers or
may possess a branched conformation.

The present invention also contemplates the coupling (either by physical
1020 attachment of separate molecules, or through the use of genetic engineering to construct
a fusion protein between an antibody of the invention (e.g., m36) and an adduct protein
(e.g., serum albumin-binding peptide) of adduct molecules, which can be various
polypeptides or fragments thereof which occur naturally in vivo and which resist
degradation or removal by endogenous mechanisms. Molecules which increase half life
1025 may be selected from the following: (a) proteins from the extracellular matrix, eg.
collagen, laminin, integrin and fibronectin; (b) proteins found in blood, e.g., serum
albumin, serum albumin-binding peptide (SAbp – e.g., see the fusion prepared in
accordance with Example 3), fibrinogen A, fibrinogen B, serum amyloid protein A,
heptaglobin, protein, ubiquitin, uteroglobulin, β -2 microglobulin, plasminogen,
1030 lysozyme, cystatin C, alpha-1-antitrypsin and pancreatic kypsin inhibitor; (c) immune
serum proteins, e.g. IgE, IgG, IgM and their fragments e.g. Fc; (d) transport proteins,
e.g. retinol binding protein; (e) defensins, e.g. beta-defensin 1, neutrophil defensins 1, 2
and 3; (f) proteins found at the blood brain barrier or in neural tissues, e.g. melanocortin
receptor, myelin, ascorbate transporter; (g) transferrin receptor specific ligand-
1035 neuropharmaceutical agent fusion proteins, brain capillary endothelial cell receptor,
transferrin, transferrin receptor, insulin, insulin-like growth factor 1 (IGF 1) receptor,
insulin-like growth factor 2 (IGF 2) receptor, insulin receptor; (h) proteins localised to
the kidney, e.g. polycystin, type IV collagen, organic anion transporter K1, Heymann's
antigen; (i) proteins localized to the liver, e.g. alcohol dehydrogenase, G250; (j) blood
1040 coagulation factor X; (k) α -1 antitrypsin; (l) HNF 1 α .; (m) proteins localised to the
lung, e.g. secretory component (binds IgA); (n) proteins localised to the heart, eg. HSP

27; (o) proteins localised to the skin, eg, keratin; (p) bone specific proteins, such as bone morphogenic proteins (BMPs) e.g. BMP-2, -4, -5, -6, -7 (also referred to as osteogenic protein (OP-1) and -8 (OP-2)); (q) tumour specific proteins, eg. human
1045 trophoblast antigen, herceptin receptor, oestrogen receptor, cathepsins eg cathepsin B (found in liver and spleen); (r) disease-specific proteins, eg. antigens expressed only on activated T-cells: including LAG-3 (lymphocyte activation gene); osteoprotegerin ligand (OPGL) see Kong YY et al Nature (1999) 402, 304-309; OX40 (a member of the TNF receptor family, expressed on activated T cells and the only costimulatory T cell
1050 molecule known to be specifically up-regulated in human T cell leukaemia virus type-I (HTLV-I)-producing cells--see Pankow R et al J. Immunol. (2000) Jul. 1;165(1):263-70; metalloproteases (associated with arthritis/cancers), including CG6512 Drosophila, human paraplegin, human FtsH, human AFG3L2, murine ftsH; angiogenic growth factors, including acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor
1055 (FGF-2), Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), transforming growth factor- α (TGF- α), tumor necrosis factor-alpha (TNF- α), angiogenin, interleukin-3 (IL-3), interleukin-8 (IL-8), platelet derived endothelial growth factor (PD-ECGF), placental growth factor (PIGF), midkine platelet-derived growth factor-BB (PDGF), fractalkine; (s) stress proteins (heat shock proteins); and (t)
1060 proteins involved in Fc transport.

In a particular aspect, an antibody of the invention, e.g., m36, can be coupled with CD4 or a fragment or mimic thereof to increase the effectiveness of the binding of the antibody with its cognate HIV epitope, e.g., a CD4i epitope. It was also been
1065 surprisingly found that soluble CD4 or fragments or mimics thereof can be co-administered with an antibody of interest of the invention (e.g. m36) to synergistically improve the effectiveness of the antibody's neutralization capability. (see Example 3 for further discuss). CD4 mimics are known in the art and can be found described, for example, in U.S. Publication Nos. 2006/0073576, 2008/0096187, each of which are incorporated herein by reference.

1070 In another aspect, the domain antibodies of the invention may be multimerized, as for example, hetero- or homodimers, hetero- or homotrimers, hetero- or

homotetramers, or higher order hetero- or homomultimers. Multimerisation can increase the strength of antigen binding, wherein the strength of binding is related to the sum of the binding affinities of the multiple binding sites. The domain antibodies can
1075 be multimerized in another aspect by binding to an additional one, two, three or more polypeptide which function to stabilize the dAb against degradation. Such polypeptides may include common blood proteins, such as, albumin, or fragments thereof. Example 3 discusses the construction of m36CH3 as an example of preparing a multimerized antibody of the invention.

1080 In certain aspects, linker may be used to join (either through physical coupling or through a genetic engineering approach) an antibody of the invention, e.g., m36, with a suitable or appropriate adduct molecule or other desired fusion partner. As defined herein, a “fusion partner” can be any molecule, such as an adduct molecule for enhancing the stability of an antibody, that is fused through recombinant means or
1085 through physical means to an antibody of interest of the invention.

In yet another aspect, modifications relating to enhancing or modifying antibody activity are contemplated by the present invention. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating a condition, infection or disorder. For
1090 example cysteine residue(s) may be introduced in the domain antibody polypeptide, thereby allowing interchain disulfide bond formation in a multimerized form of the inventive antibodies. The homodimeric or heterodimeric (or multimeric) antibodies may include combinations of the same domain antibody polypeptide chains or different domain antibody polypeptide chains, such that more than one epitope is targeted at a
1095 time by the same construct. Such epitopes can be proximally located in the target (e.g. on the HIV target) such that the binding of one epitope facilitates the binding of the multimeric antibody of the invention to the second or more epitopes. The epitopes targeted by multimeric antibodies can also be distally situated.

The invention also contemplates modifying the domain antibodies of the
1100 invention to form immunoconjugates comprising the domain antibodies of the invention conjugated to cytotoxic agents, such as a chemotherapeutic agents, toxin (e.g., an

enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), radioactive isotopes (i.e., a radioconjugate), or antiviral compounds (e.g. anti-HIV compounds).

1105 The term “**cytotoxic agent**” as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term can include radioactive isotopes (e.g., I₁₃₁, I₁₂₅, Y₉₀ and Re₁₈₆), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

1110 A “**chemotherapeutic agent**” is a type of cytotoxic agent useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Taxotere (docetaxel), Busulfan, Cytosin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C,
1115 Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins, Melphalan and other related nitrogen mustards.

 The invention also contemplates immunoconjugation with enzymatically active toxins or fragments thereof. Enzymatically active toxins and fragments thereof which
1120 can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, croton, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin,
1125 enomycin and the tricothecenes.

 Where the inventive antibodies are intended to target viruses, bacteria or other pathogens, the invention also contemplates immunoconjugation of the domain antibodies with anti-viral, anti-bacterial or other chemicals and/or compounds that might improve or increase the effectiveness of the domain antibodies of the invention
1130 against intended targets, such as, for example, HIV.

For example, the inventive antibodies can be immunoconjugated, or in the alternative, co-administered with, an antibacterial compound, such as, for example, a macrolide (e.g., tobramycin (TOBI®)), a cephalosporin (e.g., cephalexin (KEFLEX®), cephradine (VELOSEF®), cefuroxime (CEFTIN®), cefprozil (CEFZIL®), cefaclor (CECLOR®), cefixime (SUPRAX®) or cefadroxil (DURICEF®), a clarithromycin (e.g., clarithromycin (BIAXIN®)), an erythromycin (e.g., erythromycin (EMYCIN®)), a penicillin (e.g., penicillin V (V-CILLIN K® or PEN VEE K®)) or a quinolone (e.g., ofloxacin (FLOXIN®), ciprofloxacin (CIPRO®) or norfloxacin (NOROXIN®)), aminoglycoside antibiotics (e.g., apramycin, arbekacin, bambarmycins, butirosin, dibekacin, neomycin, neomycin, undecylenate, netilmicin, paromomycin, ribostamycin, sisomicin, and spectinomycin), amphenicol antibiotics (e.g., azidamfenicol, chloramphenicol, florfenicol, and thiamphenicol), ansamycin antibiotics (e.g., rifamide and rifampin), carbacephems (e.g., loracarbef), carbapenems (e.g., biapenem and imipenem), cephalosporins (e.g., cefaclor, cefadroxil, cefamandole, cefatrizine, cefazedone, ceftazidime, cefpimizole, cefpiramide, and cefpirome), cephamycins (e.g., cefbuperazone, cefmetazole, and cefminox), monobactams (e.g., aztreonam, carumonam, and tigemonam), oxacephems (e.g., flomoxef, and moxalactam), penicillins (e.g., amdinocillin, amdinocillin pivoxil, amoxicillin, bacampicillin, benzylpenicillinic acid, benzylpenicillin sodium, epicillin, fenbenicillin, floxacillin, penamcillin, penethamate hydriodide, penicillin o-benethamine, penicillin 0, penicillin V, penicillin V benzathine, penicillin V hydrabamine, penimepicycline, and phencihicillin potassium), lincosamides (e.g., clindamycin, and lincomycin), amphomycin, bacitracin, capreomycin, colistin, enduracidin, enviomycin, tetracyclines (e.g., apicycline, chlortetracycline, clomocycline, and demeclocycline), 2,4-diaminopyrimidines (e.g., brodimoprim), nitrofurans (e.g., furaltadone, and furazolium chloride), quinolones and analogs thereof (e.g., cinoxacin,, clinafloxacin, flumequine, and grepagloxacin), sulfonamides (e.g., acetyl sulfamethoxyprazine, benzylsulfamide, noprilsulfamide, phthalylsulfacetamide, sulfachrysoidine, and sulfacytine), sulfones (e.g., diathymosulfone, glucosulfone sodium, and solasulfone), cycloserine, mupirocin and tuberin.

In another example, the inventive antibodies can be immunoconjugated, or in the alternative, co-administered with, an antiviral compound, such as, for example, a zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, and pleconaril.

Methods for modifying the domain antibodies of the invention with the various cytotoxic agents, chemotherapeutic agents, toxins, antibacterial compounds, and antiviral compounds, etc. mentioned above are well known in the art. For example, immunoconjugates of the antibody and cytotoxic agents can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

The domain antibodies can also be modified with useful detectable agents, such as, for example, fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. The domain antibody construct may also be derivatized with detectable enzymes such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When the domain antibody construct is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. The domain antibody construct may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

The skilled artisan will appreciate it may be advantageous to couple any of the aforementioned molecular entities to the domain antibodies of the invention through flexible linkers, such as flexible polypeptide chains. Such linkers may be required to avoid a loss in activity of the domain antibodies, or to avoid sterically restricting the domain antibodies such that they lose their effectiveness in binding to cognate epitopes, in particular, those epitopes which themselves may be sterically restricted, e.g. HIV CD4i epitopes.

Another type of covalent modification contemplated by the present invention involves chemically or enzymatically coupling glycosides to the domain antibodies of the invention. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of any carbohydrate moieties present on the antibodies of the invention may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Hakimuddin, et al. Arch. Biochem. Biophys. 259:52 (1987) and by Edge et al. Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. Meth. Enzymol. 138:350 (1987).

1220

Antigens

The present invention, in another embodiment, relates to antigens of interest that may be used to screen the domain antibody library of the invention to identify useful and effective domain antibodies of the invention.

1225 In one preferred aspect, the antigens of the invention are HIV antigens. The
antigens can be from any HIV isolate, e.g. 92UG, Bal, JR-FL, JRCSF, IIIB, 89.6, R2,
NL4-3, GXG, Z2Z6, or GXE. The HIV source of antigens can also be from any known
clade of HIV, including clade A, B, C, D or E. It will be appreciated that HIV is
different in structure from other retroviruses. It is about 120 nm in diameter and
1230 roughly spherical. It is composed of two copies of positive single-stranded RNA that
codes for nine genes enclosed by a conical capsid composed of 2,000 copies of the viral
protein p24. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and
enzymes needed for the development of the virion such as reverse transcriptase,
proteases, ribonuclease and integrase. A matrix composed of the viral protein p17
1235 surrounds the capsid ensuring the integrity of the virion particle. The viral envelope,
which forms the outer shell of the virus, is composed of a phospholipid bilayer derived
from the membrane of a human cell when outwardly budding from the cell. Embedded
in the viral envelope are proteins from the host cell and about 70 copies of the Env
“spike” complex. Env consists of a cap made of three molecules called glycoprotein
1240 (gp) 120 (gp120) and a stem consisting of three gp41 molecules that anchor the
structure into the viral envelope. This glycoprotein complex enables the virus to attach
to and fuse with target cells to initiate the infectious cycle.

Regarding the HIV genome, of the nine genes that are encoded within the RNA
genome, three of these genes, *gag*, *pol*, and *env*, contain information needed to make the
1245 structural proteins for new virus particles. For example, *env* codes for a protein called
gp160 that is broken down by a viral enzyme to form gp120 and gp41. The six
remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are
regulatory genes for proteins that control the ability of HIV to infect cells, replicate, or
cause disease. The protein encoded by *nef*, for instance, appears necessary for the virus
1250 to replicate efficiently, and the *vpu*-encoded protein influences the release of new virus

particles from infected cells. The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as switches to control production of new viruses and can be triggered by proteins from either HIV or the host cell.

1255 The HIV antigens can be any of those indicated above, and especially, those for which the neutralization of by antibody would inhibit or cease the virus's ability to replicate, ability to infect cells, ability to assemble, or its ability to process its viral genetic or protein material, etc. In one embodiment, the antigen is the Env protein complex. It will be appreciated that the primary neutralization target on the HIV-1
1260 virion is the envelope glycoprotein (Env), which promotes virus entry by catalyzing fusion between the virion and target cell membranes. Env is a major focus for humoral vaccine and antibody-based immunotherapeutic strategies against HIV-1 (see Parren et al., 2001; Wyatt et al., 1998, for further detail regarding strategies for neutralization by anti-Env antibodies, each of which are incorporated herein by reference). One problem
1265 in the art, however, is that efforts to develop effective neutralizing antibodies against Env have been frustrated by the difficulties in eliciting antibodies with potent neutralizing activities against genetically diverse HIV-1 isolates. (Dey et al., 2003).

 In a preferred embodiment, the antigen is an inducible antigen. As used herein, the term "**inducible antigen**" or "**inducible epitope**" refers to those antigens or
1270 epitopes which are present on a polypeptide which, initially are sterically unavailable for antibody interaction, but through the binding of the polypeptide to a second interacting molecule, such as a drug, second polypeptide, ligand, receptor or nucleic acid, becomes exposed as a result of an induced conformational change in the polypeptide caused by the interaction between the polypeptide and the second
1275 interacting molecule. In one particular aspect, the inducible antigen is CD4i, which is an antigen comprising an epitope on gp120, the exposure of which is induced by binding to its cognate cellular receptor, CD4. See Labrijn et al., 2003, which is incorporated herein by reference.

 While not wishing to be bound by theory, the skilled artisan will appreciate that
1280 HIV-1 entry into host cells is initiated by the binding of the gp120 subunit of the viral

Env complex (the “spike” complex) to the host cell receptor (CD4). This interaction induces conformational changes in gp120 resulting in the exposure of a conserved high-affinity binding site for the coreceptor (the chemokine receptors CCR5 or CXCR4) (Sattentau et al., 1991; Sattentau et al., 1993).

1285 A second binding step between the gp120-CD4 complex and the coreceptor is then thought to induce additional conformational changes that ultimately result in the fusion of viral and host cell membranes (Dimitrov, 2004; Jones et al., 1991). Not wishing to be bound by theory, neutralizing antibodies are believed to act, at least in part, by binding to the exposed Env surface and obstructing the initial interaction
1290 between a trimeric array of gp120 molecules on the virion surface and receptor molecules on the target cell.

 In response, HIV-1 has evolved a number of strategies to evade recognition by neutralizing antibodies, particularly those directed to the conserved CD4 and coreceptor binding sites of Env. The extent of protection of these sites from antibody recognition is
1295 limited by the necessity to preserve the accessibility for receptor interaction. In the case of the binding site of CD4 to gp120 (the “CD4bs”), this has led to the following structural features: (i) it is partially obscured from antibody recognition by the V1/V2 loop and associated carbohydrate structures; (ii) the flanking residues are variable and modified by glycosylation; (iii) it is recessed to an extent that limits direct access by an
1300 antibody variable region; (iv) clusters of residues within the CD4bs that do not directly interact with CD4 are subject to variation among virus strains; (v) many gp120 residues interact with CD4 via main-chain atoms, allowing for variability in the corresponding amino acid side chains; and (vi) there is considerable conformational flexibility within the CD4-unbound state of gp120, and antibody binding therefore requires relatively
1305 large entropic decreases, thus “conformationally masking” the conserved CD4bs (Kwong et al., 2002; Myszka et al., 2000).

 The coreceptor binding site on gp120 is thought to be composed of a highly conserved element on the β 19 strand and parts of the V3 loop (Labrijn et al., 2003). These elements are masked by the V1/V2 variable loops in the CD4-unbound state and
1310 largely unavailable for antibody binding (Labrijn et al., 2003). Upon CD4 binding,

conformational changes are induced. These changes include displacement of the V1/V2 stem-loop structure and consequent exposure of the coreceptor binding site (Labrijn et al., 2003). Binding studies with variable loop-deleted mutants suggest that CD4 induces additional rearrangement or stabilization of the gp120 bridging sheet near the 19 strand to form the final coreceptor binding surface (Labrijn et al., 2003). Since the binding to CD4 occurs at the virus-cell interface, the exposed coreceptor binding site is optimally positioned for interaction with the coreceptor.

A highly conserved discontinuous structure on gp120 associated with the coreceptor binding site is recognized by monoclonal antibodies (MAbs) that bind better to gp120 upon ligation with CD4. These so-called CD4-induced (CD4i) antibodies, such as 17b and 48d (Labrijn et al., 2003), recognize a cluster of gp120 epitopes that are centered on the β 19 strand and partially overlap the coreceptor binding site (Labrijn et al., 2003). Although such CD4i MAbs can neutralize some T-cell line adapted HIV-1 strains, they are generally poorly neutralizing for primary isolates (Labrijn et al., 2003; Poignard et al., 2001). Recently, an antibody Fab fragment, X5, from a phage display library and directed to a CD4i epitope, neutralized a wide variety of primary isolates (Labrijn et al., 2003; Moulard et al., 2002). Also recently, the Fab X5 antibody fragment was studied against other CD4i MAbs at a molecular level and found the smaller fragments were more able to neutralize different HIV-1 isolates (Labrijn et al., 2003).

Accordingly, in one embodiment, the domain antibodies of the present invention bind to or are specifically immunoreactive against the HIV-1 CD4i epitope.

Analytical and preparative methods

Once an antibody in accordance with the invention is identified or obtained, for example, by any of the methods herein described, for example, including by panning of the dAb library of the invention and expressing same in a host, it may be preferable to carry out further steps to characterize and/or purify and/or modify the antibody. For example, it may be desirable to prepare a purified, high-titer composition of the desirable antibody or to test the immunoreactivity of the identified antibody. The present invention contemplates any known and suitable methods for characterizing,

purifying, or assaying the antibodies of the present invention and it is expected the any person of ordinary skill in the art to which the invention pertains will have the requisite level of technical know-how and resources, e.g. technical manuals or treatises, to accomplish any further characterization, purification and/or assaying of the antibodies
1345 of the invention without undue experimentation.

For example, any useful means to describe the strength of binding (or affinity) between a domain antibody of the invention and an antigen of the invention (e.g. CD4i antigen) can be used. For example, the dissociation constant, K_d ($K_d = k_2/k_1 =$
[antibody][antigen] / [antibody-antigen complex]) can be determined by standard
1350 kinetic analyses that are known in the art. It will be appreciated by those of ordinary skill in the art that the dissociation constant indicates the strength of binding between an antibody and an antigen in terms of how easy it is to separate the complex. If a high concentration of antibody and antigen are required to form the complex, the strength or affinity of binding is low, resulting in a higher K_d . It follows that the smaller the K_d (as
1355 expressed in concentration units, e.g. molar or nanomolar), the stronger the binding.

Affinity can be assessed and/or measured by a variety of known techniques and immunoassays, including, for example, enzyme-linked immunospecific assay (ELISA), Bimolecular Interaction Analysis (BIA) (e.g., Sjolander and Urbaniczky, Anal. Chem. 63:2338-2345, 1991; Szabo, et al., Curr. Opin. Struct. Biol. 5:699-705, 1995, each
1360 incorporated herein by reference), and fluorescence-activated cell sorting (FACS) for quantification of antibody binding to cells that express antigen. BIA is a technology for analyzing biospecific interactions in real time, without labeling any of the interactants (e.g., BIACORETM). BIAcore is based on determining changes in the optical phenomenon surface plasmon resonance (SPR) in real-time reactions between
1365 biological molecules, such as, an antibody of the invention and an antigen of interest, e.g. CD4i. References relating to BIAcore technology can be further found in U.S. Published Application Nos: 2006/0223113, 2006/0134800, 2006/0094060, 2006/0072115, 2006/0019313, 2006/0014232, and 2005/0199076, each of which are incorporated herein in their entirety by reference.

1370 The domain antibodies of the invention may be assayed for immunospecific
binding by any suitable method known in the art. Assays involving an antibody and an
antigen are known as "immunoassays," which can be employed in the present invention
to characterize both the antibodies and the antigens of the invention. The
immunoassays which can be used include but are not limited to competitive and non-
1375 competitive assay systems using techniques such as western blots, radioimmunoassays,
ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays,
immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions,
immunodiffusion assays, agglutination assays, complement-fixation assays,
immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to
1380 name but a few. Such assays are routine and well known in the art (see, e.g., Ausubel et
al., eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc.,
New York, which is incorporated by reference herein in its entirety) and can be
performed without undue experimentation.

Western blot analysis generally comprises preparing protein samples,
1385 electrophoresis of the protein samples in a polyacrylamide gel (e.g., 8% 20% SDS-
PAGE depending on the molecular weight of the antigen), transferring the protein
sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or
nylon, blocking the membrane in blocking solution (e.g., PBS with 3% BSA or non-fat
milk), washing the membrane in washing buffer (e.g., PBS-Tween 20), blocking the
1390 membrane with primary antibody (the antibody of interest) diluted in blocking buffer,
washing the membrane in washing buffer; blocking the membrane with a secondary
antibody (which recognizes the primary antibody, e.g., an anti-human antibody)
conjugated to an enzymatic substrate (e.g., horseradish peroxidase or alkaline
phosphatase) or radioactive molecule (e.g., ^{32}P or ^{125}I) diluted in blocking buffer,
1395 washing the membrane in wash buffer, and detecting the presence of the antigen. One of
skill in the art would be knowledgeable as to the parameters that can be modified to
increase the signal detected and to reduce the background noise. For further discussion
regarding western blot protocols see, e.g., Ausubel et al., eds, 1994, Current Protocols
in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1, which is

1400 incorporated herein by reference.

ELISAs typically comprise preparing antigen, coating the well of a 96 well microtiter plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e.g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of the antigen of interest to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs see, e.g., Ausubel et al., eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1, which is incorporated herein by reference.

Any suitable method for purifying antibodies is contemplated herein. For example, chromatographic methods, such as, for example, immuno-affinity chromatography (immobilized ligand to bind and trap antibody of interest), affinity chromatography, protein precipitation, ion exchange chromatography, hydrophobic interaction chromatography, size-exclusion chromatography, as well as electrophoresis, can be found described in the technical literature, for example, in Methods in Enzymology, Volume 182, Guide to Protein Purification, Eds. J. Abelson, M. Simon, Academic Press, 1st Edition, 1990, which is incorporated herein by reference. Thus, suitable materials for performing such purification steps, such as chromatographic steps, are known to those skilled in the art. Such methods are suitable for purification of any of the antibodies, antigens or any fragments thereof that are in accordance with the invention as described herein.

Certain embodiments may require the purification or isolation of expressed proteins or antibodies or fragments thereof from a host cell or a portion thereof.

1430 Conventional procedures for isolating recombinant proteins from transformed host cells
are contemplated by the present invention. Such methods include, for example,
isolation of the protein or fragments of interest by initial extraction from cell pellets or
from cell culture medium, followed by salting-out, and one or more chromatography
1435 steps, including aqueous ion exchange chromatography, size exclusion chromatography
steps, high performance liquid chromatography (HPLC), and affinity chromatography
may be used to isolate the recombinant protein or fragment. Guidance in the procedures
for protein purification can be found in the technical literature, including, for example,
Methods in Enzymology, Volume 182, Guide to Protein Purification, Eds. J. Abelson,
M. Simon, Academic Press, 1st Edition, 1990, which is already incorporated by
1440 reference.

Nucleic acid molecules, vectors, and host cells

In another aspect, the present invention relates to nucleic acid molecules, e.g. the
phagemid clones of the invention, comprising the nucleotide sequences encoding the
dAbs of the invention. These nucleic acid molecules may be used, for example, to
1445 express quantities of the antibodies for therapeutic or diagnostic use.

Nucleic acid molecules, e.g. phagemid clones, of the present invention may be
isolated from host cells, free of other cellular components such as membrane
components, proteins, and lipids according to any known or suitable method in the art.
Nucleic acid molecules may be isolated using standard nucleic acid purification
1450 techniques, or synthesized using an amplification technique such as the polymerase
chain reaction (PCR), or by using an automatic synthesizer. Methods for isolating
polynucleotides are routine and are known in the art. Any such technique for obtaining
a polynucleotide may be used to obtain isolated polynucleotides encoding antibodies of
the invention. For example, restriction enzymes and probes may be used to isolate
1455 polynucleotides which encode antibodies.

Antibody-encoding cDNA molecules may be made with standard molecular
biology techniques, using mRNA as a template. Thereafter, cDNA molecules may be
replicated using molecular biology techniques known in the art and disclosed in
manuals such as Sambrook, et al., (Molecular Cloning: A Laboratory Manual, (Second

1460 Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989, Vol. 1-
3, incorporated herein by reference). An amplification technique, such as PCR, may be
used to obtain additional copies of the polynucleotides.

To express a polynucleotide encoding an antibody, the polynucleotide may be
inserted into an expression vector that contains the necessary elements for the
1465 transcription and translation of the inserted coding sequence. Methods that are well
known to those skilled in the art may be used to construct expression vectors containing
sequences encoding antibodies and appropriate transcriptional and translational control
elements. These methods include *in vitro* recombinant DNA techniques, synthetic
techniques, and *in vivo* genetic recombination. Such techniques are described, for
1470 example, in Sambrook, et al. (1989) and in Ausubel, et al., (Current Protocols in
Molecular Biology, John Wiley & Sons, New York, N.Y., 1995, incorporated herein by
reference).

A variety of expression vector/host systems may be utilized to contain and
express sequences encoding antibodies. These include, but are not limited to,
1475 microorganisms, such as bacteria (e.g. *E. coli*) transformed with recombinant
bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with
yeast expression vectors; insect cell systems infected with virus expression vectors (e.g.,
baculovirus); plant cell systems transformed with virus expression vectors (e.g.,
cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV); or bacterial expression
1480 vectors (e.g., Ti or pBR322 plasmids), or animal cell systems.

The control elements or regulatory sequences are those non-translated regions of
the vector -- enhancers, promoters, 5' and 3' untranslated regions -- which interact with
host cellular proteins to carry out transcription and translation. Such elements may vary
in strength and specificity. Depending on the vector system and host utilized, any
1485 number of suitable transcription and translation elements, including constitutive and
inducible promoters, may be used. For example, when cloning in bacterial systems,
inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid
(Stratagene, LaJolla, Calif.), pSPORT1 plasmid (Life Technologies), or the like can be
used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or

1490 enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and
storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences)
may be cloned into the vector. In mammalian cell systems, promoters from mammalian
genes or from mammalian viruses may be used. If it is necessary to generate a cell line
that contains multiple copies of a nucleotide sequence encoding an antibody, vectors
1495 based on SV40 or EBV may be used with an appropriate selectable marker.

General texts describing additional molecular biological techniques useful
herein, including the preparation of antibodies include Berger and Kimmel (Guide to
Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press,
Inc.); Sambrook, et al., (Molecular Cloning: A Laboratory Manual, (Second Edition,
1500 Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.; 1989) Vol. 1-3);
Current Protocols in Molecular Biology, (F. M. Ausabel et al. [Eds.], Current Protocols,
a joint venture between Green Publishing Associates, Inc. and John Wiley & Sons, Inc.
(supplemented through 2000)); Harlow et al., (Monoclonal Antibodies: A Laboratory
Manual, Cold Spring Harbor Laboratory Press (1988), Paul [Ed.]); Fundamental
1505 Immunology, (Lippincott Williams & Wilkins (1998)); and Harlow, et al., (Using
Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1998)), all of
which are incorporated herein by reference.

Use of the dAbs of the invention

In yet another embodiment, the present invention provides a pharmaceutical
1510 composition comprising an effective amount of the domain antibody of the invention,
together with a pharmaceutically acceptable carrier or diluent.

In a particular embodiment, the present invention provides a method for treating
and/or preventing an HIV infection by administering an effective amount of the domain
antibody, m36 (or derivative thereof) of the invention, together with a pharmaceutically
1515 acceptable carrier or diluent. Administration can occur before or after HIV infection.

Some terms relating to the use of the dAbs of this invention are defined as
follows.

The term “**treatment**” includes any process, action, application, therapy, or the
like, wherein a subject (or patient), including a human being, is provided medical aid

1520 with the object of improving the subject's condition, directly or indirectly, or slowing
the progression of a condition or disorder in the subject, or ameliorating at least one
symptom of the disease or disorder under treatment.

The term "**combination therapy**" or "**co-therapy**" means the administration of
two or more therapeutic agents to treat a disease, condition, and/or disorder. Such
1525 administration encompasses "**co-administration**" of two or more therapeutic agents in
a substantially simultaneous manner, such as in a single capsule having a fixed ratio of
active ingredients or in multiple, separate capsules for each inhibitor agent. In addition,
such administration encompasses use of each type of therapeutic agent in a sequential
manner. The order of administration of two or more sequentially co-administered
1530 therapeutic agents is not limited.

The phrase "**therapeutically effective amount**" means the amount of each
agent administered that will achieve the goal of improvement in a disease, condition,
and/or disorder severity, and/or symptom thereof, while avoiding or minimizing adverse
side effects associated with the given therapeutic treatment.

1535 The term "**pharmaceutically acceptable**" means that the subject item is
appropriate for use in a pharmaceutical product.

The antibodies of this invention are expected to be valuable as therapeutic
agents, e.g. anti-HIV antibody based therapies. Accordingly, an embodiment of this
invention includes a method of treating and/or preventing a particular condition (e.g.
1540 HIV infection) in a patient which comprises administering to said patient a composition
containing an amount of an antibody of the invention that is effective in treating the
target condition.

The present invention can be used to screen and identify dAb that can be used in
the treatment or prevention of a variety of diseases and/or conditions, which include, for
1545 example, cancer, such as, carcinomas of the kidney, esophagus, breast, cervix, colon,
and lung, and which also includes viral infections (e.g. HIV), bacterial infections, and
fungal infections.

The domain antibodies of the present invention may be administered alone or in
combination with one or more additional therapeutic agents. Combination therapy

1550 includes administration of a single pharmaceutical dosage formulation which contains
an antibody of the present invention and one or more additional therapeutic agents, as
well as administration of the antibody of the present invention and each additional
therapeutic agents in its own separate pharmaceutical dosage formulation. For example,
an antibody of the present invention and a therapeutic agent may be administered to the
1555 patient together in a single oral dosage composition or each agent may be administered
in separate oral dosage formulations.

Where separate dosage formulations are used, the antibody of the present
invention and one or more additional therapeutic agents may be administered at
essentially the same time (e.g., concurrently) or at separately staggered times (e.g.,
1560 sequentially). The order of administration of the agents is not limited.

For example, in one aspect, co-administration of a domain antibody or antibody
fragment of the invention together with one or more anti-HIV agents to potentiate the
effect of either the antibody or the anti-HIV agent(s) or both is contemplated for use in
treating HIV infections.

1565 The one or more anti-cancer agents can include any known and suitable
compound in the art, such as, for example, chemoagents, other immunotherapeutics,
cancer vaccines, anti-angiogenic agents, cytokines, hormone therapies, gene therapies,
and radiotherapies. A chemoagent (or "anti-cancer agent" or "anti-tumor agent" or
"cancer therapeutic") refers to any molecule or compound that assists in the treatment of
1570 a cancer. Examples of chemoagents contemplated by the present invention include, but
are not limited to, cytosine arabinoside, taxoids (e.g., paclitaxel, docetaxel), anti-tubulin
agents (e.g., paclitaxel, docetaxel, epothilone B, or its analogues), macrolides (e.g.,
rhizoxin) cisplatin, carboplatin, adriamycin, tenoposide, mitozantron, discodermolide,
eleutherobine, 2-chlorodeoxyadenosine, alkylating agents (e.g., cyclophosphamide,
1575 mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU), lomustine
(CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C,
and cis-dichlorodiamine platinum (II) (DDP) cisplatin, thio-tepa), antibiotics (e.g.,
dactinomycin (formerly actinomycin), bleomycin, mithramycin, anthramycin),
antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine,

1580 flavopiridol, 5-fluorouracil, fludarabine, gemcitabine, dacarbazine, temozolamide),
asparaginase, Bacillus Calmette and Guerin, diphtheria toxin, hexamethylmelamine,
hydroxyurea, LYSODREN.RTM., nucleoside analogues, plant alkaloids (e.g., Taxol,
paclitaxel, camptothecin, topotecan, irinotecan (CAMPTOSAR, CPT-11), vincristine,
vinca alkaloids such as vinblastine), podophyllotoxin (including derivatives such as
1585 epipodophyllotoxin, VP-16 (etoposide), VM-26 (teniposide)), cytochalasin B, colchicine,
gramicidin D, ethidium bromide, emetine, mitomycin, procarbazine, mechlorethamine,
anthracyclines (e.g., daunorubicin (formerly daunomycin), doxorubicin, doxorubicin
liposomal), dihydroxyanthracindione, mitoxantrone, mithramycin, actinomycin D,
procaine, tetracaine, lidocaine, propranolol, puromycin, anti-mitotic agents, abrin, ricin
1590 A, pseudomonas exotoxin, nerve growth factor, platelet derived growth factor, tissue
plasminogen activator, aldesleukin, allutamine, anastrozole, bicalutamide, biaomycin,
busulfan, capecitabine, carboplatin, chlorabutil, cladribine, cytarabine, daclinomycin,
estramusine, floxuridhe, gemcitabine, gosereine, idarubicin, itosfamide, lauprolide
acetate, levamisole, lomusline, mechlorethamine, megestrol, acetate, mercaptopurino,
1595 mesna, mitolanc, pegaspargase, pentoslatin, picamycin, riuxlmab, campath-1,
straplozocin, thioguanine, tretinoin, vinorelbine, or any fragments, family members, or
derivatives thereof, including pharmaceutically acceptable salts thereof. Compositions
comprising one or more chemoagents (e.g., FLAG, CHOP) are also contemplated by the
present invention. FLAG comprises fludarabine, cytosine arabinoside (Ara-C) and G-
1600 CSF. CHOP comprises cyclophosphamide, vincristine, doxorubicin, and prednisone.

The chemoagent can be an anti-angiogenic agent, such as, for example,
angiostatin, bevacizumab (Avastin®), sorafenib (Nexavar®), baculostatin, canstatin,
maspin, anti-VEGF antibodies or peptides, anti-placental growth factor antibodies or
peptides, anti-Flk-1 antibodies, anti-Flt-1 antibodies or peptides, laminin peptides,
1605 fibronectin peptides, plasminogen activator inhibitors, tissue metalloproteinase
inhibitors, interferons, interleukin 12, IP-10, Gro- β , thrombospondin, 2-
methoxyoestradiol, proliferin-related protein, carboxiamidotriazole, CM101,
Marimastat, pentosan polysulphate, angiopoietin 2, interferon-alpha, herbimycin A,
PNU145156E, 16K prolactin fragment, Linomide, thalidomide, pentoxifylline,

1610 genistein, TNP-470, endostatin, paclitaxel, accutin, cidofovir, vincristine, bleomycin, AGM-1470, platelet factor 4 or minocycline. Without being bound by theory, the coadministration of an anti-angiogenic agent advantageously may lead to the increase in MN expression in a tumor, thereby making the tumor more susceptible to the antibodies and antibody conjugates of the invention.

1615 In one aspect, said chemoagent is gemcitabine at a dose ranging from 100 to 1000 mg/m²/cycle. In one embodiment, said chemoagent is dacarbazine at a dose ranging from 200 to 4000 mg/m² cycle. In another aspect, said dose ranges from 700 to 1000 mg/m²/cycle. In yet another aspect, said chemoagent is fludarabine at a dose ranging from 25 to 50 mg/m²/cycle. In another aspect, said chemoagent is cytosine
1620 arabinoside (Ara-C) at a dose ranging from 200 to 2000 mg/m²/cycle. In still another aspect, said chemoagent is docetaxel at a dose ranging from 1.5 to 7.5 mg/kg/cycle. In yet another aspect, said chemoagent is paclitaxel at a dose ranging from 5 to 15 mg/kg/cycle. In a further aspect, said chemoagent is cisplatin at a dose ranging from 5 to 20 mg/kg/cycle. In a still further aspect, said chemoagent is 5-fluorouracil at a dose
1625 ranging from 5 to 20 mg/kg/cycle. In another aspect, said chemoagent is doxorubicin at a dose ranging from 2 to 8 mg/kg/cycle. In yet a further aspect, said chemoagent is epipodophyllotoxin at a dose ranging from 40 to 160 mg/kg/cycle. In yet another aspect, said chemoagent is cyclophosphamide at a dose ranging from 50 to 200 mg/kg/cycle. In a further aspect, said chemoagent is irinotecan at a dose ranging from
1630 50 to 150 mg/m²/cycle. In a still further aspect, said chemoagent is vinblastine at a dose ranging from 3.7 to 18.5 mg/m²/cycle. In another aspect, said chemoagent is vincristine at a dose ranging from 0.7 to 2 mg/m²/cycle. In one aspect, said chemoagent is methotrexate at a dose ranging from 3.3 to 1000 mg/m²/cycle.

In another aspect, the anti-MN antibodies and/or antibody fragments of the
1635 present invention are administered in combination with one or more immunotherapeutic agents, such as antibodies or immunomodulators, which include, but are not limited to, HERCEPTIN®, RETUXAN®, OvaRex, Panorex, BEC2, IMC-C225, Vitaxin, Campath I/H, Smart MI95, LymphoCide, Smart I D10, and Oncolym, rituxan, rituximab, gemtuzumab, or trastuzumab.

1640 The invention also contemplates administering the domain antibodies and/or
antibody fragments of the present invention with one or more anti-angiogenic agents,
which include, but are not limited to, angiostatin, thalidomide, kringle 5, endostatin,
Serpine (Serine Protease Inhibitor) anti-thrombin, 29 kDa N-terminal and a 40 kDa C-
terminal proteolytic fragments of fibronectin, 16 kDa proteolytic fragment of prolactin,
1645 7.8 kDa proteolytic fragment of platelet factor-4, a β -amino acid peptide corresponding
to a fragment of platelet factor-4 (Maione et al., 1990, *Cancer Res.* 51:2077), a 14-
amino acid peptide corresponding to a fragment of collagen I (Tolma et al., 1993, *J.*
Cell Biol. 122:497), a 19 amino acid peptide corresponding to a fragment of
Thrombospondin I (Tolsma et al., 1993, *J. Cell Biol.* 122:497), a 20-amino acid peptide
1650 corresponding to a fragment of SPARC (Sage et al., 1995, *J. Cell. Biochem.* 57:1329-),
or any fragments, family members, or derivatives thereof, including pharmaceutically
acceptable salts thereof.

Other peptides that inhibit angiogenesis and correspond to fragments of laminin,
fibronectin, procollagen, and EGF have also been described (See the review by Cao,
1655 1998, *Prog. Mol. Subcell. Biol.* 20:161). Monoclonal antibodies and cyclic
pentapeptides, which block certain integrins that bind RGD proteins (i.e., possess the
peptide motif Arg-Gly-Asp), have been demonstrated to have anti-vascularization
activities (Brooks et al., 1994, *Science* 264:569; Hammes et al., 1996, *Nature Medicine*
2:529). Moreover, inhibition of the urokinase plasminogen activator receptor by
1660 antagonists inhibits angiogenesis, tumor growth and metastasis (Min et al., 1996,
Cancer Res. 56:2428-33; Crowley et al., 1993, *Proc Natl Acad. Sci. USA* 90:5021). Use
of such anti-angiogenic agents is also contemplated by the present invention.

In another aspect, the domain antibodies and/or antibody fragments of the
present invention are administered in combination with a regimen of radiation.

1665 The domain antibodies and/or antibody fragments of the present invention can
also be administered in combination with one or more cytokines, which includes, but is
not limited to, lymphokines, tumor necrosis factors, tumor necrosis factor-like
cytokines, lymphotoxin- α , lymphotoxin- β , interferon- β , macrophage inflammatory
proteins, granulocyte monocyte colony stimulating factor, interleukins (including, but

1670 not limited to, interleukin-1, interleukin-2, interleukin-6, interleukin-12, interleukin-15, interleukin-18), OX40, CD27, CD30, CD40 or CD137 ligands, Fas-Pas ligand, 4-1BBL, endothelial monocyte activating protein or any fragments, family members, or derivatives thereof, including pharmaceutically acceptable salts thereof.

The domain antibodies and/or antibody fragments of the present invention can also be administered in combination with a cancer vaccine, examples of which include, but are not limited to, autologous cells or tissues, non-autologous cells or tissues, carcinoembryonic antigen, alpha-fetoprotein, human chorionic gonadotropin, BCG live vaccine, melanocyte lineage proteins (e.g., gp100, MART-1/MelanA, TRP-1 (gp75), tyrosinase, widely shared tumor-associated, including tumor-specific, antigens (e.g., 1680 BAGE, GAGE-1, GAGE-2, MAGE-1, MAGE-3, N-acetylglucosaminyltransferase-V, p15), mutated antigens that are tumor-associated (β -catenin, MUM-1, CDK4), nonmelanoma antigens (e.g., HER-2/neu (breast and ovarian carcinoma), human papillomavirus-E6, E7 (cervical carcinoma), MUC-1 (breast, ovarian and pancreatic carcinoma). For human tumor antigens recognized by T-cells, see generally Robbins and Kawakami, 1996, Curr. Opin. Immunol. 8:628. Cancer vaccines may or may not be 1685 purified preparations.

In yet another embodiment, the domain antibodies and/or antibody fragments of the present invention are used in association with a hormonal treatment. Hormonal therapeutic treatments comprise hormonal agonists, hormonal antagonists (e.g., 1690 flutamide, tamoxifen, leuprolide acetate (LUPRON), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, and steroids (e.g., dexamethasone, retinoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), antigestagens (e.g., mifepristone, onapristone), and antiandrogens (e.g., cyproterone acetate).

1695 The antibodies described herein may be provided in a pharmaceutical composition comprising a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be non-pyrogenic. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier including, but

1700 not limited to, saline, buffered saline, dextrose, and water. A variety of aqueous carriers may be employed including, but not limited to saline, glycine, or the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well-known sterilization techniques (e.g., filtration).

Generally, the phrase "pharmaceutically acceptable carrier" is art recognized and
1705 includes a pharmaceutically acceptable material, composition or vehicle, suitable for administering compounds of the present invention to mammals. The carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of
1710 being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc;
1715 excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water;
1720 isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be
1725 present in the antibody compositions of the invention.

Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin,

1730 propyl gallate, alpha-tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, and the like. The concentration of the antibody of the invention in such
1735 pharmaceutical formulation may vary widely, and may be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected. If desired, more than one type of antibody may be included in a pharmaceutical composition (e.g., an antibody with different K_d for MN binding).

1740 The compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which may be used pharmaceutically. Pharmaceutical compositions
1745 of the invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means.

The compositions of the invention additionally contemplate suitable
1750 immunocarriers, such as, proteins, polypeptides or peptides such as albumin, hemocyanin, thyroglobulin and derivatives thereof, particularly bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), polysaccharides, carbohydrates, polymers, and solid phases. Other protein-derived or non-protein derived substances are known to those skilled in the art.

1755 Formulations suitable for parenteral, subcutaneous, intravenous, intramuscular, and the like; suitable pharmaceutical carriers; and techniques for formulation and administration may be prepared by any of the methods well known in the art (*see, e.g.*, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 20th edition, 2000). Liquid dosage forms for oral administration of the compounds of the invention

1760 include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions,
syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may
contain inert diluent commonly used in the art, such as, for example, water or other
solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol,
ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-
1765 butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and
sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid
esters of sorbitan, and mixtures thereof.

The phrases "parenteral administration" and "administered parenterally" as used
herein means modes of administration other than enteral and topical administration,
1770 usually by injection, and includes, without limitation, intravenous, intramuscular,
intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal,
intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular,
subarachnoid, intraspinal and intrasternal injection and infusion.

The determination of a therapeutically effective dose is well within the
1775 capability of those skilled in the art. A therapeutically effective dose refers to the
amount of an antibody that may be used to effectively treat a disease (e.g., cancer)
compared with the efficacy that is evident in the absence of the therapeutically effective
dose.

The therapeutically effective dose may be estimated initially in animal models
1780 (e.g., rats, mice, rabbits, dogs, or pigs). The animal model may also be used to
determine the appropriate concentration range and route of administration. Such
information may then be used to determine useful doses and routes for administration in
humans.

Therapeutic efficacy and toxicity (e.g., ED₅₀ - the dose therapeutically effective
1785 in 50% of the population and LD₅₀ - the dose lethal to 50% of the population) of an
antibody may be determined by standard pharmaceutical procedures in cell cultures or
experimental animals. The dose ratio of toxic to therapeutic effects is the therapeutic
index, and it may be expressed as the ratio, LD₅₀/ED₅₀. The data obtained from animal
studies may be used in formulating a range of dosage for human use. The dosage

1790 contained in such compositions may be within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage may be determined by the practitioner, in light of factors
1795 related to the patient who requires treatment. Dosage and administration may be adjusted to provide sufficient levels of the antibody or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to
1800 therapy.

The antibodies of the invention may also be administered by introducing genetically engineered bacteria which express and release the domain antibodies of the invention once the bacteria are present in the patient. This format might be suitable for treating HIV infections. The antibody-expressing bacteria can be introduced into mucus
1805 membranes of the throat, for example, or in other mucosal regions in which HIV might be found. Methods for constructing and/or engineering such recombinant bacteria are well known in the art.

Polynucleotides encoding antibodies of the invention may be constructed and introduced into a cell either *ex vivo* or *in vivo* using well-established techniques
1810 including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

1815 Effective *in vivo* dosages of an antibody are in the range of about 5 µg to about 500 µg/kg of patient body weight. For administration of polynucleotides encoding the antibodies, effective *in vivo* dosages are in the range of about 100 ng to about 500 µg of DNA.

1820 The antibodies of the present invention can also be delivered in a microsphere or
microsome bodies.

The mode of administration of antibody-containing pharmaceutical
compositions of the present invention may be any suitable route which delivers the
antibody to the host. As an example, pharmaceutical compositions of the invention may
be useful for parenteral administration (e.g., subcutaneous, intramuscular, intravenous,
1825 or intranasal administration, or microsomal or lipid microsome bodies).

The above disclosure generally describes the present invention. A more
complete understanding can be obtained by reference to the following specific
examples, which are provided for purposes of illustration only and are not intended to
limit the scope of the invention.

1830 **EXAMPLES**

The structures, materials, compositions, and methods described herein are
intended to be representative examples of the invention, and it will be understood that
the scope of the invention is not limited by the scope of the examples. Those skilled in
the art will recognize that the invention may be practiced with variations on the
1835 disclosed structures, materials, compositions and methods, and such variations are
regarded as within the ambit of the invention.

Example 1. Identification of VH framework

A naïve human Fab library (1.5×10^{10} members) was constructed from peripheral
blood B cells of 22 healthy donors, spleens of 3 donors, and lymph nodes of healthy 34
1840 donors. This library was used for selection of Fabs against HIV-1 antigen gp140 which
was conjugated to magnetic beads (Dynabeads M-270 epoxy; DYNAL Inc., New Hyde
Park, N.Y.) as described (Zhu Z et al. J Virol. 2006, 80(2):891-9). Amplified libraries of
 3.8×10^{12} phage-displayed Fabs were incubated with 20, 20, 10, and 10 μ g of gp140 in 1
ml volume for 2 h at room temperature during the first, second, third, and fourth rounds
1845 of biopanning, respectively. After incubation the beads were washed 5 times for the first
round and 10 times for the later rounds with PBST to remove nonspecifically bound
phages. Bound phages were rescued by mixing the beads with *E. coli* TG1 cells for 45
min at 37°C and a phage library was prepared for the next round of biopanning. Ninety

1850 five clones were randomly picked from the infected TG1 cells in the third and fourth round, respectively, and subjected to monoclonal phage-based enzyme-linked immunosorbent assay (monoclonal phage ELISA). A positive Fab, designated R3H1, was identified with a stop codon in the light chain. Its reading frame was corrected subsequently. R3H1 was expressed and purified as shown in Figure 4.

1855 The gene fragment of R3H1 VH domain (m0) was amplified from R3H1 Fab phagemid and subcloned into phagemid vector (e.g. pComb3x or pZYD). The phagemids containing m0 genes were prepared and transformed to *E. coli* HB2151 chemical competent cells. Soluble m0 were expressed and purified as described (Zhu Z et al. J Virol. 2006, 80(2):891-9). The SDS-PAGE of purified m0 was shown in Figure 4. The amino acid and nucleotide sequences of m0 are shown in Figure 5.

1860 **Example 2. Construction and screening of domain antibody library**

Highly diverse antibody libraries have become important sources for selection of antibodies with high affinity and novel properties. Combinatorial strategies provide efficient ways of creating antibody libraries containing a large number of individual clones. These strategies include the reassembly of naturally occurring genes encoding 1865 the heavy and light chains from either immune or nonimmune B-cell sources and introduction of synthetic diversity to either the framework regions (FRs) or the complementarity determining regions (CDRs) of the variable domains of antibodies.

This Example describes the identification of a human heavy chain-only antibody and its use as a scaffold for construction of a phage-displayed VH library as well as an 1870 approach to introduce genetic diversity in this library in which natural human CDR2, CDR3, and synthetic CDR1 repertoires are combined into a single human VH framework scaffold. The usefulness of the library has been demonstrated by the successful selection of high-affinity binders to viral and cancer-related antigens.

1875 The following methods and materials were employed in this Example. The results of this Example are subsequently discussed below.

Methods and materials:

Amplification of CDR repertoire and FR fragments

Primers used for PCR amplification of gene fragments are described in Table 1, below.

1880 Table 1. Primers used for PCR amplification of gene fragments.

Primer description	Name	Sequence	Target
H1 antisense	H1R	5'-GCG GAC CCA GCT CAT TTC ATA <u>AKM</u> <u>AKM</u> GAA <u>AKM</u> GAA <u>AKM</u> AGA GGC TGC ACA GGA GAG (SEQ ID NO: 5) ^c	CDR1
H1 sense	H1-F	5'-GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCC AGG TGC AGC TGG TGC- 3' (SEQ ID NO: 6)	CDR1
H2 sense	H2F1	5'-GAA ATG AGC TGG GTC CGC CAG GCT CCA GGA CAA SGS CTT GAG TGG (SEQ ID NO: 7)	VH1-2 ^a , VH1-3, VH1-8, VH1-18, VH1-45, VH1-46, VH1-58, VH1-69, VH1-C, VH6-1, VH7- ^{*b}
	H2F2	GAA ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG GCC CTG GAG TGG (SEQ ID NO: 8)	VH2-*
	H2F3	5'-GAA ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG GGN CTR GAG TGG (SEQ ID NO: 9)	VH1-24, VH1-F, VH3-*, VH4-*, VH5-*
H2 antisense	H2R1	ATT GTC TCT GGA GAT GGT GAC CCT KYC CTG RAA CTY	VH1-*

		(SEQ ID NO: 10)	
	H2R2	5'-ATT GTC TCT GGA GAT GGT GAA TCG GCC CTT CAC NGA (SEQ ID NO: 11)	VH3-*, VH6-1
	H2R3	5'-ATT GTC TCT GGA GAT GGT GAC TMG ACT CTT GAG GGA (SEQ ID NO: 12)	VH2-*, VH4-*
	H2R4	5'-ATT GTC TCT GGA GAT GGT GAC STG GCC TTG GAA GGA (SEQ ID NO: 13)	VH5-*
	H2R5	5'-ATT GTC TCT GGA GAT GGT AAA CCG TCC TGT GAA GCC (SEQ ID NO: 14)	VH7-*
H3 sense	H3F1	5'-ACC CTG AGA GCC GAG GAC ACR GCY TTR TAT TAC TGT (SEQ ID NO: 15)	VH3-9, VH3-20, VH3-43
	H3F2	5'-ACC CTG AGA GCC GAG GAC ACA GCC AYR TAT TAC TGT (SEQ ID NO: 16)	VH1-45, VH2-*, VH5-*, VH7-81
	H3F3	5'-ACC CTG AGA GCC GAG GAC ACR GCY GTR TAT TAC TGT (SEQ ID NO: 17)	Other than above
H3 antisense	H3R	5'-GTG GCC GGC CTG GCC ACT TGA GGA GAC GGT GAC C (SEQ ID NO: 18)	FR4

FR3 sense	FR3F	5'-ACC ATC TCC AGA GAC AAT TCC (SEQ ID NO: 19)	FR3
FR3 antisense	FR3R	5'-GTC CTC GGC TCT CAG GGT G (SEQ ID NO: 20)	FR3
Extension #1	FR1F	5'-TGG TTT CGC TAC CGT GGC CCA GGC GGC CCA GGT GCA GCT GGT G (SEQ ID NO: 21)	FR1
Extension #2	HISR	5'-GTC GCC GTG GTG GTG GTG GTG GTG GCC GGC CTG GCC ACT TG (SEQ ID NO: 22)	5' end of H3R primer

^a Sub-groups of human antibody heavy chain genes.

^b All members in the groups.

^c Underlined codons indicates primer degeneracy.

For amplification of CDR1 repertoire and FR3 fragment, the master human VH
1885 m0, as described in Example 1 and shown in Figure 5, was used as the template. The
degenerate primer H1R (see Table 1) enables randomization of four amino acids in
CDR1 to A/D/S/Y. Three non-immune (IgM-derived from healthy non-infected non-
immunized donors) human antibody phage display libraries that have recently been
constructed according to the procedure described previously (de Haard HJ et al. 1999. J.
1890 Biol. Chem. 274:18218-30) and one immune (IgG-derived from HIV-infected donors)
library constructed from HIV patients have been used as templates for amplification of
CDR2 and CDR3 repertoires (See Figure 6A). The libraries used included (a) a naïve
human Fab library (5×10^9 members) constructed from peripheral blood B cells of 10
healthy donors (Zhu et al., J. Virol., 2006, which is incorporated herein by reference);
1895 (b) a naïve human Fab library (1.5×10^{10} members) constructed from peripheral blood
B cells of 22 healthy donors, spleens of 3 donors, and lymph nodes of healthy 34 donors
(de Haard HJ et al. 1999. J. Biol. Chem. 274:18218-30); (c) a naïve human Fab library
(7.2×10^8 members) constructed from cord blood of 2 healthy babies (essentially the

procedure and primers described in (de Haard HJ et al. 1999. J. Biol. Chem. 274:18218-30) were used but with one-step overlapping PCR cloning (Zhu Z and Dimitrov DS, in press, Therapeutic antibodies, A. Dimitrov, Ed., Methods in Molecular Biology, Humana Press; and (d) an immune human Fab library constructed from bone marrow obtained from 3 long-term nonprogressors whose sera exhibited the broadest and most potent HIV-1 neutralization among 37 HIV-infected individuals (Zhang et al., J. Immunol. Methods, 2003). To maintain maximal diversity, 8 first round PCR reactions were carried out using different primer combinations of Table 2 against each of the above libraries as a template to obtain CDR2 product repertoire. Similarly, 3 first round PCR reactions were carried out using different primer combinations of Table 2 against each of the above libraries as a template to obtain CDR3 product repertoire.

Table 2. Primer pairings used for first round amplification of gene segments

Primer pairings	Products	Targets
FR1F-H1R	FR1-CDR1-FR2 ^a	CDR1
H2F1-H2R1	FR2 ^a -CDR2-FR3 ^a	VH1-* (except VH1-24, VH1-F)
H2F1-H2R2	FR2 ^a -CDR2-FR3 ^a	VH6-1
H2F1-H2R5	FR2 ^a -CDR2-FR3 ^a	VH7-*
H2F2-H2R3	FR2 ^a -CDR2-FR3 ^a	VH2-*
H2F3-H2R1	FR2 ^a -CDR2-FR3 ^a	VH1-24, VH1-F
H2F3-H2R2	FR2 ^a -CDR2-FR3 ^a	VH3-*
H2F3-H2R3	FR2 ^a -CDR2-FR3 ^a	VH4-*
H2F3-H2R4	FR2 ^a -CDR2-FR3 ^a	VH5-*
H3F1-H3R	FR3 ^a -CDR3-FR4	VH3-9, VH3-20, VH3-43
H3F1-H3F2	FR3 ^a -CDR3-FR4	VH1-45, VH2-*, VH5-*, VH7-81
H3F1-H3F3	FR3 ^a -CDR3-FR4	Other than above
FR3F-FR3R	FR3	FR3

^a These products partially cover FRs.

PCRs were performed in a volume of 50 µl using High Fidelity PCR Master (Roche, Cat. #12140314001), 500 pM of each primer and 0.5 µg of template DNA (e.g. VH m0 or the four antibody phage display libraries described above) for 30 cycles (45 sec at 94 °C; 45 sec at 55 °C; and 1 min at 72 °C). Products for each primer combination from one template were pooled, purified from 2% agarose gel using

QIAquick Gel Extraction Kit (Qiagen, Cat. #28706), and quantified by reading the
1920 optical density (O.D.) at 260 nm (1 O.D. unit = 50 µg/ml). Finally, all products from the
four antibody phage display library templates were pooled at a molarity ratio calculated
by counting the number of donors for template libraries, e.g. 10:59:2:3.

Assembly of complete VH domain antibodies

The primers used in the first round of PCR created identical sequences in the
1925 downstream regions of the CDR1 products, the upstream regions of CDR3 products,
and both the downstream and the upstream regions of the CDR2 products. These
identical sequences are homologous to FRs and serve as the overlap for the second- and
third-round extension PCR reactions.

In the second-round PCR extension reactions (see Figure 6B), CDR1 fragments
1930 containing the whole FR1 and partial FR2 on both sides were joined to the CDR2
fragments containing partial FR2 and partial FR3 on both sides by overlapping PCRs
performed in a volume of 100 µl using both templates (in the same molarities) for 7
cycles in the absence of primers and additional 15 cycles in the presence of primers
(500 pM of FR1F and 500 pM of H2R1-5 mixture). Under the same condition, FR3
1935 fragments were joined to CDR3 fragments containing partial FR3 and the whole FR4 on
both sides by overlapping PCRs using primers FR3F and H3R.

In the third-round extension (see Figure 6C), complete VHs were formed by
annealing the products of the second-round extension reactions to each other using
overlapping PCRs with the extension primers HISR and FR1F appended with SfiI
1940 restriction sites.

Preparation of VH domain library

Gel-purified VH products formed above were digested with SfiI (New England
BioLabs, Inc., Cat. #R0123L), and cloned into a phagemid vector. The SfiI-digested
and gel-purified VH fragments and phagemid vectors, 80 µg and 230 µg respectively,
1945 were ligated in an 8-ml reaction mixture with 10000 units of T4-DNA ligase (New
England BioLabs, Inc., Cat. #M0202L) at 16 °C for 72 h. The ligation products were
then desalted and concentrated by passing through a 4-ml Amicon Ultra-4 centrifugal
filter with a cutoff of 3000 MW (Millipore, Cat. #UFC800324) at 4000 × g for 20

minutes at room temperature and washing 3 times with 4 ml of distilled water. About
1950 100- μ l of ligation product was recovered from the filter and stored at -20 °C for later
use.

Electroporation was then performed. For electroporations, 1 L of 2YT medium
containing 1% glucose (w/v) was pre-warmed at 37 °C. 100 gene pulser cuvettes (Bio-
Rad, Cat. #165-2089) with 1 mm gap were chilled on ice. The desalted ligation product
1955 and 4 ml of *E. coli* strain TG1 electroporation-competent cells (Stratagene, Cat.
#200123) were thawed together on ice. The TG1 competent cells were divided into 10
pre-chilled 1.5-ml Eppendorf tubes in 400 μ l aliquots. Ten μ l of ligation product was
added to each tube and gently mixed by pipet action. Next, 41 μ l of mixture was
transferred to each electroporation cuvette, which was gently tapped on the bench to
1960 move the mixture to the bottom of the cuvette. Electroporations were performed at 1.8
kV, 25 μ F, and 200 Ω and the cuvettes were flushed immediately with 1 ml and then
twice with 2 ml of pre-warmed 2YT medium and transferred into a 2-L flask.

After completing all electroporations, 700 ml of pre-warmed 2YT medium was
added to each flask to make a volume of 1 L in total. The cultures were incubated at 37
1965 °C with shaking at 250 rpm for 30 min. Ten μ l of the culture was 10-fold serially
diluted in 100 μ l of 2YT medium, and plated on 2YT agar plates containing 2% glucose
(w/v) and 100 μ g/ml of ampicillin. The plates were incubated overnight at 37 °C. The
total number of transformants was calculated by counting the number of colonies,
multiplying by the culture volume, and dividing by the plating volume.

1970 For preparation of the phage display library, 1 ml of 100 mg/ml ampicillin was
added to the 1-L culture and the culture was then incubated with shaking for additional
2 h at 37 °C. The cell density was measured by reading the O.D.600 of the culture and
the total number of cells was calculated by multiplying the O.D.600 value by 5×10^8
(estimated number of cells in 1 ml culture when O.D.600 reaches 1) and the culture
1975 volume (1000 in this case). The culture was infected with 10 M.O.I. of M13KO7 helper
phage (New England BioLabs, Cat. #N0315S) and incubated at 37 °C for 30 min,
shaking for homogenization every 10 min. The cells were collected by centrifuging at
5000 rpm for 10 min and resuspended in 2-L 2YT medium containing 100 μ g/ml of

ampicillin and 50 µg/ml of kanamycin. Following incubation at 250 rpm overnight at
1980 30 °C, the culture was centrifuged at 5000 rpm for 15 min at 4 °C.

The phagemids were prepared from the bacterial pellet using the Qiagen
HiSpeed Plasmid Maxi Kit (Cat. #12663). For phage precipitation, the supernatant was
transferred to 2 clean 2-L flasks, mixed well with ¼ volume of PEG8000 (20%,
w/v)/NaCl (2.5 M) solution, and incubated on ice for 3 h. The mixture was centrifuged
1985 at 14000 × g for 20 min at 4 °C and the phage pellet was resuspended in 100 ml 1 ×
PBS, pH7.4 by pipetting up and down along the side of the centrifuge bottles with a 10-
ml pipet. The phage suspension was centrifuged at 5000 rpm for 10 min at 4 °C. The
supernatant was transferred to a clean 200 ml flask, mixed well with ¼ volume of
PEG8000 (20%, w/v)/NaCl (2.5 M) solution, and incubated on ice for 1 h. The mixture
1990 was centrifuged at 14000 × g for 20 min at 4 °C and the phage pellet was resuspended in
50 ml 1 × PBS, pH7.4. The phage suspension was centrifuged at 5000 rpm for 10 min
at 4 °C and the supernatant was transferred to a clean 200 ml flask. The concentration
of phage was measured by reading O.D.280 (1 O.D.280 = 2.33 × 10¹²/ml). For long-
term storage, the phages were mixed with the same volume of autoclaved glycerol,
1995 aliquoted to make sure that each contains phage particles at least 100 times of the total
number of transformants, and stored at -80 °C.

Sequence diversity analysis

To evaluate sequence diversity of the VH domain library, clones were randomly
selected from the library and sequenced. The amino acid sequences were deduced from
2000 those clones with complete nucleotide sequences. For analysis of CDR1 sequence
diversity, the frequency of A/D/S/Y usage in each position mutated was calculated (see
Figure 8A).

The origins of CDR2s were determined and the numbers of mutations in amino
acid sequences were calculated by comparing to the germlines of human VHs from
2005 IMGT database (http://imgt.cines.fr/textes/IMGTrepertoire/Proteins/protein/human/IGH/IGHV/Hu_IGHVallgenes.html). The FR sequences on both sides of CDR2, e.g.
residue #53-55 (IMGT numbering) and residue #70-76, are highly diversified among 7

groups of human VH germlines, thus, could be used as markers in addition to CDR2 sequences themselves to determine CDR2 origins. See Figure 8B and 8C.

2010 The length of CDR3 was calculated one by one and the length distribution was compared to that of human heavy chain CDR3 in vivo. The pairing between CDR2 origins and lengths of CDR3 was also plotted. See Figure 8D.

Measurement of VH folding

2015 The folding of phage-displayed VHs with a fixed VH3 scaffold was measured by their ability binding to protein A. The use of Protein A as a marker for proper folding of human VH3 is well known. The library of $\sim 10^{13}$ phage particles was blocked in 2% non-fat dry milk in PBS for 1 h at room temperature and passed through a chromatography column (Bio-Rad, Cat. #731-1550) loaded with 300 ml of nProtein A Sepharose 4 Fast Flow (GE Healthcare, Cat. #17-5280-02). The column was washed 3
2020 times with 10 ml of PBS containing 0.05% (v/v) Tween-20 (PBST) each. Bound phages were eluted by incubation at room temperature for 10 min with 1 ml of 100 mM acetic acid (pH 3.0) followed by neutralization with 0.1 ml of 1 M Tris-HCl (pH 9.0). Eluted phage were rescued by infection of *E. coli* TG1 cells and a phage library was prepared for the next round of selection. In the fourth round of selection, TG1 cells
2025 were infected with the eluted phages, serially diluted and plated on 2YT agar plates. Clones were randomly selected from these plates and sequenced. The origins of CDR2s were determined as described above.

Panning the library to screen for domain antibodies of interest

2030 The library of $\sim 10^{13}$ phage particles was blocked in 1 ml PBS containing 2% non-fat dry milk and incubated with 10 μ g of gp120-CD4, 5 μ g of gp140, 5 μ g of gp120-CD4, and 5 μ g of gp140 conjugated to magnetic beads (Dynabeads M-270 epoxy, Invitrogen, Cat. #143-01) for 2 h at room temperature during the first, second, third and fourth rounds of biopanning, respectively. After incubation the beads were washed 5 times for the first round and 15 times for the later rounds with PBST to
2035 remove nonspecifically bound phages. Bound phage were rescued by mixing the beads with *E. coli* TG1 cells for 45 min at 37°C and a phage library was prepared for the next round of biopanning. Clones were randomly picked from the infected TG1 cells in the

third and fourth round and subjected to monoclonal phage-based enzyme-linked immunosorbent assay (monoclonal phage ELISA) to identify clones of phage displaying
2040 VHs with binding activity as described (Zhu et al., J. Virol., 2006, which is incorporated herein by reference).

Each of the selected clones was inoculated into 100 μ l of 2YT medium containing 100 μ g/ml ampicillin and 0.2% glucose in 96-well plates. When the bacterial cultures reached an O.D. 600 of 0.5, 50 μ l of fresh 2YT medium containing
2045 helper phage M13KO7 at M.O.I. of 10 and kanamycin at 50 μ g/ml (final concentration) was added to each well, and the plates were further incubated at 30°C overnight in a shaker at 250 rpm. 50 μ l of each phage supernatant was moved to a Corning high-binding 96-well plate (Sigma, Cat. #CLS3690) coated with 1 μ g/ml of gp120-CD4 and blocked with 3% non-fat dry milk in PBS, and incubated for 2 h at room temperature.
2050 The plates were washed 4 times with PBST and incubated with 50 μ l of horse radish peroxidase (HRP)-conjugated anti-M13 antibody (GE Healthcare, Cat. # 27-9421-01) 1:5000 diluted in PBS at room temperature for 1 h. After incubation the plates were washed 4 times with PBST and 50 μ l of substrate solution ABST (Roche, Cat. #1684302) was added to each well. The solution absorbance at 405 nm (A405) was
2055 measured following incubation at 37°C for 15-30 min.

Expression and purification of VH domain antibodies

The selected clones resulting from the panning and screening process, each phagemid clone containing a VH gene of interest, were prepared and transformed into *E. coli* HB2151 chemical competent cells. These selected phagemid clones were
2060 referred to as c3, c6, d1, d7, b4, c11, d10, b3, b5 (also referred to herein as m36), b7, g6 and e11. Soluble VHs were expressed as described previously (Zhu et. al., J. Virol., 2006, which is incorporated herein by reference). The bacterial pellet was collected after centrifugation at 8000 \times g for 10 min and resuspended in PBS buffer containing 0.5 mU polymixin B (Sigma, Cat. #P0972). After 30 min incubation with rotation at 50
2065 rpm at room temperature, it was centrifuged at 25000 \times g for 25 min at 4°C, and the supernatant was used for Hexahistidine-tagged VH purification by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA resin (Qiagen, Cat. # 30230)

according to manufacturer's protocols (which are incorporated herein by reference). The purity of each VH was determined by running SDS-PAGE (see Figure 7 or Figure 11C) and measuring O.D. at 280.

Binding of soluble VH domain antibodies

ELISA was performed by using Corning high-binding 96-well plates coated with 1 µg/ml of antigens and blocked with 3% non-fat dry milk in PBS. Microplate wells were then inoculated with 50 µl of serially diluted soluble VHs for 2 h at room temperature. After 4 washes with PBST, FLAG-tagged VHs were detected by adding 50 µl of 1:5000 diluted HRP-conjugated anti-FLAG antibody (Sigma, Cat. #A8592) to each well. Following incubation with the antibody for 1 h at room temperature, the plates were washed 4 times with PBST and the assay was developed at 37°C with ABST substrate and monitored at 405 nm as described.

Measurement of VH domain antibody oligomerization

Superdex75 column was calibrated with protein molecular mass standard of 13.7 (ribonuclease A), 25 (chymotrypsin), 44 (ovalbumin), 67 (albumin), 158 (aldolase), 232 (catalase), 440 (ferritin) and 669 (thyroglobulin) kDa. Purified VH domain antibodies in PBS were loaded onto the column that had been pre-equilibrated with PBS. The proteins were eluted with PBS at 0.5 ml/min.

Pseudovirus neutralization assay

Viruses pseudotyped with Envs from HIV-1 primary isolates representing HIV-1 group M, clades A–E (4) were used in this study. Briefly, pseudotyped viruses were prepared by cotransfection of 70–80% confluent 293T cells with pNL4-3.luc.E-R- and pSV7d-env plasmid using the PolyFect transfection reagent, according to manufacturer's instruction (Qiagen, Cat. #301105). Pseudotyped viruses were obtained after 24 h by centrifugation and filtration of cell culture through 0.45-µm filters, mixed with different concentrations of antibodies for 1 h at 37 °C, and then added to 1.5×10^4 HOS-CD4-CCR5 cells grown in each well of 96-well plates. Luminescence was measured after 2 days, using the Bright-Glo Luciferase Assay System (Promega, Cat. #E2610) and a LumiCount microplate luminometer (Turner Designs). Mean relative light units (RLU) for triplicate wells were determined. Percentage inhibition was

calculated by the following formula: $(1 - \text{average RLU of antibody-containing wells} / \text{average RLU of virus-only wells}) \times 100$. IC₅₀ and IC₉₀ of neutralization were
2100 assigned for the antibody concentration at which 50% and 90% neutralization were
observed, respectively.

A more detailed description of the specific materials that could be employed in
carrying out the methods of Example 2 are outlined as follows. Equivalent materials
can be employed as will be understood by one of ordinary skill in the art. This specific
2105 listing of materials is not meant to limit the present invention in any way.

Detailed list of materials:

The following list of materials were used in connection with the human VH
genes and phagemid vectors

1. VH framework m0 is shown in Figure 20A.
- 2110 2. Phagemid vector is shown in Figure 20B.

The following list of materials were used in connection with the isolation of
lymphocytes.

- 2115 1. Defibrinated or anticoagulant-treated human peripheral blood stored at 4 °C was
used as soon as possible. Total RNA, PolyA+ RNA, and cDNA of human blood and
other immune tissues such as bone marrow, spleen, and lymph node are commercially
available, e.g. from Clontech.
2. Ficoll-Paque Plus reagents (Amersham Bioscience, Piscataway, NJ) were used.
3. Solution A: 0.1% (w/v) anhydrous D-glucose, 0.05 mM CaCl₂, 0.98 mM MgCl₂,
5.4 mM KCl, and 145 mM Tris was used. To prepare, Solution A was dissolved in
2120 approximately 950 ml double distilled water (ddH₂O) and add 10 N HCl until pH is 7.6.
The volume was adjusted to 1 L with ddH₂O.
4. Solution B: 140 mM NaCl in ddH₂O was used.
5. Balanced salt solution (ready to use) was employed. To prepare, 1 volume of
Solution A was mixed with 9 volumes of Solution B. The balanced salt solution should
2125 be prepared fresh each week. Equivalent salt solution may be employed, e.g.,
phosphate buffer solution (PBS), pH 7.4.

6. Eppendorf centrifuge 5804R (Eppendorf, Westbury, NY) was used for centrifugation. Any equivalently refrigerated centrifuge producing up to at least 400 g and maintaining temperature of 18-20 °C may be employed.

2130 7. BD Falcon™ Conical Tubes (BD Biosciences, San Jose, CA) we used. Any equivalent tubes with a volume of ~15 ml and internal diameter ~1.3 cm may be used.

8. Pasteur pipettes, 3 ml, were used.

9. Hemacytometer (Sigma, St. Louis, MO) was used.

10. 0.4% trypan blue stain (Sigma, St. Louis, MO) was used.

2135 The following list of materials were used in connection with the isolation of total RNA and the synthesis of cDNA.

1. RNeasy Mini Kits (Qiagen, Valencia, CA) were employed.

2. QIAshredder (Qiagen, Valencia, CA) kits were employed.

2140 3. SuperScript. III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was employed.

4. Corning® PCR tubes, free of RNase and DNase (Sigma, St. Louis, MO) were employed.

5. 1.5 ml Eppendorf tubes, treated with distilled water containing 0.05% (v/v) DEPC at 37 °C overnight, dried in an oven, and autoclaved, were employed.

2145 6. Ultra pure water (Quality Biologicals, Gaithersburg, MD), free of RNase and DNase was employed.

7. Eppendorf centrifuge 5417R (Eppendorf, Westbury, NY), or other refrigerated centrifuges with adapters for 1.5 ml Eppendorf centrifugal tubes were employed.

2150 8. Bio-Rad PTC-100 thermal cycler (Bio-Rad, Hercules, CA) was employed. Any equivalent thermal cycler with a hot bonnet heated lid may be employed.

The following list of materials were used in connection with the PCR amplification of CDRs and FRs and the assembly of entire VHs.

1. High Fidelity PCR Master (Roche, Indianapolis, IN) was used. Other high-fidelity PCR systems may be used.

2155 2. Primers for PCR amplification of CDRs can be found in Table 1 above. It is noted that to construct a highly diverse antibody library, it is essential for the primers to

be able to cover as many human antibody genes as possible. To design those primers, it is necessary to possess some sequence information and try to understand how human antibody genes are organized. It is further noted that the products of CDR1 primers and CDR2 primers will cover FR1 and FR2, respectively, so that there are only four fragments instead of six (FR1-3 and CDR1-3) for the entire VH assembly. The number of fragments for assembly should be reduced to help decrease reading frame shifts. It was found that highly efficient digestion of VH products with restriction enzymes is critical for the construction of a large library. PCR amplification using the extension primers will result in long overhangs at both 5' and 3' ends of VH products so that there is an obvious difference in length change after digestion with restriction enzyme SfiI, which can be observed clearly on agarose gel.

The following list of materials were used in connection with the digestion of VHs and ligation of VHs with phagemids.

1. Restriction enzymes SfiI, 20000 units/ml (BioLabs, Ipswich, MA) were used.
2. T4 DNA Ligase, 400000 units/ml (BioLabs, Ipswich, MA) was used.

The following list of materials were used in connection with the concentration and desalting of ligations.

1. Centrifugal filter: Amicon Ultra-4 with a cutoff of 3000 MW (Millipore, Billerica, MA).

The following list of materials were used in connection with cell electroporations.

1. TG1 electroporation-competent cells (Stratagene, La Jolla, CA).
2. Gene Pulser/MicroPulser Cuvettes (Bio-Rad, Hercules, CA).
3. Gene Pulser (Bio-Rad, Hercules, CA)

The following list of materials were used in connection preparing the VH library.

1. 2YT medium: 0.5% (w/v) NaCl, 1% (w/v) yeast extract, 1.6% (w/v) tryptone in distilled water. Autoclaved and stored at room temperature.
2. 20% (w/v) glucose in distilled water. Sterilized using 0.22 μ m pore size filter (Nalgene, Rochester, NY).

3. M13KO7 helper phage (BioLabs, Ipswich, MA).
4. Antibiotics: 100 mg/ml ampicillin and 100 mg/ml kanamycin.

A more detailed description of the specific methods that could be employed in
2190 Example 2 are outlined as follows. Equivalent methods can be employed as will be
understood by one of ordinary skill in the art. This specific listing of methods is not
meant to limit the present invention in any way.

Detailed list of methods:

To construct a high-quality (high diversity, low mutation rate, and very few of
2195 reading frame shifts) antibody library, it is important to optimize each step before next
step can be performed.

Lymphocyte isolation by Ficoll-Paque Plus reagents

1. To a 15 ml BD Falcon tube, add 2 ml of defibrinated or anticoagulant-treated
2200 blood and 2 ml of balanced salt solution. Mix by drawing the blood and buffer in and
out of a Pasteur pipette. Note that tissue culture plasticware or pretreated glassware
should be used. All glassware which comes in contact with the samples should be
siliconized before use. The glassware should be immersed in a 1% silicone solution for
10 seconds, washed with distilled water (6 times) and then dried in an oven.
2. Invert the Ficoll-Paque Plus bottle several times to ensure thorough mixing.
2205 Pipette 3 ml of the reagents into a new 15 ml BD Falcon tube. Carefully layer the
diluted blood sample (4 ml) onto the Ficoll-Paque Plus. When layering the sample do
not mix the reagents and the diluted blood sample.
3. Centrifuge at 400 g for 30-40 minutes at 18-20 °C. Note that it is important to
2210 maintain exactly 18-20 °C temperature in the centrifuge. Lower temperature will result
in precipitates in the layer of plasma making the lymphocyte layer unclear.
Optimization of the duration of centrifuging is also recommended to yield a clear
lymphocyte layer. This can be accomplished through a practice using irrelevant blood
samples. After centrifugation, generally four layers can be clearly observed including
plasma, lymphocyte, Ficoll-Paque Plus, and granulocyte/erythrocyte layer from top to
2215 bottom, respectively. Draw off the upper layer of plasma using a clean Pasteur pipette,
leaving the lymphocyte layer undisturbed at the interface.

4. Transfer the lymphocyte layer to a clean 15 ml BD Falcon tube using a clean Pasteur pipette. It is critical to remove the entire interface but with a minimum amount of Ficoll-Paque Plus and plasma. Removing excess plasma causes contamination by platelets and plasma proteins. Removing excess Ficoll-Paque Plus results in unnecessary granulocyte contamination.
5. Add at least 3 volumes of balanced salt solution to the lymphocytes. Suspend the cells by gently drawing them in and out of a Pasteur pipette.
6. Centrifuge at 400 g for 10 minutes at 18-20 °C. Remove the supernatant and resuspend the lymphocytes in 6-8 ml balanced salt solution by pipetting them gently in and out.
7. Determine the number of living cells by using hemacytometer: Mix 50 µl cell suspension with 50 µl trypan blue stain, load 20 µl of the mixture to the hemacytometer, count the total number of living cells (i.e., the unstained cells, since only the live cells have intact membrane that is not permeable for the dye), and calculate the total cell quantity according to the hemacytometer instructions.
8. Centrifuge the cell suspension at 400 g for 10 minutes at 18-20 °C. Remove the supernatant. The lymphocyte pellet can be used immediately for RNA extraction or stored at -80 °C for later use.

2235 Extraction of total RNA from lymphocytes

To extract the total RNA we used RNeasy Mini Kit from Qiagen following the basic protocol provided by the manufacturer. Note that this kit provides enrichment for mRNA by eliminating most RNAs shorter than 200 nucleotides such as 5.8S rRNA, 5S rRNA, and tRNAs. Thus, it may be more efficient for the products to be retro-transcribed to cDNA. Below are described a few modifications in this protocol that may improved the yield and quality of the extracted RNA.

1. Thaw the lymphocyte pellet at room temperature if it is stored at -80 °C. Note that the lymphocytes should not be frozen but used for RNA extraction directly. Also it will be better for RNA products to be retro-transcribed to cDNA without freeze-thaw cycle. Finish these steps within one day. Gently tap the bottom of the tube containing the lymphocyte pellet on the bench to loosen the cells.

- 2. Disrupt cells (up to 5×10^6) by addition of 350 μ l of Buffer RLT from RNeasy Mini Kit. Note that there are limitations with the capacity of the buffer to lyse the cells and the binding capacity of the column. Thus, it is important to use appropriate number of cells in order to obtain optimal RNA yield and purity. Vortex or pipet to mix.
- 3. Pipet the lysate directly onto a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at $12000 \times$ rpm.
- 4. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting. Do not centrifuge.
- 5. Apply all sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube. Centrifuge for 15 s at $10000 \times$ rpm. Discard the flow-through.
- 6. Add 700 μ l Buffer RW1 to the RNeasy column. Centrifuge for 15 s at $10000 \times$ rpm. Discard the flow-through and collection tube.
- 7. Transfer the RNeasy column into a new 2 ml collection tube. Pipet 500 μ l Buffer RPE onto the RNeasy column. Centrifuge for 15 s at $10000 \times$ rpm. Discard the flow-through.
- 8. Add another 500 μ l Buffer RPE to the RNeasy column. Centrifuge for 2 min at $10000 \times$ rpm to dry the membrane in the column.
- 9. Transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30-50 μ l RNase-free water onto the membrane in the column. Centrifuge for 1 min at $10000 \times$ rpm to elute. Store the RNA product at -80°C and use it quickly.

Retro-transcription of RNAs to cDNAs

These instructions assume the use of SuperScript™ III First-Strand Synthesis System from Invitrogen. The following procedure is designed to convert total RNA (5 pg to 25 μ g) or mRNA (5 pg to 2.5 μ g) into first-strand cDNA.

- 1. Mix and briefly centrifuge each component in the kit before use.
- 2. Prepare two RNA/primer mixtures, one with oligo (dT)₂₀ and the other with random hexamers, in 0.2 ml Corning® PCR tubes by combining the following:

2275	Total RNA	x μ l (up to 25 μ g)
	50 μ M oligo (dT) ₂₀ or	5 μ l

	50 ng/μl random hexamers	
	10 mM dNTP mix	5 μl
	DEPC-treated water	40-x μl
2280	Total	50 μl

Incubate at 65 °C for 5 min, then place on ice for at least 1 min.

3. During the incubation, set up two tubes containing the same cDNA synthesis mixtures by adding each component in the indicated order:

	10 × RT buffer	10 μl
2285	2.5 mM MgCl ₂	20 μl
	0.1 M DTT	10 μl
	RNase OUT™ (40 u/μl)	5 μl
	Superscript™ III RT (200 u/μl)	5 μl
	Total	50 μl

2290 4. Add 50 μl cDNA synthesis mixtures to the 50 μl RNA/primer mixtures, mix gently, and collect by brief centrifugation. Incubate as follows:

For oligo (dT)₂₀ primer: 50 min at 50 °C.

For random hexamer primer: 10 min at 25 °C, followed by 50 min at 50 °C.

5. Terminate the reactions at 85 °C for 5 min. Chill on ice.

2295 6. Collect the reactions by brief centrifugation add 5 μl of RNase H to each reaction, and incubate for 20 min at 37 °C.

7. Run a 0.8% (w/v) agarose gel using 2 μl of the reaction to simply check the amount and length distribution of cDNA. An example of the results produced is shown in Figure 21. The cDNA reactions are combined and stored at -20 °C for further use.

2300 PCR amplification of CDRs and FRs

1. First round of PCR to get CDR2s

To amplify the CDR2s from cDNA samples, perform eight amplifications. Set up one PCR tube for each primer combination as the following:

	ddH ₂ O	23-x μl
2305	2 × High Fidelity PCR Master	25 μl
	Forward primer (25 μM)	1 μl

Reverse primer (25 μ M)	1 μ l
cDNA	x μ l (~1 μ g)
Total	50 μ l

2310 Primer recombinations:

H2-F1/H2-R1; H2-F1/H2-R2; H2-F1/H2-R5; H2-F2/H2-R3; H2-F3/H2-R1; H2-F3/H2-R2; H2-F3/H2-R3; H2-F3/H2-R4

Perform the PCR under the following conditions:

Step 1: 4 min at 94 °C for initial denaturation

2315 Step 2: 45 sec at 94 °C; 45 sec at 55 °C; 1 min at 72 °C (30 cycles)

Step 3: 5 min at 72 °C

Run the products separately on a 2% agarose gel to check the specific amplification of CDR2s. An example of the results produced is shown in Figure 22A.

2320 Cut out the correct-sized bands on the gel and purify the DNA using, for example, QIAquick Gel Extraction Kit (Qiagen, Cat. #28706). Running the eight products separately on the gel is strongly recommended to make observation. When different templates are used, products may not be observed on the gel for one or two primer combinations. This should be due to very limited number of templates for the specific primer combinations so it is not necessary to repeat the reaction. However, it is

2325 recommended to cut out the correct-sized gel and purify the limited number of DNA for these primer combinations as is done to those with clear bands. This essentially helps maintain the diversity of the repertoires. Then, pool the purified DNA and quantify it by reading the optical density (O.D.) at 260 nm (1 O.D. unit = 50 μ g/ml). Store the sample at -20 °C for later use.

2330 2. First round of PCR to get CDR3s

Three amplifications are performed to obtain CDR3. Set up the reaction for each primer combination and perform the PCR as it is described above for CDR2 amplification except for the use of different primers.

Primer combinations:

2335 H3-F1/H3R; H3-F2/H3R; H3-F3/H3R

Run the products separately on a 2% agarose gel to check the specific amplification of CDR3s. An example of the results produced is shown in Figure 22B. Purify the DNA. Note that when running the CDR3 products on 2% agarose gel, the bands will not be so sharp due to their highly diverse lengths. Thus, cut as wide a gel as you can to make
 2340 sure it covers those CDR3s with long or short lengths. Next, pool and quantify it by reading the optical density (O.D.) at 260 nm (1 O.D. unit = 50 µg/ml). Store the sample at -20 °C for later use.

3. First round of PCR to get CDR1s

Only one reaction is needed for CDR1 amplification as the following.

2345	ddH ₂ O	46-x µl
	2 × High Fidelity PCR Master	50 µl
	H1-F (25 µM)	2 µl
	H1-R (25 µM)	2 µl
	m0	x µl (~0.1 µg)
2350	Total	100 µl

Perform the PCR under the same conditions as for the CDR2 amplification. At least 1 µg of purified DNA is required in order to proceed further.

4. First round of PCR to get FR3

Only one reaction is needed for FR3 amplification as the following

2355	ddH ₂ O	46-x µl
	2 × High Fidelity PCR Master	50 µl
	FR3-F (25 µM)	2 µl
	FR3-R (25 µM)	2 µl
	m0	x µl (~0.1 µg)
2360	Total	100 µl

Perform the PCR under the same conditions as for the CDR2 amplification. At least 1 µg of purified DNA is required in order to proceed further.

2365 Assembly of entire VHs

1. Perform a second round of PCR (overlap extension) to get CDR1s and CDR2s together

The primers in the first round of PCR create identical sequences in the downstream regions of the CDR1s and the upstream regions of CDR2s. These identical
2370 sequences serve as the overlap for the second-round extension.

Set up a reaction without primers as the following. The CDR1s and CDR2s should be added in the same molarities.

	ddH ₂ O	46-x-y μl
	2 × High Fidelity PCR Master	50 μl
2375	CDR1s	x μl (~100 ng)
	CDR2s	y μl (~120 ng)
	Total	100 μl

Perform the PCR under the following conditions:

Step 1: 4 min at 94 °C for initial denaturation

2380 Step 2: 45 sec at 94 °C; 45 sec at 55 °C; 1 min at 72 °C (7 cycles)

Step 3: 5 min at 72 °C

After the cycling, add primers to the reaction: 2 μl H1-F (25 μM) and 2 μl H2-R1-5 mixture (25 μM). Then perform another 15 cycles of PCR under the same condition. Note that other than the major correct-sized fragments, this procedure could
2385 generate minor nonspecific products for some reasons. Optimization is may be helpful to minimize nonspecific amplification. Increasing the annealing temperature from 55 °C to 60 °C and reducing the number of cycles from 7 to 5, 15 to 12 before and after addition of primers, respectively, may help.

2. Second round of PCR (overlap extension) to get FR3 and CDR3s together

2390 The procedure is almost the same as above except the use of different gene fragments and primers for overlap. The reaction after addition of primers contains the following:

	ddH ₂ O	46-x-y μl
	2 × High Fidelity PCR Master	50 μl
2395	FR3	x μl (~100 ng)

CDR3s	y μ l (~120 ng)
FR3-F (25 μ M)	2 μ l
H3-R (25 μ M)	2 μ l
Total	100 μ l

2400 Run the products on a 2% agarose gel, purify the DNA, and quantify it. At least 2 μ g each of the purified CDR1-CDR2 and FR3-CDR3 DNA is required to proceed.

3. Third round of PCR (final overlap extension) to get whole-length VHs.

Also, the procedure is almost the same as above except the use of resultant gene fragments from Step 1 (CDR1-CDR2) and 2 (FR3-CDR3) above, and extension primers

2405 for overlap. The reaction after addition of primers contains the following:

ddH ₂ O	46-x-y μ l
2 \times High Fidelity PCR Master	50 μ l
CDR1-CDR2	x μ l (~100 ng)
FR3-CDR3	y μ l (~100 ng)
2410 H1-F (25 μ M)	2 μ l
HISR	2 μ l
Total	100 μ l

Run the products on a 2% agarose gel, purify the DNA with gel extraction kit (elute the DNA with ultra pure water in this step instead of elution buffer provided with
2415 the kit), and quantify it. At least 50 μ g of purified VHs is needed to make a library with a size of up to 10^{10} . If the yields are too low, repeat the final overlap PCR and pool the end products.

Digestion of VHs and phagemid vector, and ligation of same

1. Digestion of VHs and phagemid vector

2420 The reaction for VH digestion should contain:

ddH ₂ O	870-x μ l
10 \times Buffer 2	100 μ l
VHs	x μ l (up to 50 μ g)
SfiF (20 units/ μ l)	20 μ l
2425 BSA	10 μ l

	Total	1000 μ l
Set up two reactions for phagemid digestion. Each should contain:		
	ddH ₂ O	870-x μ l
	10 \times Buffer 2	100 μ l
2430	Phagemid vector/phagemid vector	x μ l (up to 100 μ g)
	SfiF (20 units/ μ l)	20 μ l
	BSA (100 \times)	10 μ l
	Total	1000 μ l

2435 Incubate both digests at 50 °C for 3 hours. Run the digested products on agarose gels (2% for VHs and 1% for phagemids), purify the DNA with gel extraction kit (elute the DNA with ultra pure water), and quantify it. Note that the digestion of phagemid vectors may not be complete due to quality of DNA. To address this problem, additional treatment may be needed to further purify the phagemids before digestion, or use more SfiI to digest for longer time, for example, overnight.

2440 2. Ligation of VHs with phagemid vector.

Before large-scale ligation can be performed, it is recommended to test the ligations. One test can be to assess the suitability of the vector and inserts for high-efficiency ligation and transformation. This can be accomplished through assembling small reactions either with vector only (test for vector self-ligation) or with both vector and insert, and transforming chemical competent cells like DH5 α . Another test can be to determine the optimal ratio between insert and vector for the highest efficiency of ligation. This can be accomplished through assembling small reactions with insert and vector in different molarity ratios such as 3:1, 2:1, and 1:1, and transforming chemical competent cells. The highest ligation efficiency may be obtained by using insert in two-
2450 fold molar excess.

Assemble the reaction as the following:

	ddH ₂ O	1750-x-y μ l
	10 \times T4 ligase buffer	200 μ l
	SfiI-digested VHs	x μ l (~30 μ g)
2455	SfiI-digested pCom3bX	y μ l (~90 μ g)

T4 ligase (200 units/ μ l)	50 μ l
Total	2000 μ l

Incubate at 16 °C for 72 hours.

Concentration and desalting of ligated products

2460 Concentrate and desalt the reactions by passing through a 4 ml Amicon Ultra-4 centrifugal filter with a cutoff 3000 MW:

1. Add all 2000 μ l reactions into the filter; centrifuge at 4000 \times g for 20 minutes at room temperature, Remove the flow-through to a 15 ml Falcon tube (do not discard the flow-through at this moment just in case most of DNA is lost due to the broken
2465 membrane of the filter).

2. Add 3.5 ml ultra pure water into the filter and centrifuge under the same condition for 30 min, remove the flow-through to a 15 ml Falcon tube.

3. Repeat Step 2 at least twice. Note that the desalting of DNA samples is an important step to the success of electroporations. High concentration of ions in the
2470 DNA solution will result in a long and intense pulse in electroporations, which causes cell damage or rupture. At least 1000-time dilution of DNA solution can be needed to generate time constants of 4.6-5.0 s in electroporations that generally gave the highest efficiency. In the last repeat, centrifuge for a longer time, making sure that about 50 μ l reactions remain in the filter.

2475 4. Gently pipette the reactions and remove them to a 1.5 ml Eppendorf tube, store at -20 °C for later use.

Electroporations and preparation of library

1. Pre-warm 1 L 2YT medium containing 1% glucose (w/v) at 37 °C. Chill 50 gene pulser cuvettes on ice. At the same time thaw, on ice, the desalted ligations and 2 ml of
2480 TG1 electroporation-competent cells.

2. Divide 2 ml of TG1 competent cells into 5 pre-chilled 1.5 ml Eppendorf tubes with 400 μ l each. Add 10 μ l ligations to each tube and pipet gently to mix. Transfer 41 μ l mixtures to each cuvette. Gently tap the cuvette on the bench to make the mixture fill out the bottom of the cuvette.

- 2485 3. Electroporate at 1.8 kV, 25 μ F, and 200 Ω . Flush the cuvette immediately with 1 ml and then twice with 2 ml of pre-warmed 2YT medium and combine the 3 ml in a 2 L flask. After all electroporations are completed, add 850 ml pre-warmed 2YT medium left to the flask.
- 2490 4. Shake at 250 rpm for 30 min at 37°C. Serially dilute 10 μ l of the culture in 100 μ l of 2YT medium, and spread on LB agar plates containing 2% glucose (w/v) and 100 μ g/ml of ampicillin. Incubate the plates overnight at 37°C. Calculate the total number of transformants by counting the number of colonies, multiplying by the culture volume, and dividing by the plating volume.
- 2495 5. Add 1 ml of 100 mg/ml ampicillin to the 1-L culture and shake for additional 2 hours at 37°C.
- 2500 6. Take 1 ml of the culture and measure the cell density by reading O.D.600. Calculate the total number of cells by multiplying the O.D.600 value by 5×10^8 (estimated number of cells in 1 ml culture when O.D.600 reaches 1) and the culture volume (1000 in this case). Add 10 M.O.I. of M13KO7 helper phage to the culture. Incubate at 37°C for 30 min, shaking for homogenization every 10 min.
7. Spin down the cells at 5000 rpm for 10 min. Resuspend in 2 L 2YT medium containing 100 μ g/ml of ampicillin and 50 μ g/ml of kanamycin. Incubate at 250 rpm overnight at 30°C.
- 2505 8. Spin at 5000 rpm for 15 min at 4°C. Save the bacterial pellet for phagemid preparation using, for example, the Qiagen HiSpeed Plasmid Maxi Kit. For phage precipitation, transfer the supernatant to a clean 2 L flask and add $\frac{1}{4}$ volume of 20% (w/v) PEG8000 and 2.5 M NaCl solution. Mix well and incubate on ice for at least 1 h.
- 2510 9. Spin at 14000 g for 20 min at 4°C. Discard the supernatant. Resuspend the phage pellet in 50 ml PBS, pH7.4 by pipetting up and down along the side of the centrifuge bottle by using a 10-ml pipette.
10. Spin at 5000 rpm for 10 min at 4°C. Transfer the supernatant to a clean 200 ml flask and add $\frac{1}{4}$ volume of 20% (w/v) PEG8000 and 2.5 M NaCl solution. Mix well and incubate on ice for 1 h.

11. Spin at 14000 g for 20 min. Discard the supernatant. Resuspend the phage
2515 pellet in 50 ml PBS, pH7.4.
12. Spin at 5000 rpm for 10 min at 4 °C. Transfer the supernatant to a clean 200 ml
flask.
13. Add the same volume of autoclaved glycerol and mix well.
14. Measure the concentration of phage by reading O.D.280 (1 O.D.280 = 2.33 ×
2520 10¹²/ml). Aliquot the phage to make sure that each contains phage particles at least 100
times of the total number of transformants (calculated in step 4). Store the phage at -80
°C. The phage library is now ready for panning.

The results of Example 2 are follows.

Results:

2525 Design of the VH library

To obtain a diverse VH library, an approach that grafts all three CDRs from
several sources to the framework scaffold of m0 could have been taken. However, the
estimated diversity of such an approach could be more than 10¹² after recombination
among three CDRs, which is difficult for artificially created phage display libraries to
2530 reach. This Example took the approach of mutating one of the CDRs, while grafting in
the remaining CDRs from a variety of different sources. Given that the CDR1s were
relatively more conserved in both their sequences and their lengths, this Example
focused on grafting in CDR2s and CDR3s from a variety of sources, while randomly
mutating four putative solvent-accessible positions of the CDR1 of m0 to A/D/S/Y (the
2535 solvent-accessible positions being amino acid residue #27, 29, 31 and 32 under the
IMGT numbering system) (see Figure 5) in the original CDR1 of m0 to A/D/S/Y. ? not
sure whether you meant our 2008 PNAS paper or this one – you could retain it but the
way it is cited appears like ours.

In order to access as many different human VH gene segments as possible, a
2540 new set of primers (see Table 1) was designed for amplification of CDR2 and CDR3
repertoires, respectively, based on human VH germline sequences loaded in the IMGT
database (<http://imgt.cines.fr/textes/IMGTrepertoire/Proteins/alleles/human/>

HuA1_list.html). The target sequences (see italicized nucleotide sequences flanking CDR2 and CDR3 regions in the m0 template of Figure 5) for these primers are mostly conserved within each group of germlines. Thus, these primers, in combination with each other (see Table 2), should allow efficient amplification of all commonly used human VH gene segments.

To make the resultant library more suited for selection of antibodies against a wide range of antigens including, pathogen and cancer-related antigens, an IgM-derived library from cord blood was used as a template additionally, which we assumed should provide more naïve CDR repertoires. Given the special case of HIV-1, which has mostly evolved to escape the human immune system, an immune library from HIV-1 patients was also used.

Construction of the VH library

The VH library was constructed in three steps. In the first step, 8 and 3 PCRs were performed for amplification of CDR2 and CDR3 gene segments from each library template, respectively (see Figure 6A). Products of CDR2s from different libraries were pooled. Products of CDR3 amplifications were also pooled. CDR1 repertoire was amplified from m0 master VH using a degenerate primer covering the whole CDR1. FR3 segment was also obtained from m0 for assembly of entire VHs. In the second step, overlapping PCRs were performed to join CDR1s to CDR2s and FR3 to CDR3s, respectively (see Figure 6B). In the third step, entire VHs were assembled from the products of the second step by overlapping PCR (see Figure 6C). The products were cloned into phagemid vectors and a library of around 2.5×10^{10} members was obtained by performing 100 electroporations as described above.

Sequence diversity of the library

To assess the sequence diversity of the library, 190 clones were randomly selected from the library and sequenced. 166 complete sequences were obtained, of which 143 have correct reading frames. The 143 sequences were aligned, and the occurrence of A/D/S/Y in each mutated position within CDR1s, the origins of CDR2s and their mutations, and the length distribution of CDR3s were tabulated. The results showed that these sequences were totally different from each other after recombination

among three CDRs although several identical CDR1s and CDR2s were found due to the limited diversity or conservation themselves.

2575 For CDR1s, A/D/S/Y was well scattered in each position while Y dominated slightly (see Figure 8A). CDR2s were derived from VH1, 2, 3, 4, 5 and 7 of germlines (see Figure 8B). VH4-derived CDR2s were dominant by covering around 50% of the 143 sequences. No CDR2s originated from VH6, which is the smallest one, were found. More than 40% of CDR2s are completely identical to the germline sequences, 2580 while there are members with many mutations, regardless of those induced by PCR process (see Figure 8C). For the CDR3s, the lengths vary from 5 to 24 amino acid residues with a central peak from 12 to 14 residues and a side peak at 17 residues (see Figure 8D). In comparison with the length distribution of CDR3s in vivo (Wu et al., Proteins, 1993), there is relatively limited number of CDR3s with length shorter than 8 2585 or longer than 19 residues. Moreover, CDR2s from different origins were paired with CDR3s with varying length (see Figure 9). These data suggest that the library should have high sequence diversity.

Folding of phage-displayed VHs

The folding of phage-displayed VHs was evaluated by measuring their activity 2590 binding to protein A since a VH3 framework scaffold m0 was used in library construction. The library was cycled through 4 rounds of selection to enrich for members that were capable of binding to protein A, and thus, were likely to be folded as natural VH3 structures. 95 clones were randomly picked from the fourth round of enrichment and sequenced and 79 complete and clear sequences were obtained. In the 2595 143 clones from the original library, all 4 positions mutated in CDR1s bias slightly toward residue Y (see Figure 8A) and VH3-originated CDR2s cover about 20% (see Figure 8B). In contrast, all 79 sequences picked after the binding selection process against protein A were shown to have CDR2s derived from VH3. Within CDR1, residue D apparently dominates in position #27, 29 and 32 (see Figure 10). CDR3 of 2600 these clones are much diversified and the length varies from 7 to 17 residues.

VH expression and solubility

We evaluated the expression and solubility of VHs by measuring the yield of soluble proteins purified from the soluble fraction of *E. coli* periplasm. First, 2 clones each, one with relatively short CDR3 and the other long CDR3, were selected from those with CDR2s derived from 6 different groups of human VH germlines, respectively, expressed and purified as described above. The SDS-PAGE result showed that 11 of 12 could be expressed in the form of soluble proteins and the estimated yield ranged from 0.5 to 24 mg l⁻¹ (see Figure 11A). There was no significant relation between the yield and the CDR2 origins or CDR3 lengths of VHs tested.

Then, 12 clones (named c3, c6, d1, d7, b4, c11, d10, b3, b5 (also referred to hereas m36), b7, g6 and e11) with CDR3 length from 7 to 24 residues were picked from those with VH4-derived CDR2s and measured for soluble protein expression. 10 of 12 clones gave yield varying from 0.5 to 15 mg l⁻¹ and there was no obvious connection between the yield and the lengths of CDR3 either (see Figure 11B). Last, we evaluated the solubility of VHs selected after three and four rounds of biopanning of the library with HIV-1 antigen gp120-CD4 fusion protein and gp140 (described further below).

Twelve positive clones on monoclonal phage ELISA with A405 of >1.5 were expressed and purified. As shown in Figure 11C (which is a duplicate of the gel image of Figure 7 showing the identify of clone b5 or “m36”), 11 of 12 clones gave high-level yield from 5.0 to 30 mg l⁻¹. These clones have CDR2s that originated from VH3, VH4 and VH7, which were determined to be dominant in the library (see Figure 8B). These clones were also shown to have medium length CDR3s.

These results indicate that the framework scaffold used for library construction could be flexible enough to harbor CDR2s derived from a variety of human VH germlines and CDR3s with length as short as 7 residues and as long as 24 residues.

Selection of VHs against viral and cancer-related antigens

To assay the performance of the library and given the constant need for new and better therapeutics, the library was panned against three viral antigens and one cancer-related antigen (see Table 3).

Table 3. Panning of the library with viral and cancer-related antigens.

Antigen	No. of selections	Positive clones/ tested clones	No. of unique clones/ sequenced clones	EC ₅₀ of the best binder (µg/ml)
gp120-CD4 (HIV-1 antigen)	4	188/190	14/14	ND
gp120-CD4/ gp140 (HIV-1 antigen)	4	17/190	7/7	0.040 ^a
B5R (vaccinia antigen)	3	6/95	6/6	0.013
Her2 (cancer antigen)	3	5/95	5/5	1.1

^a Binding activity with gp120-CD4.

2635 Selections were carried out using antigens conjugated on magnetic beads as described above. In the sequential panning with HIV-1 antigens (alternate panning between HIV gp120-CD4 and gp140), gp120-CD4 was used for the first round and third round, and gp140 the second round and fourth round. Enrichment was achieved in all selections and positive clones were identified on monoclonal phage ELISA.

2640 A handful of clones having a high value of A405 following the ELISA were sequenced. All of the sequenced clones were mosaic, especially in CDR1 and CDR3 (see Table 4).

Table 4. CDR diversity of phage-displayed VHs selected from biopanning.

<u>Antigen</u>	<u>Round No.</u>	<u>Clone</u>	<u>CDR1</u>	<u>CDR2</u>		<u>CDR3</u>
				<u>Sequence</u>	<u>Origin</u>	
gp120-CD4	3	a9	D-H-HS--	INHSGIT (SEQ ID NO: 23)	4-4, 4-34	AIVDTAMVWDY (SEQ ID NO: 44)
		b2	S-A-SD--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AASGSYSYDY (SEQ ID NO: 45)
		b3	A-D-SY--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	ATHDYGDSFES (SEQ ID NO: 46)
		c3	D-S-YS--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	ARIGDGGFFSDAFEI (SEQ ID NO: 47)
		c6	D-Y-DY--	IDNSGST (SEQ ID NO: 25)	4-28, 4-30-2, ect.	AGDYGSGSEFEN (SEQ ID NO: 48)
		d1	D-D-YD--	INTDGDIP (SEQ ID NO: 26)	7-4-1	AKYTWNSDSGWGEL (SEQ ID NO: 49)
		d7	Y-D-YD--	IYHRGNT (SEQ ID NO: 27)	4-4, 4-30-2, ect.	VGYGADQDDC (SEQ ID NO: 50)

<u>Antigen</u>	<u>Round No.</u>	<u>Clone</u>	<u>CDR1</u>	<u>CDR2</u>		<u>CDR3</u>
				27)		
		e10	D-A-DD--	INHTGST(SEQ ID NO: 28)	4-34	ATHDYGDSFES (SEQ ID NO: 51)
		g2	D-D-DY--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AADTGNAFDI (SEQ ID NO: 52)
	4	a11	Y-D-DD--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AGSSGWLHEY (SEQ ID NO: 53)
		b4	D-D-AS--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	ATDQAGIEH (SEQ ID NO: 54)
		c8	S-S-AD--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	ATSVGYEEL (SEQ ID NO: 55)
		c11	A-S-DY--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AMSDGYSATDV (SEQ ID NO: 56)
		d10	A-A-DD--	ITGSGDTT (SEQ ID NO: 29)	3-23	ALTDSSSYDY (SEQ ID NO: 57)
gp120-CD4/gp140	3	a8	D-D-SD--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AFYMRGAILEY (SEQ ID NO: 58)
		b3	Y-D-SS--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AAFYDFWGSYPY (SEQ ID NO: 59)
		b5	A-D-SD--	INDSGNT (SEQ ID NO: 30)	4-34, 4-55	AIYGGNSGGEY (SEQ ID NO: 60)
		b7	D-S-DY--	VGGSGERT (SEQ ID NO: 31)	3-23	ARIDRDGDEH (SEQ ID NO: 61)
		c6	S-D-DY--	INHSGST (SEQ ID NO: 24)	4-4, 4-34	AGSGSYSDY (SEQ ID NO: 62)
	4	g6	D-A-DY--	INSNGSVT (SEQ ID NO: 32)	3-74	ARDWGYSPEL (SEQ ID NO: 63)
		e11	Y-D-YD--	ISYDGSIK (SEQ ID NO: 33)	3-30, 3-30-3	N/A
B5R	3	z1	S-Y-DY--	IYHSGST (SEQ ID NO: 34)	4-30-2	ARTPPRIAAAGMRYFDL (SEQ ID NO: 64)
		z2	S-D-AD--	INSSSYI (SEQ ID NO: 35)	3-21	ARDWGYSPEL (SEQ ID NO: 65)
		z3	D-D-YS--	ISGDGGAT (SEQ ID NO: 36)	3-23	ARADYRSTDH (SEQ ID NO: 66)
		z4	D-S-YD--	IYYSYGST (SEQ ID NO: 37)	4-39	ARQVAAPV (SEQ ID NO: 67)
		z5	D-S-YY--	IKQDGSVV (SEQ ID NO: 38)	3-7	ARDWGYSPEL (SEQ ID NO: 68)
		z6	Y-D-YA--	ISYDGSNK (SEQ ID NO: 39)	3-30, 3-30-3	VRDWGYNPED (SEQ ID NO: 69)
Her2	3	z7	Y-D-SS--	IYSGGTT (SEQ ID NO: 40)	3-53, 3-66	VRDWGYNPED (SEQ ID NO: 70)
		z8	Y-Y-DY--	ISNSGGTI	3-11	ARGTGLHDYGDY

<u>Antigen</u>	<u>Round No.</u>	<u>Clone</u>	<u>CDR1</u>	<u>CDR2</u>		<u>CDR3</u>
				(SEQ ID NO: 41)		WAHTEFDY (SEQ ID NO: 71)
		z9	D-A-AD--	IYSGGST (SEQ ID NO: 42)	3-53, 3-66	ARDWGYSPED (SEQ ID NO: 72)
		z10	D-D-YD--	IRYDGSNK (SEQ ID NO: 43)	3-30, 3-30-3, ect.	ARGVDYGDYGGY FDY (SEQ ID NO: 73)

For the panning against HIV-1 antigens, however, most of selected clones have
 2645 CDR2s originated from VH4, in particular VH4-4 or VH4-34 sub-group. Their CDR3s
 vary and lengths range from 9 to 14 residues. In contrast, most and all clones selected
 from B5R and Her2 panning, respectively, have VH3-derived CDR2s. Some of them,
 for example, z2, z5 and z6, have similar CDR3s.

Some clones were expressed and purified, and their binding and specificity were
 2650 confirmed by ELISA against the antigen used for its selection, as well as several
 unrelated antigens (e.g. BSA or antigens which are significantly heterologous to the one
 used in the panning could be used as unrelated antigens. For example, in the Her2
 panning HIV-1 antigen Bal gp120-CD4 could used as a negative control while in the
 Bal gp120-CD4 panning Her2 could be used as an unrelated antigen).

2655 In the sequential panning, an antibody with affinity in nanomolar range ($EC_{50} =$
 0.040 μ g/ml), designated b5 (see Figure 11C) (later designated m36) was identified as
 binding to HIV-1 antigen gp120-CD4 but neither gp120 nor CD4 alone.

Further characterization showed that b5 (m36) was a potent neutralizing
 antibody which targeted an epitope on HIV-1 gp120 whose accessibility appeared to be
 2660 induced upon CD4 binding to HIV-1 gp120. In addition, b5 (m36) was determined to
 be cross-reactive against HIV-1 isolates from clade A, B, C, and D (see Figure 17).

Regarding panning results for vaccinia antigen B5R, a specific VH, z1, was
 found with an EC_{50} of 0.013 μ g/ml. Binders against human self-antigen Her2 could also
 be selected, albeit at a somewhat lower frequency and with lower affinity (see Table 3).

2665 VH oligomerization

Size exclusion chromatography was performed to measure the oligomerization
 of VHs using superdex75 column. 5 purified VHs were randomly selected from 12

positive clones (c3, c6, d1, d7, b4, c11, d10, b3, b5, b7, g6 and e11) against HIV-1 antigen gp120 on monoclonal phage ELISA and tested for oligomerization. 2 of 5 VHs, e.g. b7 and d10, have CDR2s originated from human VH3 while the CDR2s of the other 3 from human VH4 (see Figure 11C).

Before conducting the experiments, all 12 VHs (c3, c6, d1, d7, b4, c11, d10, b3, b5, b7, g6 and e11) had already been stored at 4°C for 4 weeks and no precipitation was observed with these protein solutions. The data shown in Figure 12 demonstrates the existence of a monomeric state of all 5 VHs (~15 kDa) and lack of higher order oligomers.

The results of Example 2 are further discussed as follows.

Discussion:

Domain antibodies (dAbs) are of great interest for biomedical application due to their special biophysical properties. However, the poor solubility and stability of most dAbs, particularly those derived from human antibodies, severely limits the successful development of dAbs and dAb libraries. Camels produce functional antibodies devoid of light chains, V_HHs, which are adapted replacing aliphatic residues in the former light chain interface by hydrophobic residues, packing against the framework and stabilizing the global V_HH fold by long CDR3s (Holt et al., Trends Biotech., 2003; Muyldermans et al., Trends in Biochem. Sciences, 2001). It has been demonstrated that the variable domains of human VH3 are significantly more soluble and stable than those from any other human VH and VL families in the absence of light chains (Holt et al., Trends Biotech., 2003).

Jirholt et al. used a camelized human VH3 germline, DP47, as a master framework for construction of a dAb library of 9×10^6 members but no further characterization was described in terms of solubility and functionality of dAbs from the library (Jirholt et al., Gene, 1998).

The present inventors have constructed a large non-immune human Fab library (~ 1.5×10^{10} members) from the lymph nodes, spleen and peripheral blood lymphocytes of 50 donors. One of these Fabs (R3H1, see Figure 4) had a stop codon in the light chain but was still isolated from library panning and was functional as a heavy chain

alone. The VH domain of this antibody was cloned to form the VH domain antibody, m0 (as shown in Figure 5). It was found that m0 exhibits high levels of expression and high solubility.

m0, a natural VH domain and belonging to the VH3 family, was used as a framework to construct a large human VH domain library ($\sim 2.5 \times 10^{10}$ members) by grafting in vivo-formed CDR2s and CDR3s from our other human antibody libraries and mutating four residues in CDR1. The library exhibited a high degree of variability and most of VHs were highly soluble. The quality of the library was also validated by selection of VHs against a panel of antigens, including HIV-1 antigens (Bal gp120-CD4; Bal gp120-CD4 / R2 gp140), a vaccinia antigen (B5R), and a cancer antigen (Her2).

Without wishing to be bound by theory, naturally occurring and proofread CDRs are advantageous as compared to synthetic repertoire CDRs because they are processed and adapted by the immune system with respect to their functionality and immunogenicity. Abergel and Claverie (Eur. J. Immunol., 1991) showed that VH genes rearranged in vivo have CDR3s largely derived from the D-segments encoding amino acids with a propensity for loop formation. By comparison with synthetic CDR3s, therefore, naturally occurring CDRs may be more likely to fold properly or produce usefully shaped binding sites. Furthermore, there is evidence that antibodies derived from recombination of natural CDRs exhibit low immunogenicity (Soderlind et al., Nature Biotech., 2000). A scFv library containing 2×10^9 members has recently been constructed by randomly shuffling all six in-vivo formed CDRs into a single master framework using human VH DP47 and VL DPL3. Ten scFvs selected from the library specific for different antigens did not show an increased presence of T-cell epitopes, and they contained even fewer T-cell epitopes than the natural antibody variable regions.

The size and sequence diversity are thought to be key features of high-quality libraries. A finding consistent with theoretical considerations is that the affinity of antibodies selected is proportional to the size of the library, with K_{ds} ranging from 10^{6-7} for the smaller libraries to 10^9 for the larger ones, and antibodies with affinity

comparable to those obtained from immune libraries can be selected from naïve libraries that are large enough (Andrew et al., *J. Immunol. Methods*, 2004).

2730 Aiming in part at generating a library with enormous diversity, the present invention relates to the design of a new set of primers to allow amplification of as many VH genes as possible. The complexity of the library formed according to the invention was determined by sequencing 190 clones randomly selected (see Figure 8 and Figure 9). The 143 complete sequences have CDR2s derived from 6 (VH1-5 and 7) of 7
2735 human VH germlines (see Figure 8B). Since VH6 is the smallest group of germlines, we assume that CDR2s with VH6 origin could also be found if more clones are sequenced. The length, sequence, and pairing of CDR3s with different germline-derived CDR2s were varied simultaneously (see Figures 8D and 9). These data suggest that this library should have high sequence diversity.

2740 In a scFv library previously constructed, primers were designed for amplification of naturally occurring CDRs, but they were specific for DP47 and DPL3 frameworks only (Soderlind et al., *Nature Biotech.*, 2000; Jirholt et al., *Gene*, 1998). By contrast, the primers used in the present invention advantageously may cover CDRs derived from all groups of human VH germlines, as described above. They may,
2745 therefore, the present invention, in part, facilitates more rapid and efficient construction of large human VH domain libraries.

 While the combinatorial strategy provides a powerful tool for creating tremendous diversity, the m0 framework scaffold used in the library construction plays a challenging role because it must support the CDRs from various non-VH3 germlines
2750 while maintain a stable tertiary fold. Structural incompatibility between these foreign CDRs and the fixed framework could potentially prevent the formation of stable and soluble VHs. Thus, it is surprising and unexpected that the domain antibodies of the invention specific against various antigens and having a diversity of non-VH3 CDRs possess good folding characteristics.

2755 Protein A binding is considered a marker for proper folding of human VH3 (Potter et al., *J. Immunol.*, 1996). To see whether recombinant VHs with a fixed m0 which is close to VH3 scaffold could retain activity, the library was cycled through four

rounds of selection against protein A. All 79 clones randomly picked from the fourth round of selection had CDR2s derived from VH3, suggesting that VH3-derived CDR2s and their flanking FR regions are essential for their property of protein A binding, in agreement with previous studies (Potter et al., J. Immunol., 1996; Randen et al., Eur. J. Immunol., 1993). Interestingly, position #27 and 29 in CDR1s significantly biases toward residue D in those clones after protein A selection while residue Y dominates in these positions of VHs with VH3-derived CDR2s before selection (see Figure 10). There is also a slight increase in the usage of D in position #32 and this does not happen to position #31. Given D is a small and polar residue, and Y an aromatic residue, the polarity of the residues in these positions could significantly impact the folding of VH3 with respect to protein A binding activity.

It has recently been demonstrated that this property can be used to monitor the structural stability and soluble expression of VH3 (Bond et al., J. Mol. Biol., 2003). Thus, the fact that VHs with non-VH3 CDR2s lack the property further raises a doubt whether these VHs can be soluble and stable. To address this issue, the yield of soluble VHs having non-VH3 CDR2s and CDR3s ranging from 7 to 24 residues was measured (Figure 11). The results show that most of VHs have favorable yield of soluble proteins and the yield is not significantly related to CDR2 origin and CDR3 length.

In addition to proper folding, the domain antibodies of the invention were evaluated as to whether they possessed any tendency to form dimers and higher order multimers – which, for steric considerations, may not be desirable. Antibodies in the format of scFv have the tendency to form dimers and higher order multimers in a clone-dependent and relatively unpredictable way (Abergel et al. 1991; Andrew et al., 2004; Potter et al., 1996). The oligomerization of 5 VHs randomly selected was measured by size exclusion chromatography and they all appeared to be monomeric (see Figure 12). There is evidence that VHs are prone to aggregate upon concentration or prolonged standing at 4°C (Kortt et al., 1995; Ewert et al., 2002). The 5 VHs tested for oligomerization were concentrated to get concentrations of as high as 10 mg/ml. After being stored at 4°C for more than 8 weeks no precipitation was observed with these 5 protein solutions.

2790 These data suggest that the m0 scaffold not only is capable of presenting diverse CDRs, but also has desirable properties for biotechnological application, including, high level of expression, solubility, resistance to aggregation in solution, and lack of polymerization.

2795 A central question in the evaluation of the library is whether high-affinity functional VHs could be directly selected against a panel of antigens. The library design was based on the principle that a specific single framework had the potential to include CDRs derived from other groups of germline genes and the small antigen-binding surface was capable of presenting structural diversity enough to form paratopes for a wide range of antigens.

2800 Biopanning of the library showed that VHs with estimated affinity in nanomolar range could be obtained against viral antigens (see Table 3). Binders against human self-antigens could also be selected, albeit at a somewhat lower frequency and with lower affinity. These binders are much diversified in CDRs (see Table 4) suggesting that a number of different antibody specificities could be generated. Thus, this Example demonstrates that this library could be useful for selection of high-affinity binders as therapeutics, e.g., HIV therapeutics.

2805 **Example 3. Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers**

2810 This Examples describes the identification and characterization of an antibody heavy chain variable domain (VH) (domain antibody, dAb), m36, targeting highly conserved but sterically restricted CD4-induced (CD4i) structures on the Env. It is believed that M36 is the first reported representative of a novel class of potent and broadly cross-reactive HIV-1 inhibitors based on human dAbs. It has potential as a candidate therapeutic and a microbicide, and as an agent for exploration of the highly protected conserved Env structures with implications for the design of novel small molecule inhibitors, and elucidation of the mechanisms of virus entry into cells and evasion of immune responses.

2815 The following methods and materials were employed in this Example. The results of this Example are subsequently discussed below.

Methods and materials:

Cells, viruses, plasmids, gp120, gp140 and antibodies

2820 293T cells were purchased from ATCC. Other cell lines and plasmids used for
expression of various HIV-1 Envs were obtained from the NIH AIDS Research and
Reference Reagent Program (ARRRP). Recombinant gp140s were kindly provided by
C. Broder (USUHS, Bethesda, MD). Gp120_{Bal} and the single-chain fusion protein
gp120_{Bal}-CD4 (8) were gifts from T. Fouts (Institute of Human Virology, Baltimore;
2825 currently at Profectus, Baltimore, MD). Horseradish peroxidase (HRP)-conjugated anti-
FLAG tag antibody and HRP-conjugated anti-human IgG (Fc-specific) antibody were
purchased (Sigma-Aldrich, St. Louis, MO).

Library construction and selection of VHs against HIV-1 antigens

A large phage-displayed human VH library ($\sim 2.5 \times 10^{10}$ individuals) was
2830 constructed by grafting of naturally occurring heavy chain CDR2s and CDR3s from
other five template libraries to a VH framework scaffold, m0, and randomly mutating
four putative solvent-accessible residues in its CDR1 (Figure 23). These template
libraries include: (a) a naive human Fab library (5×10^9 members) from peripheral
blood B cells of 10 healthy donors (Zhu et al., 2006); (b) a naïve human Fab library (1.5
2835 $\times 10^{10}$ members) from peripheral blood B cells of 22 healthy donors, spleens of three
donors, and lymph nodes of healthy 34 donors; (c) two naïve human Fab libraries ($6 \times$
 10^8 and 7.2×10^8 members, respectively) from cord blood of two healthy babies,
respectively; and (d) an immune human Fab library from the bone marrow of three
long-term nonprogressors whose sera exhibited the broadest and most potent HIV-1
2840 neutralization among 37 HIV-infected individuals (Chen et al., J. Mol. Biol., (2008)
382:779-789, which is incorporated herein by reference in its entirety). This dAb library
was used for selection of VHs against HIV-1 antigens conjugated to magnetic beads
(Dynabeads M-270 epoxy; DYNAL Inc., New Hyde Park, NY) as described previously
(Zhu et al, 2006). For sequential panning, 10 and 5 μ g of gp120_{Bal}-CD4 was used in the
2845 first round and third round, respectively; antigens were alternated with 5 μ g of gp140_{R2}
or gp140_{JRFL} during the second round and fourth round. Clones that bound to HIV-1

antigens were identified from the third and fourth round of biopanning by using monoclonal phage ELISA as described (Zhu et al, 2006).

Construction and cloning of m36 fusion proteins

- 2850 The following primers were used:
- m36F, 5'-TGGTTTCGCTACCGTGGCCAGCCGGCCAGGTGCAGCTG
GTG-3' (sense) (SEQ ID NO: 74);
- m36F1, 5'-TGGTTTCGCTACCGTGGCCAGCCGGCCAGGTGCAGC
TGGTG-3' (sense)(SEQ ID NO: 75);
- 2855 m36R1, 5'-GTGAGTTTTGTTCGGGCCCTGAGGAGACGGTGAC-3'
(antisense)(SEQ ID NO: 76);
- m36R2, 5'-TGGTTGTGGTTGGGGTATCTTGGGTTCTGAGGAGACGGT
GAC-3' (antisense)(SEQ ID NO: 77);
- m36R3, 5'-GTCACCAAGTGGGGTTTTGAGCTCTGAGGAGACGGT
2860 GAC-3' (antisense)(SEQ ID NO: 78);
- m36R4, 5'-TTCTCGGGGCTGCCCTGAGGAGACGGTGAC-3' (antisense)(SEQ
ID NO: 79);
- m36R5, 5'-CAGGAGTTCAGGTGCTGAGGAGACGGTGAC-3' (antisense)(SEQ
ID NO: 80);
- 2865 CH3F, 5'-GGGCAGCCCCGAGAACCA-3' (sense)(SEQ ID NO: 81);
- CH3R, 5'-GTGGTGGTGGTGGTGGCCGGCCTGGCCTTTACCCGGAGA
CAG-3' (antisense)(SEQ ID NO: 82);
- FcF1, 5'-ACGTGTCCCAAATGTCCAGCACCTGAACTCCTGGGG-3' (sense)(SEQ
ID NO: 83);
- 2870 FcF2, 5'-CCGTGCCACGGTGCCAGCACCTGAACTCCTGGGG-3' (sense)(SEQ
ID NO: 84);
- FcF3, 5'-GCACCTGAACTCCTGGGG -3' (sense)(SEQ ID NO: 85);
- FcR, 5'-GTCGAGGCTGATCAGCGG-3' (antisense)(SEQ ID NO: 86);
- FcR1, 5'-CTCCTATGCGGCCGCTTTACCCGGAGACAG-3' (antisense)(SEQ ID
2875 NO: 87);
- HcF, 5'-CCCCAACCACAACCAAAAACCACAACCACAACCACAACCA

CAACCAAACCAC-3' (sense)(SEQ ID NO: 88);

HcR, 5'-TGGACATTTGGGACACGTGCATTCTGGTTCAGGTTTTG

GTTGTGGTTTTGGTTGTGG-3' (antisense)(SEQ ID NO: 89);

2880 HhF, 5'-ACCCCACTTGGTGACACAACACTCACACATGCCACGG

TGCCAGAGCCCAA-3' (sense)(SEQ ID NO: 90);

HhR, 5'-TGGGCACCGTGGGCACGGGGGAGGTGTGTCACAAGA

TTTGGGCTCTGGGCA-3' (antisense)(SEQ ID NO: 91);

HSAPR2, 5'- CTCCTATGCGGCCGCATCATCGTCGCCCCACAAACACCCCC

2885 AGCGTGGCAA-3' (antisense)(SEQ ID NO: 92);

HSAPR3, 5'- CCCCAGCGTGGCAAACATATATCTTCTGGGTGGCGCTG

GCCCTTATCGTCATC-3' (antisense) (SEQ ID NO: 93).

For construction of m36CH3, the m36 gene was amplified by PCR (primer: m36F1 and m36R4) with m36-encoding plasmid pCom36 as a template. The CH3 gene
 2890 of human IgG1 was PCR (primer: CH3F and CH3R) amplified from plasmid pSecTagB-Fc, which encoded the human IgG1 Fc portion (Figure 29). M36 fragment was joined to CH3 by overlapping PCR performed in a volume of 100 µl using both templates (in the same molarities) for 7 cycles in the absence of primers and additional 15 cycles in the presence of primers (500 pM of m36F1 and CH3R, respectively). The
 2895 m36CH3 products appended with SfiI restriction sites on both sides were digested and cloned into a phagemid vector (Figure 22). To generate m36b0Fc, m36 gene was amplified by PCR using primer m36F1 and m36R5. Human IgG1 Fc gene was obtained by PCR amplification using pSecTagB-Fc as the template and primer FcF3 and FcR1. M36 fragment was joined to Fc as described above. The products were
 2900 digested with SfiI and NotI, and cloned into pZYD-N1, which was developed by the inventors to have a NotI site after the FLAG tag. The vector pSecTagB-Fc was used for construction of m36h1Fc, m36c2Fc, and m36h3Fc. The m36 fragment was PCR amplified using primer m36F and m36R1, digested with SfiI and ApaI, and cloned into pSecTagB-Fc to generate m36h1Fc. M36c2Fc was cloned by amplifying m36 fragment
 2905 (primer: m36F and m36R2), human IgG1 Fc (primer: FcF1 and FcR), and camel IgG2 hinge (primer: HcF and HcR). The m36 fragment was fused to camel IgG2 hinge and

the product was subsequently joined to Fc by overlapping PCR. The resultant full-length m36c2Fc product was digested with SfiI and PmeI, and cloned into pSecTagB-Fc vector. In the same way m36h3Fc was constructed except for the use of primer m36F and m36R3 for m36 amplification, primer FcF2 and FcR for human IgG1 Fc amplification, and primer HhF and HhR for human IgG3 hinge amplification. To generate m36SAbp, the m36 fragment was amplified using primer m36F1 and HSAPR3, purified and further extended by PCR using primer m36F1 and HSAPR2. The products were digested with SfiI and NotI, and cloned into pZYD-N1.

2915 Expression and purification of m36 and its fusion proteins

M36, m36SAbp, m36CH3 and m36b0Fc were expressed in *E. coli* HB2151 as described previously (Zhu et al, 2006). The bacterial pellet was collected after centrifugation at $5,000 \times g$ for 10 min and resuspended in PBS (pH 7.4) containing 0.5 mU polymixin B (Sigma-Aldrich, St. Louis, MO). After 30 min incubation with rotation at 50 rpm at room temperature, it was centrifuged at $25,000 \times g$ for 25 min at 4°C. The supernatant was used for purification of m36, m36SAbp and m36CH3 by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA resin (Qiagen, Valencia, CA) according to manufacturer's protocols. For purification of m36b0Fc, nProtein A Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ) was used. M36h1Fc, m36c2Fc and m36h3Fc were expressed in 293 free style cells. CellFectin (Invitrogen, Carlsbad, CA) was used to transfect 293 free style cells according to the instructions of the manufacturer. Three days posttransfection, the culture supernatant was harvested and used for purification of m36h1Fc, m36c2Fc and m36h3Fc by using nProtein A Sepharose 4 Fast Flow.

2930 Binding ELISA

Antigens were coated on Corning EIA/RIA high-binding 96-well plates (Corning Inc., Corning, NY) at 50 ng per well overnight at 4°C and blocked with 3% nonfat milk in PBS. Threefold serially diluted antibody was added in the absence or presence of sCD4 at 2 µg/ml final concentration and incubated at room temperature for 2 h. The plates were washed with PBS containing 0.05% Tween 20. Bound m36, m36SAbp or m36CH3 was detected by HRP-conjugated anti-FLAG tag antibody

(Sigma-Aldrich, St. Louis, MO). The m36 fusion proteins with human IgG1 Fc were detected by HRP-conjugated anti-human IgG (Fc-specific) antibody (Sigma-Aldrich, St. Louis, MO). The assay was developed at 37°C with ABST substrate (Roche, Indianapolis, IN) and monitored at 405 nm. The half-maximal binding (EC_{50}) was calculated by fitting the data to the Langmuir adsorption isotherm.

Competition ELISA

Antigens were coated and blocked as described above. M36 at a concentration leading to 90% maximum binding was premixed with threefold serially diluted competitors without or with sCD4 at 2 µg/ml final concentration. Mixtures were subsequently added to each well and incubated. Bound m36 was detected and the assay was developed as described above.

Measurement of m36 oligomerization

Superdex75 column was calibrated with protein molecular mass standard of 14 (ribonuclease A), 25 (chymotrypsin), 44 (ovalbumin), 67 (albumin), 158 (aldolase), 232 (catalase), 440 (ferritin) and 669 (thyroglobulin) kDa. Purified m36 in PBS were loaded onto the column that had been pre-equilibrated. The proteins were eluted with PBS at 0.5 ml/min.

Pseudovirus neutralization assay

Viruses pseudotyped with HIV-1 Envs were prepared by cotransfection of 70–80% confluent 293T cells with pNL4-3.luc.E-R- and pSV7d constructs encoding HIV-1 Envs using the PolyFect transfection reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. Pseudotyped viruses were obtained after 24 h by centrifugation and filtration of cell culture through 0.45-µm filters. For neutralization, viruses were mixed with different concentrations of antibodies and/or sCD4 at 8 nM for 1 h at 37°C, and then the mixture was added to $\sim 1.5 \times 10^4$ HOS-CD4-CCR5 (used for all R5 and dual tropic viruses) or HOS-CD4-CXCR4 cells grown in each well of 96-well plates. Luminescence was measured after 48 h using the Bright-Glo Luciferase Assay System (Promega, Madison, WI) and a LumiCount microplate luminometer (Turner Designs). Mean relative light units (RLU) for duplicate wells were determined. Percentage inhibition was calculated by the following formula: (1 - average RLU of

antibody-containing wells/average RLU of virus-only wells) \times 100. IC₅₀ and IC₉₀ of neutralization were assigned for the antibody concentration at which 50% and 90% neutralization were observed, respectively.

2970 The results of Example 3 are provided as follows.

Results:

Selection of m36 from a newly constructed human antibody VH library.

2975 Examples 1 and 2 discuss the identification of a phage-displayed heavy chain only antibody by panning of a large (size $\sim 1.5 \times 10^{10}$) human naive IgM library against an Env. The VH of this Fab, designated as m0, was independently folded, stable, highly soluble, monomeric, and expressed at high levels in bacteria. M0 was used as a scaffold to construct a large (size $\sim 2.5 \times 10^{10}$) highly-diversified phage-displayed human VH library by grafting naturally occurring CDR2s and CDR3s of heavy chains from five human antibody Fab libraries, and randomly mutating four putative solvent-accessible
2980 residues in CDR1 (Figure 23).

A VH, m36, was selected from this library as the highest affinity binder by using the sequential antigen panning (SAP) methodology (Zhang et al., 2003) with HIV-1 Envs from clade B: a truncated Env lacking the transmembrane portion and the cytoplasmic tail from R2 (gp140_{R2}) (Quinnan et al., 1999) or from JRFL (gp140_{JRFL}),
2985 and gp120 from Bal in complex with CD4 as a fusion protein (gp120_{Bal}-CD4) (Fouts TR et al., 2000). M36 is monomeric in PBS at pH 7.4 as determined by size exclusion chromatography, and runs on SDS-PAGE gels and size exclusion chromatography with an apparent molecular weight (MW) of 14-15 kDa which is close to the calculated MW (14.972 kDa, including the His and FLAG tags) (data not shown). It is highly soluble,
2990 thermally stable, and is expressed at high levels in bacteria (~ 30 mg per L of culture) (data not shown). The m36 framework and CDR1 are closest to those encoded by the VH3-23 germline gene; the CDR2 – to the VH4-34 (Figure 24). All m36 CDRs contain negatively charged and neutral but not basic residues. The CDR3 sequence is relatively short.

2995 Potent cross-reactive neutralization of pseudotyped HIV-1 isolates by m36

To determine the potency and breadth of HIV-1 neutralization by m36, viruses pseudotyped with Envs from HIV-1 isolates representing clades A, B, C, D and E were used. M36 neutralized six isolates from clade B, one isolate from clade C, and one isolate from clade A with potency on average two-fold higher (two-fold lower IC₅₀s on molar basis) than that of the broadly cross-reactive neutralizing CD4i antibody scFv m9 (Zhang et al., 2004) (Figure 25); m9 is an in vitro matured derivative of X5 and exhibits superior neutralizing activity compared to known cross-reactive HIV-1 neutralizing antibodies (b12, 4E10, 2F5, 2G12, X5) when tested against more than 100 isolates. M36 exhibited remarkable activity against the clade C isolate GXC-44 and the clade B isolate NL4-3 with very low IC₉₀s (Figure 32). It exhibited lower neutralization activity against the clade B isolate 89.6 and the clade-D isolate Z2Z6 compared to scFv m9. M36 and scFv m9 did not neutralize the clade E isolate GXE at concentrations up to 667 nM. M36 was also on average more potent than the peptide C34 (Figure 25); C34 (10) is a gp41-derived peptide which exhibits HIV-1 entry inhibitory activity comparable to or higher than that of the FDA approved peptide entry inhibitor T20 (DP178, FUZEON™) which shares significant sequence homology with C34 although inhibits entry by somewhat different mechanism involving binding to multiple sites (Liu S et al., 2005). The inhibitory activity of m36 was dose-dependent (Figure 26A). Complete (100%) inhibition of 4 out of 7 clade B isolates and the isolate from clade C was achieved at 667 nM concentration; note that at the equivalent molar concentration C34 did not completely inhibit any of the isolates tested, and m9 completely inhibited 3 out of 7 clade B isolates (Figure 26B). These results suggest that m36 is a potent cross-reactive neutralizing antibody with potency and breadth of neutralization for this panel of isolates on average better than that of scFv m9 and C34. These three inhibitors exhibit differential neutralization profiles and could be used in combination.

M36 binding to gp120 is enhanced by CD4 and decreased by the CD4-binding site antibody m14

To approximately localize the m36 epitope and begin to elucidate the underlying mechanisms of neutralization, we measured binding of m36 to Envs from different isolates alone and in complex with CD4 as well as the m36 competition with well

characterized antibodies. M36 bound to gp120_{Bal}-CD4 with high affinity ($EC_{50} \sim 2.5$ nM) but not to gp120_{Bal} or to soluble CD4 (sCD4) and BSA, as measured by an ELISA assay (Fig. 3A). It also bound to gp140 from a clade C isolate GXC-44 (gp140_{GXC-44}) in the presence of sCD4 but notably it did bind although weaker to gp140_{GXC-44} alone too (data not shown) in contrast to gp120_{Bal} (data not shown). Similarly, it also bound to another clade B Env, a tethered gp140 from 89.6, without complexation with CD4 but its binding was enhanced after gp140 bound to CD4 (data not shown). In both cases the binding as function on concentration deviated from a classical Langmuir-type isotherm likely due to a more complex multi-stage mechanism of antibody-antigen interactions.

3030 (data not shown) in contrast to gp120_{Bal} (data not shown). Similarly, it also bound to another clade B Env, a tethered gp140 from 89.6, without complexation with CD4 but its binding was enhanced after gp140 bound to CD4 (data not shown). In both cases the binding as function on concentration deviated from a classical Langmuir-type isotherm likely due to a more complex multi-stage mechanism of antibody-antigen interactions.

3035 As expected for a CD4-induced (CD4i) antibody, m36 competed for binding to gp120_{Bal}-CD4 with the CD4i antibody m16 (Fig. 3B) but not with the CD4 binding site (CD4bs) antibody m14 which was used as a negative control. Notably, it also competed for binding to gp140_{GXC-44} in the absence of CD4 with m14 (data not shown). These results suggest that m36 is a cross-reactive CD4i antibody which binds to an epitope

3040 localized close to the CD4 binding site.

The m36 epitope is sterically restricted

In an attempt to further elucidate possible mechanisms of neutralization, access to the m36 epitope was examined to determine whether it was sterically restricted. To answer this question several m36 fusion proteins with MWs ranging from 18 to 115 kDa (Figure 28 and Figure 29) were designed and tested. First, the binding of the m36 fusion proteins to Env complexed with CD4 was carried out to assure that the additional protein does not interfere with binding. Next, the neutralizing activity of the fusion proteins was evaluated.

3045 kDa (Figure 28 and Figure 29) were designed and tested. First, the binding of the m36 fusion proteins to Env complexed with CD4 was carried out to assure that the additional protein does not interfere with binding. Next, the neutralizing activity of the fusion proteins was evaluated.

The m36 was fused to a serum albumin binding peptide (SAbp), human IgG1 CH3 domain and Fc without a peptide linker; these fusion proteins, m36SAbp (MW ~ 18 kDa), m36CH3 (MW ~ 60 kDa) and m36b0Fc (MW ~ 80 kDa), respectively (Fig. 4), were expressed in *E. coli* HB2151 and purified. In the other three fusion proteins m36 was joined with human IgG1 Fc by a human IgG1 hinge (m36h1Fc, MW ~ 110 kDa), a camel IgG2 hinge (m36c2Fc, MW ~ 115 kDa) or a human IgG3 hinge (m36h3Fc, MW ~ 115 kDa), respectively (Figure 28), and expressed in mammalian 293 suspension

3050 CH3 domain and Fc without a peptide linker; these fusion proteins, m36SAbp (MW ~ 18 kDa), m36CH3 (MW ~ 60 kDa) and m36b0Fc (MW ~ 80 kDa), respectively (Fig. 4), were expressed in *E. coli* HB2151 and purified. In the other three fusion proteins m36 was joined with human IgG1 Fc by a human IgG1 hinge (m36h1Fc, MW ~ 110 kDa), a camel IgG2 hinge (m36c2Fc, MW ~ 115 kDa) or a human IgG3 hinge (m36h3Fc, MW ~ 115 kDa), respectively (Figure 28), and expressed in mammalian 293 suspension

3055 ~ 115 kDa), respectively (Figure 28), and expressed in mammalian 293 suspension

cells. All fusion proteins, except m36SAbp, were dimeric in PBS, pH 7.4 as shown on non-reducing SDS-PAGE gels (Figure 30). They all exhibited comparable to or higher than m36 binding to gp120_{Bal}-CD4 as measured by ELISA. M36SAbp and m36CH3 showed binding comparable to that of m36 (Figure 31A); m36b0Fc bound slightly
3060 better. The three fusion proteins expressed in mammalian cells (m36h1Fc, m36c2Fc and m36h3Fc) exhibited the highest binding strengths with EC₅₀ ~ 0.5 nM (Figure 31B). All antibodies at concentrations up to 870 nM did not show significant binding to gp120_{Bal} in the absence of CD4.

In spite of the preserved or even higher affinity (avidity), all fusion proteins,
3065 except m36SAbp, exhibited significantly weaker neutralizing activity when compared to m36 side by side in the same experiment (Figure 25 and Figure 32). The increased size of m36CH3 resulted in loss of neutralization against 7 of 10 isolates compared to m36, and for those neutralized (IIIB, 89.6 and NL4-3) the IC₅₀s were significantly higher than the corresponding ones for m36. With an additional increase in molecular
3070 size, m36b0Fc further lost neutralization against the T cell line adapted (TCLA) isolate, IIIB, and had a decreased inhibitory activity against 89.6 and NL4-3. Notably, the three fusion proteins with long flexible linkers neutralized HIV-1 significantly better than the bacterially expressed m36b0Fc which does not have a linker and m36CH3 which has much smaller molecular size (Figure 25 and Figure 32). They neutralized five isolates,
3075 one of them (89.6) even with potency higher than that of m36 likely due to their bivalency leading to avidity and other effects. These isolates were neutralized equally well by m36h1Fc, m36c2Fc and m36h3Fc indicating that further increase in the length of the linker may not affect the neutralization activity. Fusion of m36 with the relatively much smaller SAbp resulted only in slight but not significant decrease of the
3080 neutralizing activity (Figure 25).

Next, it was investigated whether the access to the m36 epitope on intact virions before entry into cells could be enhanced by CD4. M36 and its fusion proteins were preincubated with pseudovirus and in the presence of a low concentration of sCD4. The m36h1Fc fusion protein alone at up to 870 nM and sCD4 alone at 8 nM or combined
3085 with a control domain antibody-Fc fusion protein exhibited low neutralizing activity of

about 25% and 10%, respectively (Figure 33). Preincubation of the Bal pseudovirus with both m36h1Fc and sCD4 resulted in a dramatic increase in neutralization – up to 100% (Figure 33); similar increase was also observed with JRFL pseudovirus. Taken together these results suggest that the m36 epitope is sterically obstructed and fully
3090 accessible during virus entry only by relatively small size molecules.

The results of Example 3 are further discussed as follows.

Discussion:

Example 3 demonstrates that dAbs could bind to conserved structures that are inaccessible or partially accessible during virus entry for molecules of larger size
3095 comparable to that of full-size antibodies generated by the human immune system. Such antibodies could not only be potentially useful as candidate therapeutics against viruses, including HIV-1, which can protect highly conserved structures that are vital for virus replication, but can also help identify those conserved structures with implications for the development of small molecule inhibitors, and elucidation of mechanisms of entry
3100 and evasion of immune responses.

The binding of the Env to receptor and coreceptor molecules may result in the exposure of conserved structures that could be used as antigens for selection of cross-reactive neutralizing antibodies. The identification and characterization of the potent broadly cross-reactive human Fab X5 (Moulard et al., 2002) provided supporting
3105 evidence. The crystal structure of its complex with gp120-CD4 enabled the localization of the highly conserved epitope overlapping the putative coreceptor binding site very close to the CD4 binding site (Huang et al, 2005). However, unexpectedly, IgG1 X5 on average exhibited lower potency than Fab and scFv likely due to its larger size. Because the crystal structure of Fab X5 complexed with gp120-CD4 suggested that only
3110 its heavy chain contacts gp120, decreasing the size to a single VH could further increase the potency of X5. However, efforts to isolate stable VH X5 or VH X5-like dAbs by rational design, mutagenesis and screening have failed. Similarly, efforts to develop a stable highly soluble VH dAb based on an HIV-1 gp120-specific heavy chain only
3115 antibody have also failed likely due to certain extent of hydrophobicity that is important for its structural stability.

As disclosed in the present application, including in the above Examples, the present inventors identified another heavy chain only antibody which as an isolated VH, m0, exhibited high stability and solubility. As outlined in the present application, including in Example 2, a large highly diversified library using as a scaffold m0 was
3120 constructed. The high diversification of the library was achieved by two strategies – grafting highly diverse CDR2s and CDR3s from five separate libraries including one from HIV-1-infected individuals, and randomly mutating four residues in the CDR1 to residues frequently found in antibody CDRs. This library was used for selection of m36 and could also be useful for isolation of dAbs against other antigens.

3125 Access of full-size antibodies to CD4i epitopes can be restricted during virus entry into cells. The crystal structures of two CD4i antibodies, X5 and 17b, in complex with gp120 and sCD4, indicate that access to their epitopes requires long protruding heavy chain CDR3s. Most of the known CD4i antibodies have long CDR3s that could play an important role in accessing sterically restricted areas. Their CDR3s are highly
3130 acidic (Figure 24) and the closest germline VH gene for this group of antibodies is VH1-69. A smaller group of CD4i antibodies have relatively short CDR3s, acidic CDR2s and VH1-24 gene usage. M36 appears to be the only representative of a third group characterized with short CDR3, acidic CDR1 and VH3-23 gene usage (Figure 24). Because of its small size it may not need a long CDR3 for access to sterically
3135 restricted structures.

M36 exhibited on average higher neutralizing activity than scFv m9, which has been recently shown to be superior to the best characterized cross-reactive HIV-1 neutralizing antibodies b12, 2G12, 2F5 and 4E10, and than C34, a peptide similar to the fusion inhibitor T20 (FUZEON™) which is in clinical use. It was hypothesized that a
3140 dimer of m36 could have even higher potency due to avidity effects and used CH3 as a dimerization domain. However, m36CH3 was significantly weaker than m36 indicating that the m36 epitope is fully accessible during virus entry only by antibody domains or smaller molecules (Figure 25). A larger fusion protein, M36b0Fc, was poorer inhibitor most likely due to the increased molecular size. However, a fusion protein with a
3145 human IgG1 hinge region as a linker between m36 and Fc, m36h1Fc, neutralized better

several isolates than m36CH3 and m36b0Fc despite an increase in size (Figure 25). For some isolates (89.6, NL4-3 and GXC-44) its potency was as high as that of m36. It was further hypothesized that the long linker may provide a flexibility needed for the m36 to reach its epitope combined with an increased binding due to avidity effects resulting from the m36h1Fc bivalency. However, fusion proteins with even longer hinge regions (from camel antibodies, m36cFc, or from human IgG3, m36h3Fc) did not exhibit higher potency than m36h1Fc (Figure 25) possibly due to compensation of the flexibility effect by an increase in the effective hydrodynamic size of the molecules leading to a decrease in the accessibility of the m36 epitope.

The neutralizing activity of the m36 fusion proteins was dramatically increased for viruses sensitized (pre-triggered) by sCD4 (soluble CD4) to expose the m36 epitope (Figure 33). These data not only provide evidence for the restricted nature of the m36 epitope during entry but also suggest the possibility to develop novel m36-based potential therapeutics, e.g. fusion proteins of m36 with sCD4 or small molecule mimics of CD4. These molecules could neutralize the virus before it binds to cell surface-associated CD4 while m36 is likely to exert its major neutralizing activity after the virus binds to the cell surface-associated CD4 which triggers the conformational changes in gp120 leading to exposure of its epitope.

To our knowledge m36 is the first identified and characterized HIV-1 neutralizing human dAb. It could have potential as a therapeutic adding a new target to the growing family of entry inhibitors. Although its half-life in vivo is likely to be very short our findings that a fusion protein with an SAbp (serum albumin-binding peptide) retains about the same neutralizing activity as m36 indicates the possibility to improve its pharmacokinetics. We found that the m36SAbp binds to serum albumins from human (HSA), bovine (BSA) and mouse (MSA) (data not shown) indicating that such possibility is realistic. The epitope of m36 is sterically restricted and may not be directly used to develop potential vaccine immunogens. However, it is highly conserved and therefore could be also useful as a tool to explore mechanisms of entry and to understand how HIV-1 guards its conserved structures and evade neutralizing immune responses.

Having thus described in detail preferred embodiments of the present invention, it is to be understood that the invention defined by the above paragraphs is not to be limited to particular details set forth in the above description as many apparent
3180 variations thereof are possible without departing from the spirit or scope of the present invention.

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3335

WHAT IS CLAIMED IS:

1. An isolated domain antibody or fragment thereof according to the amino acid sequence of m36, or an amino acid molecule having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with the amino acid sequence of m36.

3340 2. The domain antibody of claim 1, wherein the domain antibody or fragment is immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive isotopes, or antiviral agents.

3. The domain antibody of claim 2, wherein the antiviral agent is zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

3345 4. An isolated domain antibody or fragment thereof comprising (a) the m0 framework amino acid sequence or an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto and (b) the CDR3 amino sequence of m36 or an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto.

3350 5. The domain antibody of claim 4, wherein the domain antibody or fragment thereof is immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive isotopes, or antiviral agents.

3355 6. The domain antibody of claim 5, wherein the antiviral agent is zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

3360 7. An isolated domain antibody or fragment thereof comprising (a) the m0 framework amino acid sequence or an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto and (b) at least one of CDR1, CDR2 or CDR3 of m36 or an amino acid sequence having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto.

3365 8. The domain antibody of claim 7, wherein the domain antibody or

fragment thereof is immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive isotopes, or antiviral agents.

3370 9. The domain antibody of claim 8, wherein the antiviral agent is zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

3375 10. A domain antibody framework for the construction of a domain antibody library, said domain antibody framework comprising the framework sequence of m0 or an amino acid sequence having at least 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto.

3380 11. A fusion protein comprising a domain antibody or fragment thereof and a fusion partner, wherein the domain antibody or fragment thereof is the amino acid sequence of m36, or an amino acid molecule having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with the amino acid sequence of m36.

12. The fusion protein of claim 11, wherein the fusion protein is immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or synthetic toxins, radioactive isotopes, or antiviral agents.

3385 13. The fusion protein of claim 12, wherein the antiviral agent is zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir, ritonavir, adefovir, clevadine, entecavir, or pleconaril.

14. The fusion protein of claim 12, wherein the fusion partner is fused to the m36 or fragment thereof via a linker.

3390 15. The fusion protein of claim 11, wherein the fusion partner increases the stability of m36 or fragment thereof as compared to the m36 or fragment thereof alone.

16. The fusion protein of claim 11, wherein the fusion partner induces a CD4i epitope on Env.

3395 17. The fusion protein of claim 15, wherein the fusion partner is serum albumin-binding protein or peptide..The fusion protein of claim 16, wherein the fusion

partner is CD4 or a fragment or mimic thereof.

18. The fusion protein of claim 14, wherein the linker is an IgG hinge region.

19. A fusion protein comprising a domain antibody or fragment thereof and
3400 a fusion partner, wherein the domain antibody or fragment thereof comprises (a) the
m0 framework amino acid sequence or an amino acid sequence having at least 60%,
80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto and (b) at
least one of CDR1, CDR2 or CDR3 of m36 or an amino acid sequence having at least
60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto.

20. The fusion protein of claim 20, wherein the fusion protein is
3405 immunoconjugated to one or more cytotoxic agents, chemotherapeutic agents, natural or
synthetic toxins, radioactive isotopes, or antiviral agents.

21. The fusion protein of claim 21, wherein the antiviral agent is zidovudine,
acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin, as well as
3410 foscarnet, amantadine, rimantadine, saquinavir, indinavir, amprenavir, lopinavir,
ritonavir, adefovir, clevadine, entecavir, or pleconaril.

22. The fusion protein of claim 21, wherein the fusion partner is fused to the
domain antibody or fragment thereof via a linker.

23. The fusion protein of claim 21, wherein the fusion partner increases the
3415 stability of the fusion protein.

24. The fusion protein of claim 21, wherein the fusion partner induces a
CD4i epitope on Env of HIV.

25. The fusion protein of claim 24, wherein the fusion partner is serum
albumin-binding protein.

26. The fusion protein of claim 25, wherein the fusion partner is CD4 or a
3420 fragment or mimic thereof.

27. The fusion protein of claim 21, wherein the linker is an IgG hinge
region.

28. A pharmaceutical composition comprising a therapeutically effective
3425 amount of a domain antibody or a fusion protein in accordance with any of the

preceding claims and a pharmaceutically acceptable salt.

29. A method of treating and/or preventing an HIV infection in a patient in need thereof comprising, administering a therapeutically effective amount of a (a) domain antibody of any of claims 1-10, (b) a fusion protein of any of claims 11-28, or
3430 (c) a pharmaceutical composition of claim 29..

30. The method of claim 30, further comprising co-administering a cytokine, anti-angiogenic agent, immunotherapeutic agent, anti-cancer agent, anti-bacterial agent, or anti-viral agent.

31. The method of claim 30, further comprising co-administering a
3435 therapeutically effective amount of soluble CD4 (sCD4) or functional fragment or mimic thereof.

32. A domain antibody library comprising a plurality of unique clones wherein each unique clone comprises the framework sequence of m0 or an amino acid sequence having at least 90%, 95%, 96%, 97%, 98% or 99% sequence identity thereto.

33. The domain antibody library of claim 33, wherein library comprises at
3440 least 10^8 unique clones.

34. The domain antibody library of claim 33, wherein library comprises at least 10^9 unique clones.

35. The domain antibody library of claim 33, wherein library comprises at
3445 least 10^{10} unique clones.

36. A method of constructing a dAb library comprising the steps of (a) obtaining an isolated VH framework nucleotide sequence and (b) introducing a CDR1, CDR2 and CDR3 repertoire into the isolated VH framework nucleotide sequence, and (b) optionally mutagenizing one or more CDR sequences by PCR, thereby forming a
3450 dAb library.

37. The method of claim 37, wherein the step of introducing the CDR1, CDR2 and CDR3 repertoire is achieved by graft replacement of the corresponding CDR sequences of the VH framework with the CDR1, CDR2 and CDR3 repertoire.

38. The method of claim 38, wherein the CDR1, CDR2 and CDR3 repertoire
3455 sequences are obtained by PCR amplification using the primers of Table 1 and a non-

immunized or immunized human antibody library templates.

39. The method of claim 38, wherein the graft replacement is achieved by extension reactions between the PCR CDR products and the VH framework.

40. The method of claim 37, wherein the dAb library is a phage-display
3460 library.

41. A method of identifying a dAb that binds to an antigen of interest, comprising panning the dAb phage-display library of claim 41 with the antigen of interest.

42. The method of claim 42, wherein the antigen of interest is HIV-1
3465 antigen.

43. The method of claim 42, wherein the panning step is a sequential panning step using at least two antigens.

44. The method of claim 44, wherein the at least two antigens are HIV-1 antigens from different HIV-1 isolates.

3470 45. A method for treating or preventing an HIV infection comprising administering in a therapeutically effective amount a composition comprising a dAb that binds to HIV-1 epitopes with a dissociation constant (K_d) of about 100 nM to about 500 nM.

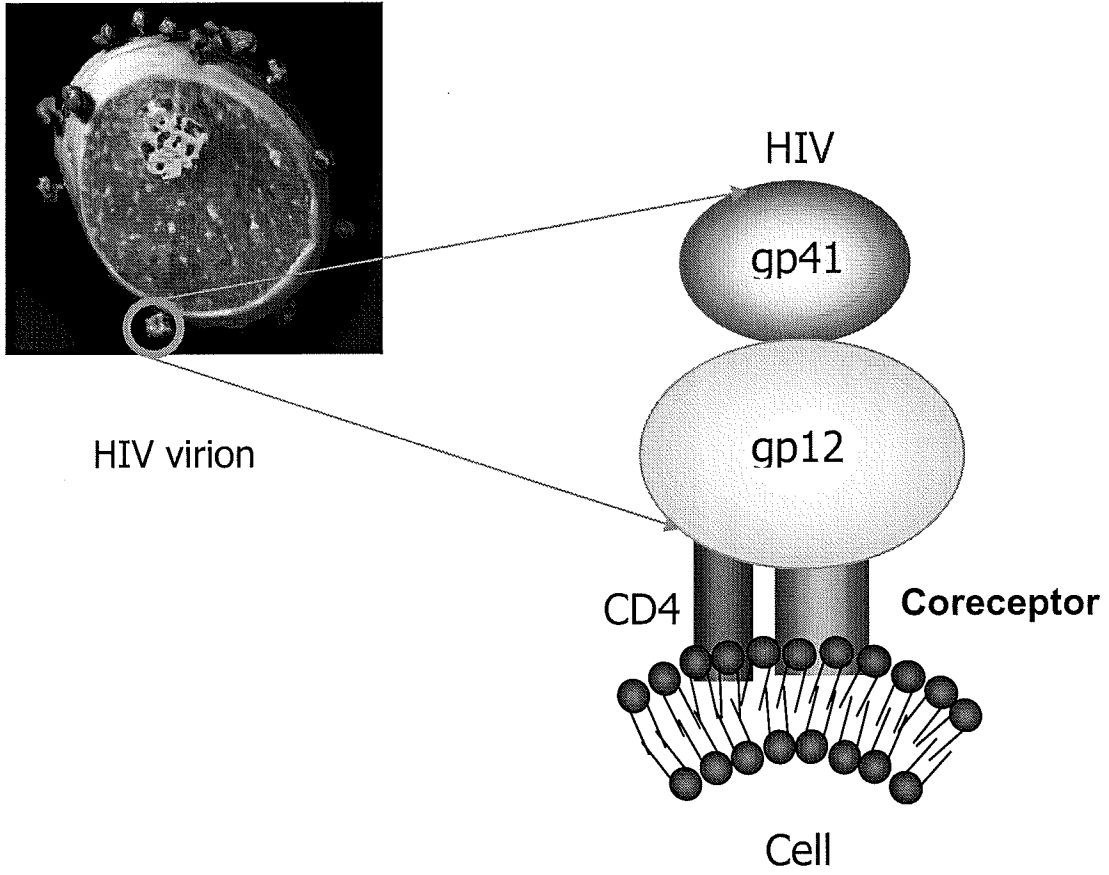


Figure 1

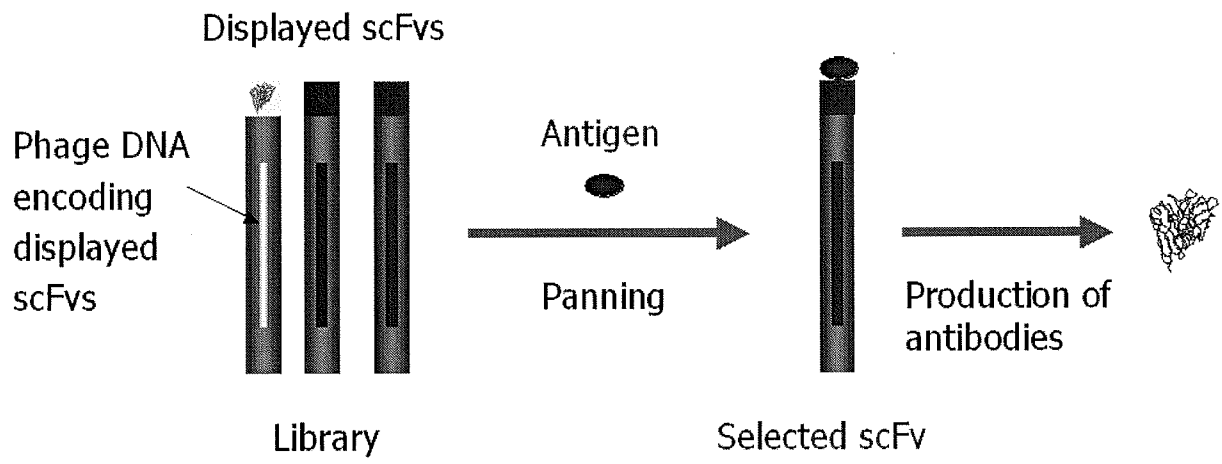


Figure 2

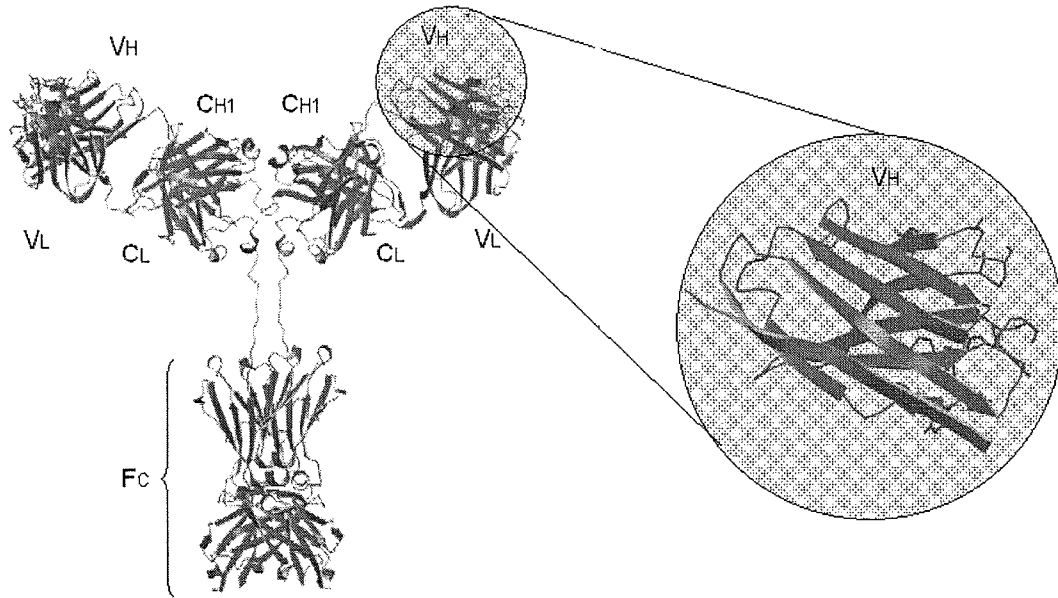


Figure 3

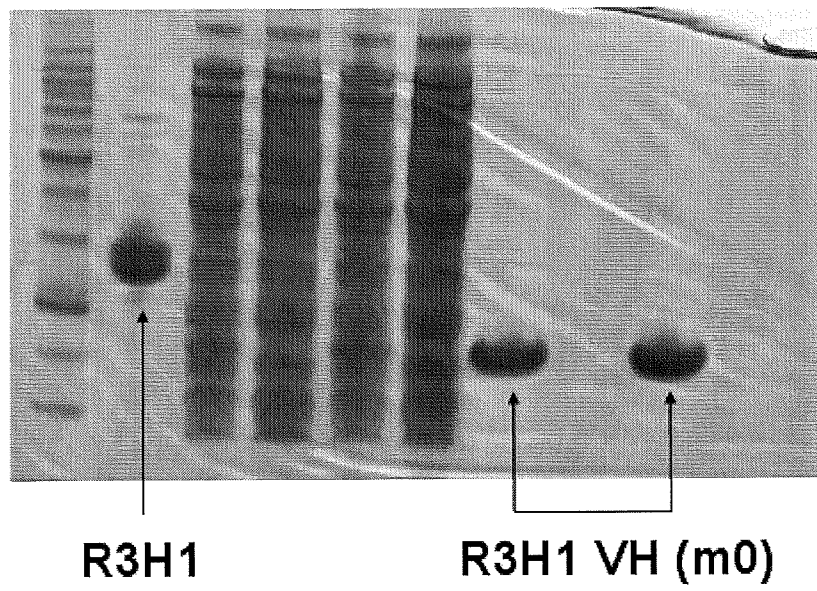


Figure 4

```

m0      1           5           10           15           20
VH3-23*04 Q V Q L V Q S G G ... G L V Q P G G S L R
caggtg cagctg gtg cagtct ggggga... ggcttg gta cagcct ggagggtcc ctg aga
E           E
g-- ... .. g-- ... .. --g ... ..

                                CDR1 - IMGT
m0      25           30           35           40
VH3-23*04 L S C A A S G F S F S T Y E ... .. M S
ctc tcc tgt gcagcctct ggattc agcttc agt act tat gaa... .. atg agc
T           S           A
... .. -c- -t -c -gc ... -cc... ..

                                CDR2 - IMGT
m0      45           50           55           60
VH3-23*04 W V R Q A P G K G L E W V S P I S G S G
tgg gtc cgccaggct cca gggaaggggctg gagtgg gtc tca cct att agt ggt agt ggt
A
... .. g-- ... ..

m0      65           70           75           80
VH3-23*04 G N S ... .. Y Y A D S V K ... G R F T I S R
ggt aac tca ... .. tac tac gcagactcc gtg aag... ggccgggtc acc atc tcc aga
S T
... -g- a-- ... ..

m0      85           90           95           100
VH3-23*04 D N S K N T L Y L Q M N T L R A E D T A
gacaat tcc aagaac acgctg tat ctg caa atg aac acc ctg agagccgaggacacggcc
S
... .. -g- ... ..

                                CDR3 - IMGT
m0      104
VH3-23*04 V Y Y C A K G P P V W S G Y Y F A D G F
gta tat tac tgt gcgaaa ggcccc ccggtt tgg agt ggt tat tat ttc gct gat ggt ttt
... .. -a

m0
-----
D I W G Q G T M V T V S S (SEQ ID NO: 94)
gat atc tgg ggccaa gggacaatg gtc acc gtc tct tca (SEQ ID NO: 95)

```

Figure 5A

Amino acid sequence of m0 framework region

QVQLVQSGGGLVQPGGSLRLS CAAS<CDR1>MSWVRQAPGKG
LEWVSP<CDR2>YYADSVKGRFTISRDN SKNTLYLQMNTLRA
EDTAVYYC<CDR3>WGQGTMTVSS (SEQ ID NO: 94)

Nucleotide sequence of m0 framework region

CAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTTGGTACAGCCTGGAGGGTCCCTGAGA
CTCTCCTGTGCAGCCTCT<CDR1>ATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCT
GGAGTGGGTCTCACCT<CDR2>TACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCT
CCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACACCCTGAGAGCCGAGGAC
ACGGCCGTATATTACTGT<CDR3>TGGGGCCAAGGGACAATGGTCACCGTCTCTTCA
(SEQ ID NO: 95)

Figure 5B

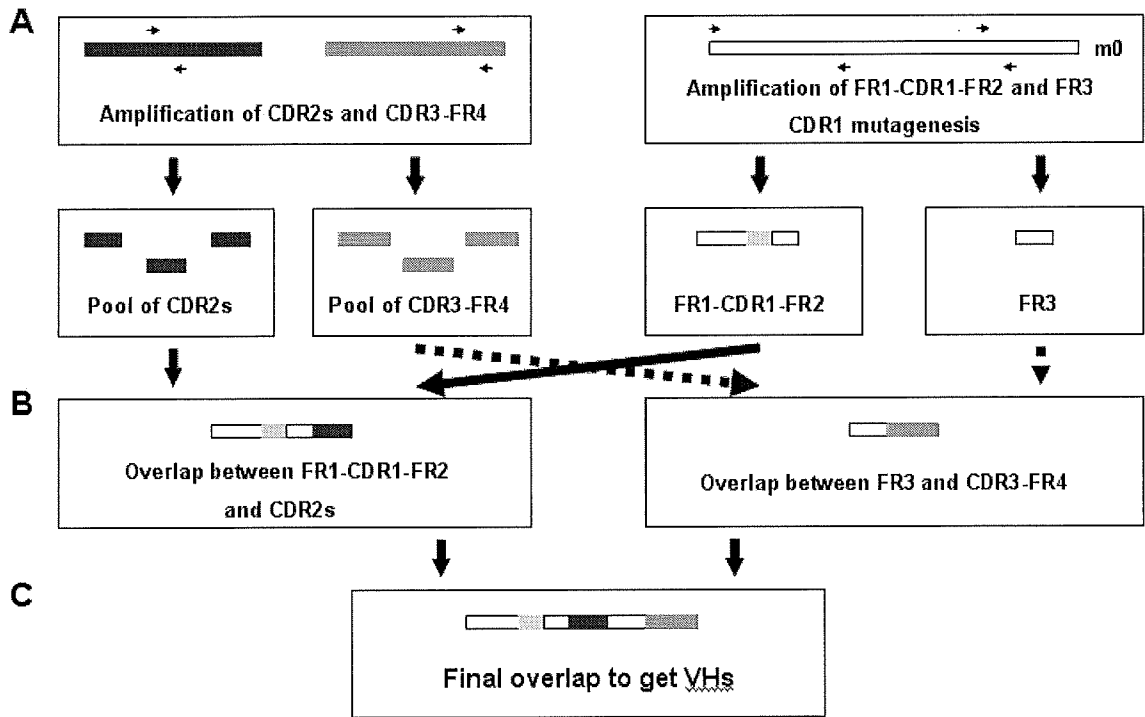


Figure 6

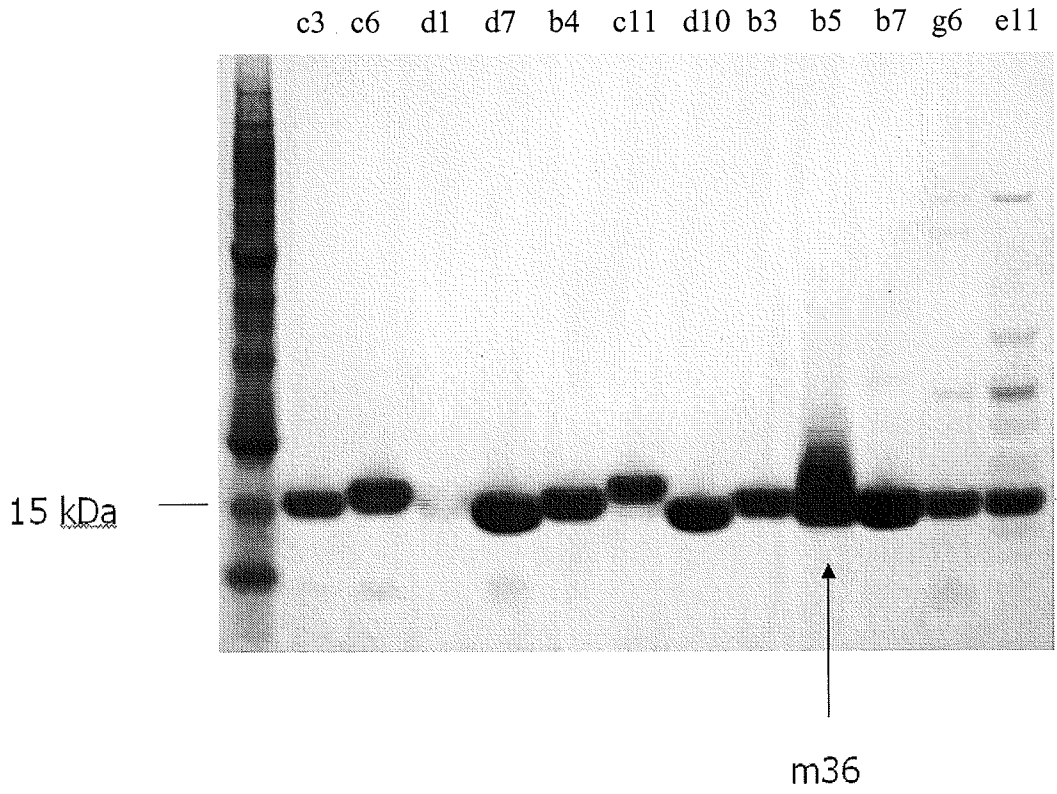


Figure 7

A

aa	Frequency (%)					
	27	28	29	30	31	32
	-	F	-	F	-	-
A	16.1		14.7		21.7	19.6
D	35.7		25.9		26.6	23.1
S	15.4		11.2		9.8	16.8
Y	32.9		48.3		42.0	40.6

B

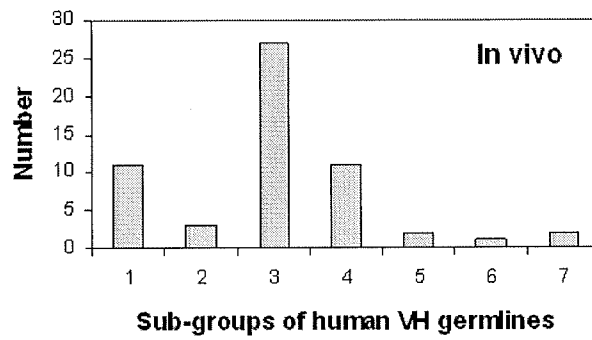
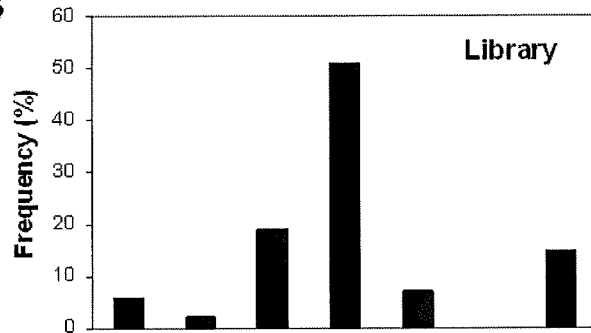


Figure 8

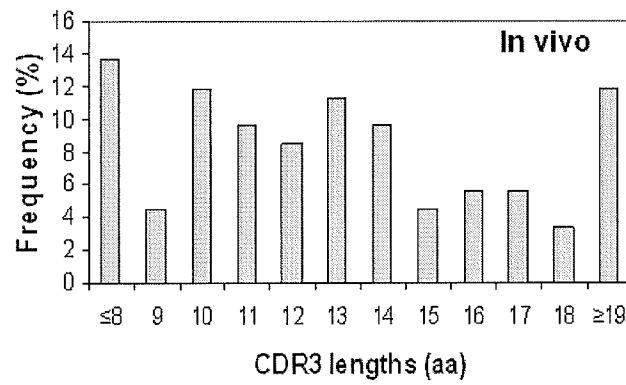
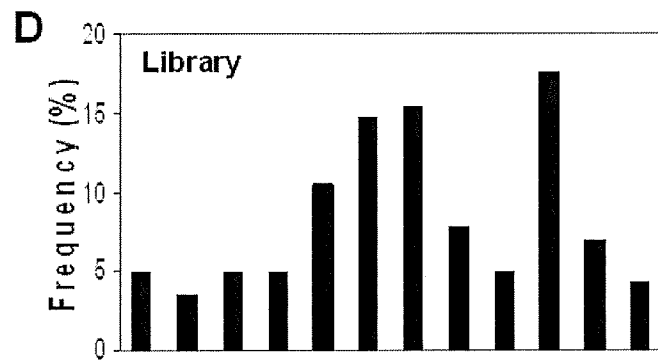
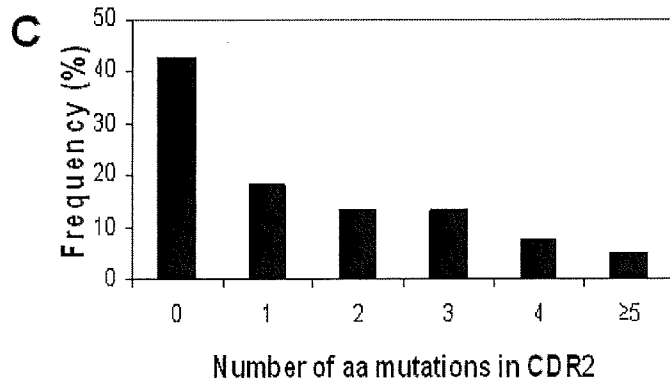


Figure 8 contd.

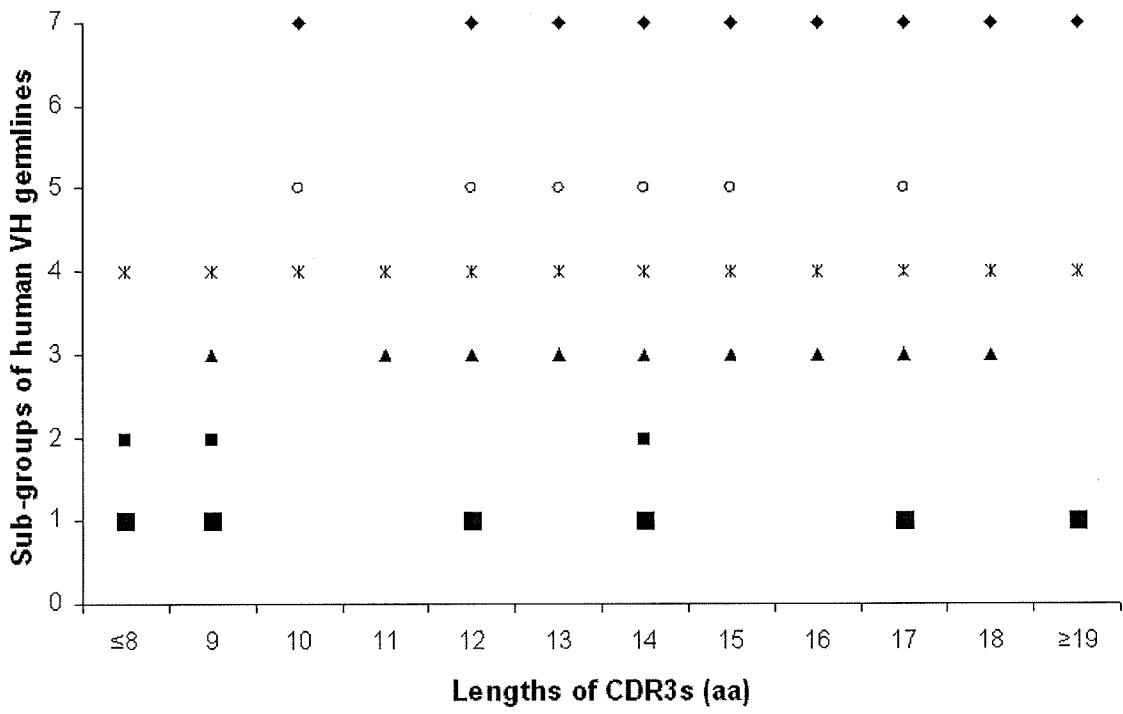


Figure 9

aa	Frequency (%)					
	27	28	29	30	31	32
	-	F	-	F	-	-
A	16.5		11.4		31.6	11.4
D	62.0		53.2		20.3	65.8
S	10.1		13.9		31.6	11.4
Y	11.4		21.5		16.5	11.4

After selection

aa	Frequency (%)					
	27	28	29	30	31	32
	-	F	-	F	-	-
A	11.1		22.2		29.6	22.2
D	33.3		18.5		18.5	40.0
S	18.5		11.1		7.4	11.1
Y	37.0		48.1		44.4	25.9

Before selection

Figure 10

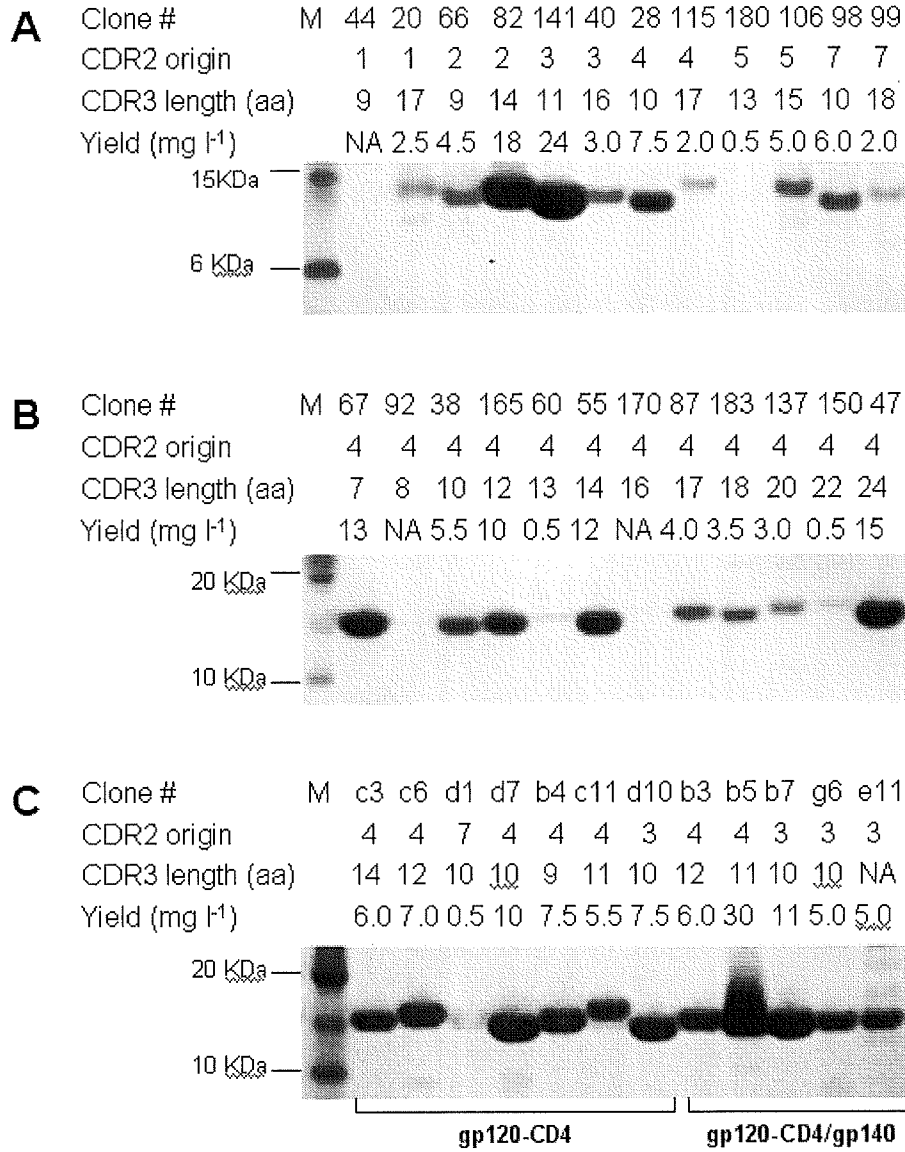


Figure 11

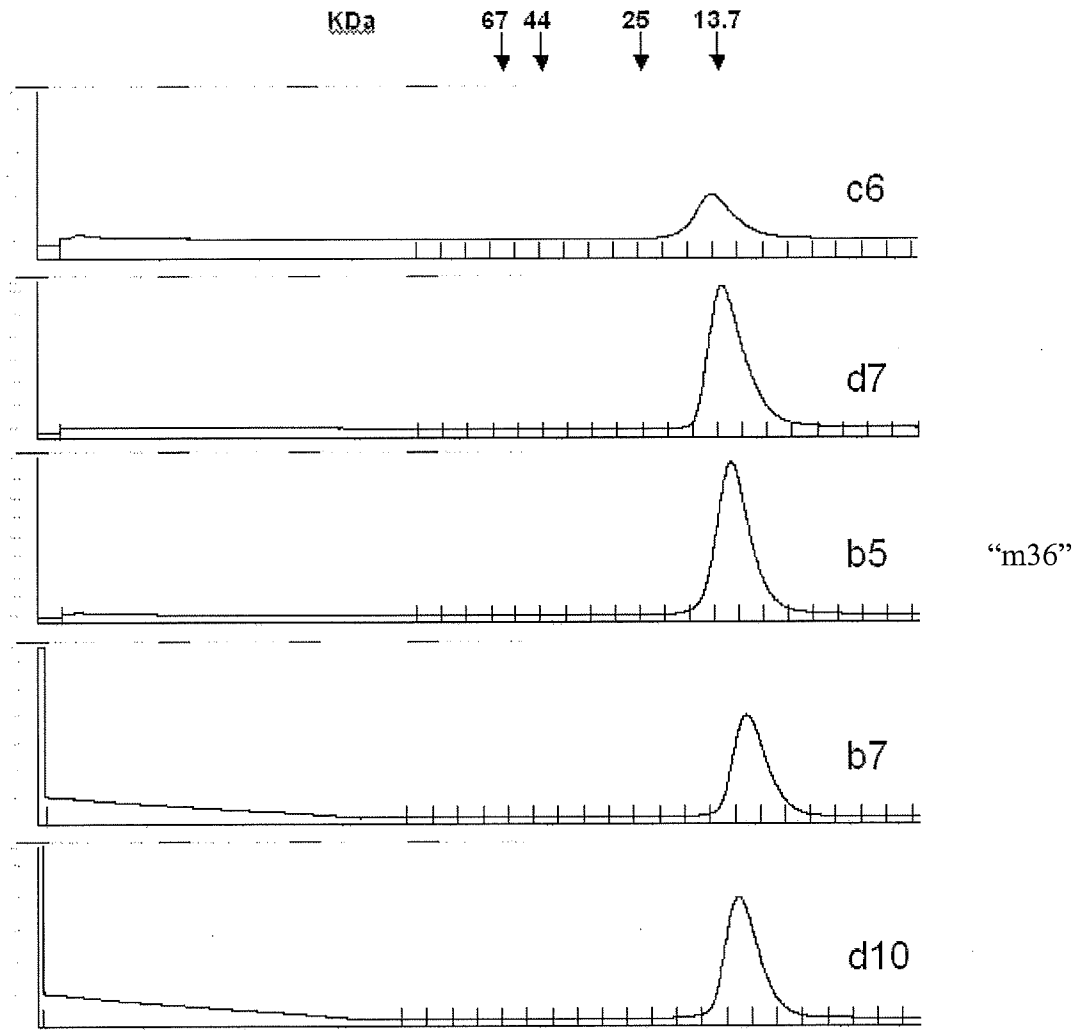


Figure 12

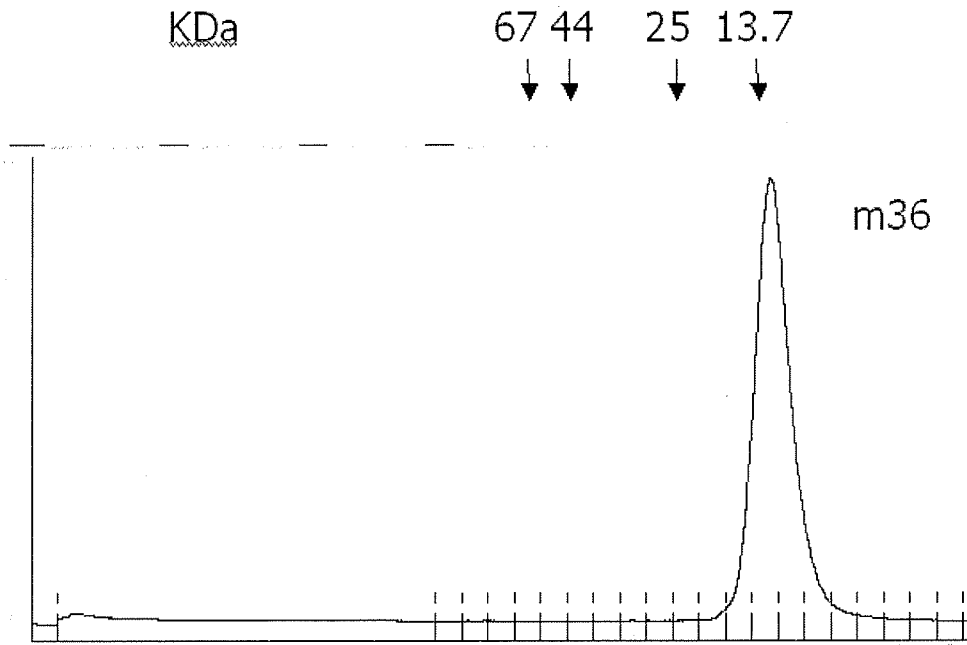


Figure 13

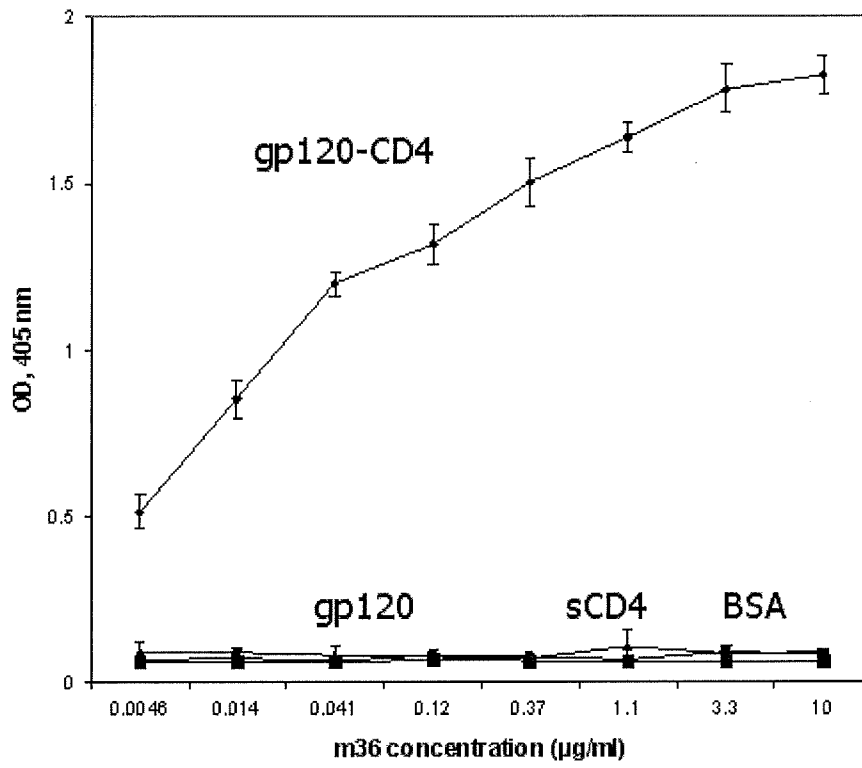


Figure 14

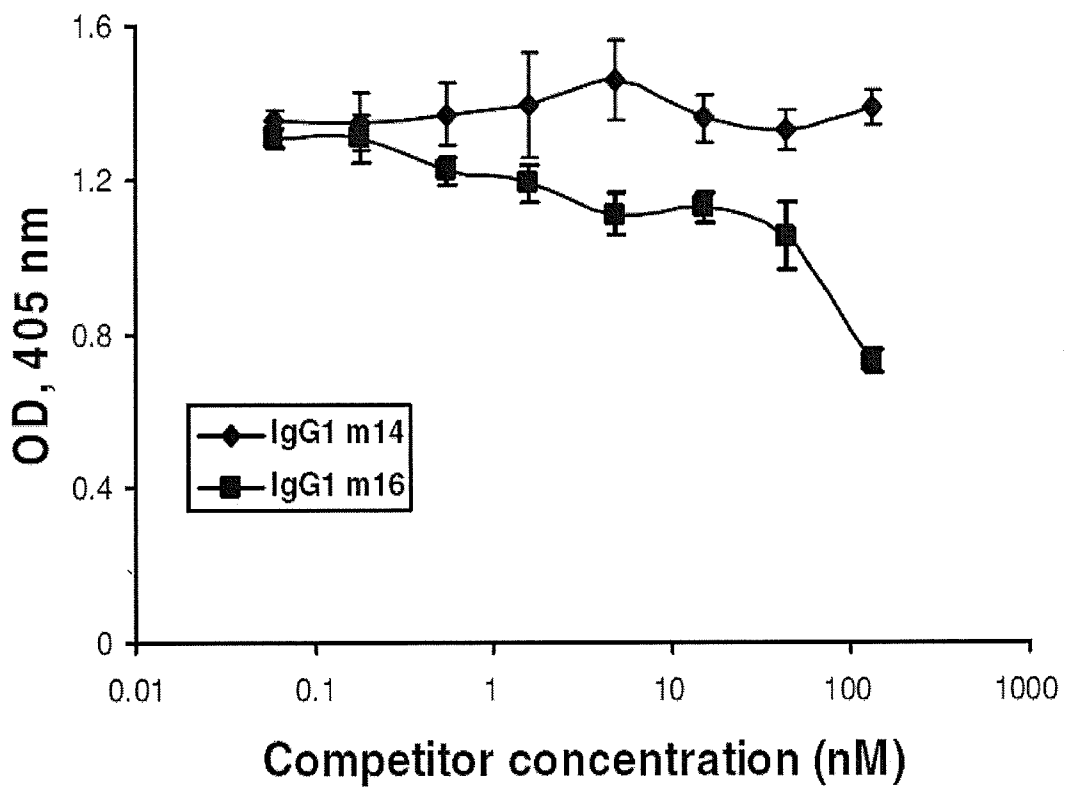


Figure 15

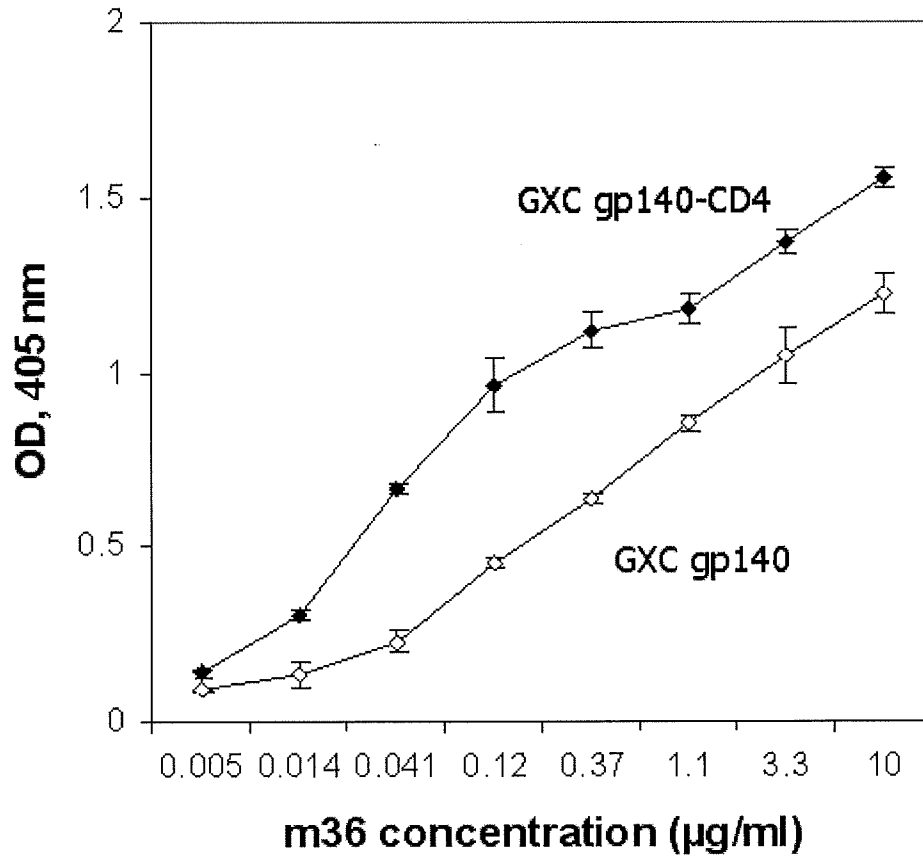


Figure 16

Sheet 19/35

Virus	clade	m36			scFv m9			c34		
		IC ₅₀	IC ₉₀	%In. ^a	IC ₅₀	IC ₉₀	%In.	IC ₅₀	IC ₉₀	%In.
92UG	A	2.2	>10	74	6.7	>20	81	1.1	>4.0	75
Bal	B	0.12	1.1	100	2.2	8.9	98	1.0	>4.0	85
JR-FL	B	0.37	3.3	96	2.3	15	95	1.0	>4.0	62
JRCSF	B	0.37	1.1	100	2.2	6.6	100	1.3	>4.0	68
IIIB	B	0.12	0.85	100	0.61	2.0	100	0.44	3.5	94
89.6	B	2.6	>10	77	0.25	>20	86	0.044	>4.0	79
R2	B	0.31	10	91	1.1	20	90	0.044	>4.0	84
NL4-3	B	<0.12	<0.12	100	0.57	2.1	100	3.4	>4.0	57
GXC	C	0.24	1.1	100	1.9	20	90	1.3	>4.0	63
Z2Z6	D	10	>10	50	0.66	5.2	97	0.72	>4.0	67
GXE	E	>10	>10	0	>20	>20	0	3.1	>4.0	64

^aPercentage inhibition for the highest concentration (m36: 10 µg/ml; scFv m9: 20 µg/ml; c34: 4 µg/ml)

Figure 17

m36 1 5 10 15 20
 Q V Q L V Q S G G ... G L V Q P G G S L R
 cagggtg cagctg gtg cagctct gggggga... ggcttg gta cagcct ggaggggtcc ctg aga
 VH3-48*03 E E
 g-- --- --- --- --- g-- --- --- --- --- --- --- --- --- --- --- ---

CDR1 - IMGT
 m36 25 30 35 40
 L S C A A S A F D F S D Y E M S
 ctc tcc tgt gcagcctct gct ttc gat ttc tct gat tat gaa... .. atg agc
 VH3-48*03 G T S N
 --- --- --- --- --- -ga --- acc--- ag- ag- --- --- --- --- --- -a-

CDR2 - IMGT
 m36 45 50 55 60
 W V R Q A P G K G L E W I G E I N D S G
 tgg gtc cgccaggtc cca gggaaggggctg gagtgg att ggggaaatc aat gat agt gga
 VH3-48*03 V S Y S S
 --- --- --- --- --- --- --- --- --- --- --- g-- tca t-c --t -g- ag- --- --t

m36 65 70 75 80
 N T I Y N P S L K ... S R V T I S R
 aacacc... .. att tac aat ccgtcc ctc aag... agt cgagtc acc atc tcc aga
 VH3-48*03 S I Y A D V G F
 -gt --- ata --- --- tac --- gcagac--t g-g --- --- g-c --- t-- --- --- ---

m36 85 90 95 100
 D N S K N T L Y L Q M N T L R A E D T A
 gacaat tcc aagaac acgctg tat ctg caa atg aac acc ctg agagccgaggacacagcc
 VH3-48*03 A S S
 --- --c g-- --- --- t-a --- --- --- --- --- -g- --- --- --- --- --g --t

CDR3 - IMGT
 m36 104
 I Y Y C A I Y G G N S G G E Y W G Q G T
 ata tat tac tgt gcgata tat ggt ggt aac tcc gggggagagtfac tgg ggccagggcacc
 VH3-48*03 V R
 g-t --- --- --- --- -g-

m36 L V T V S S (SEQ ID NO: 96)
 ctg gtc acc gtc tcc tca (SEQ ID NO: 97)

Figure 18

Nucleotide sequence of m36

caggtgcagctggtgcagctctgggggaggcttgggtacagcctggaggggtccctgagact
 ctctgtgcagcctctgctttcgatttctctgattatgaaatgagctgggtccgccagg
 ctccaggaaggggctggagtgattggggaaatcaatgatagtggaacaccatttac
 aatccgtccctcaagagtcgagtcaccatctccagagacaattccaagaacacgctgta
 tctgcaaatgaacaccctgagagccgaggacacagccatatattactgtgcgatatatg
gtggtaactccgggggagagtactggggccagggcacccctggtcaccgtctcctca

(SEQ ID NO: 97)

Amino acid sequence of m36

FR1-----|CDR1----|FR2-----|CDR2--|FR3-
 QVQLVQSGGGLVQPGSLRLSCAASAFDFSDYEMSWVRQAPGKGLEWIGEINDSGNTIY
 -----|CDR3-----|FR4-----|
 NPSLKSRVTISRDN SKNTLYLQMNTLRAEDTAIYYCAIYGGNSGGEYWGQGLVTVSS

(SEQ ID NO: 96)

Figure 19

A

```

<----- FR1 - IMGT ----->
1       5       10      15       20
Q V Q L V Q S G G       G L V Q P G G S L R L S C
cag gtg cag ctg gtg cag tct ggg gga ... ggc ttg gta cag cct gga ggg tcc ctg aga ctc tcc tg

-----> CDR1 - IMGT <----->
25       30       35       40       45
A A S G F S F S T Y E       M S W V R Q A
t gca gcc tct gga ttc agc ttc agt act tat gaa ... . . . . . atg agc tgg gtc cgc cag gct

FR2 - IMGT -----> CDR2 - IMGT -----<----->
50       55       60       65
P G K G L E W V S P I S G S G G N S       Y Y A
cca ggg aag ggg ctg gag tgg gtc tca cct att agt ggt agt ggt ggt aac tca ... . . . . . tac tac gc

----- FR3 - IMGT ----->
70       75       80       85       90
D S V K       G R F T I S R D N S K N T L Y L Q
a gac tcc gtg aag ... ggc cgg ttc acc atc tcc aga gac aat tcc aag aac acg ctg tat ctg caa

-----> CD
95       100      104
M N T L R A E D T A V Y Y C A K G P P V W S G
atg aac acc ctg aga gcc gag gac acg gcc gta tat tac tgt gcg aaa ggc ccc ccg gtt tgg agt gg

R3 - IMGT ----->
Y Y F A D G F D I W G Q G T M V T V S S (SEQ ID NO: 96)
t tat tat ttc gct gat ggt ttt gat atc tgg ggc caa ggg aca atg gtc acc gtc tct tca (SEQ ID NO: 97)

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B

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SD
caggagggaattttaaagtgaaaaagacagctatcgcgattgcagtgccactggctggtttc
M K K T A I A I A V A L A G F
OmpA leader peptide

SBI
gctaccgtggcccaggcggcc-----
A T V A Q A A

SBI
-----ggccaggccggccagcaccatcaccatcaccatggcgca
G Q A G Q [H H H H H H] G A
Hexahistidine tag

taccogtaacgacgttccggactacgcttcttaggaggggtggctctgaggg (SEQ ID NO: 98)
[Y P Y D V P D Y A S] - E G G G S E (SEQ ID NO: 99)
HA tag Amber Gene III (aa 230-406)
stop
codon

```

Figure 20

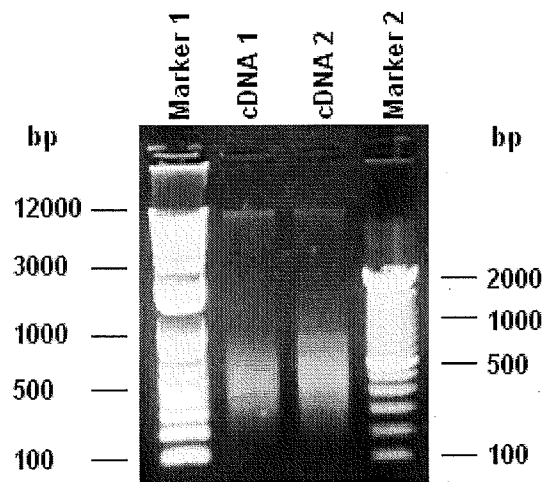


Figure 21

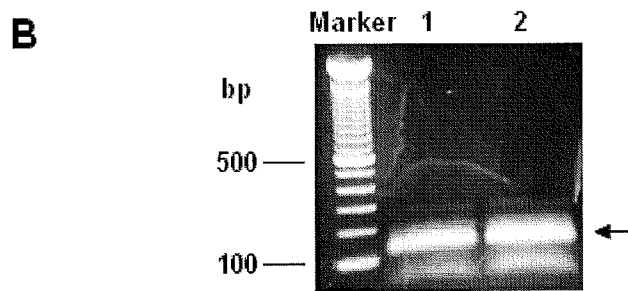
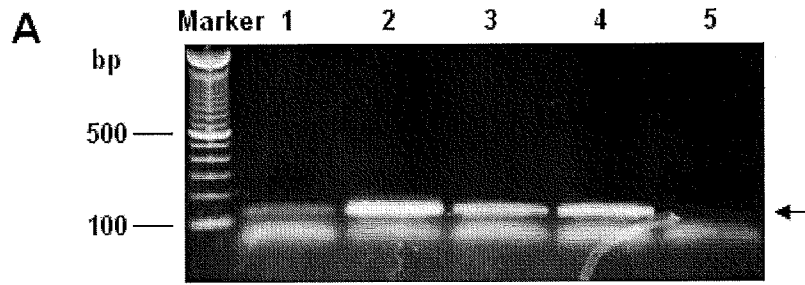


Figure 22

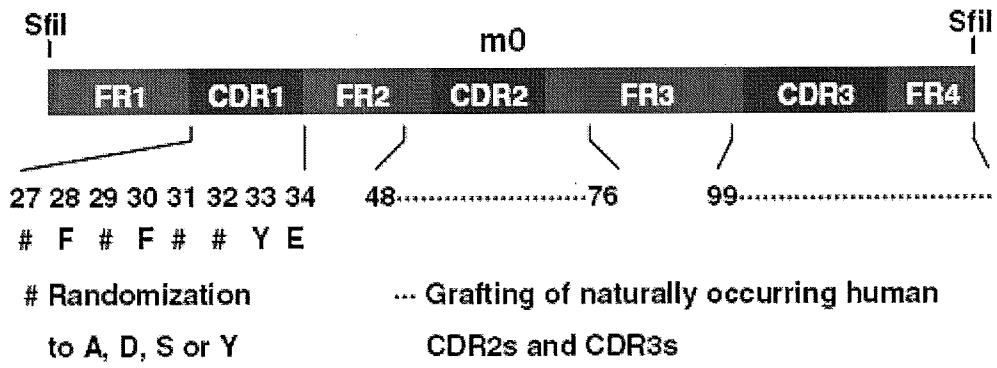


Figure 23

REPLACEMENT SHEET 26/35

m36 48d 411g 16c 17b 23e 47e 412d m16 Sb1 C12 X5 E51	CDR1										CDR2										Origin	
	1	10	20	30	40	50	60	70	1	10	20	30	40	50	60	70	FR	CDR1	CDR2			
m36	QVQLVQSGG	GLVQPGGSLR	LSCAAS	AFDFEDYE	NSWVRQAPGKGL	EWIGE	INDSGNY	LYHP	SLK	SRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH3-23*	VH4-34
48d	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-24	VH1-24
411g	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-24	VH1-24
16c	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-24	VH1-24
17b	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
23e	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
47e	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
412d	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
m16	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
Sb1	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
C12	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
X5	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69
E51	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	EVQLVQSGA	EVKRP	CAVKLS	CKAS	GYTFEDFY	NSWVRQAPGKGL	EWIMGL	DPEDADT	NYAEKRP	GRVTI	VH1-69	VH1-69

m36 48d 411g 16c 17b 23e 47e 412d m16 Sb1 C12 X5 E51	CDR3										length (aa)	m36 (SEQ ID NO: 100) 48d (SEQ ID NO: 101) 411g (SEQ ID NO: 102) 16c (SEQ ID NO: 103) 17b (SEQ ID NO: 104) 23e (SEQ ID NO: 105) 47e (SEQ ID NO: 106) 412d (SEQ ID NO: 107) m16 (SEQ ID NO: 108) Sb1 (SEQ ID NO: 109) C12 (SEQ ID NO: 110) X5 (SEQ ID NO: 111) E51 (SEQ ID NO: 112)										
	1	10	20	30	40	50	60	70	80	90			11	12	13	16	21	21	22	23	23	24
m36	SRPNSKQITL	QNTLR	RAEDT	AIYYC	AIYGGHS	GGEX	WGQG	TLVTVSS	SRPNSKQITL	QNTLR	RAEDT	AIYYC	AIYGGHS	GGEX	WGQG	TLVTVSS	11	m36 (SEQ ID NO: 100)				
48d	TADTSDT	TXLHLS	SLRSED	IAVYC	AADEH	ELNAPFV	WGQG	TLVTVSS	TADTSDT	TXLHLS	SLRSED	IAVYC	AADEH	ELNAPFV	WGQG	TLVTVSS	12	48d (SEQ ID NO: 101)				
411g	TEDTSSD	TAXHLS	SLRSD	IAVYC	ASGEQ	VAAPFDY	WGQG	TLVTVSS	TEDTSSD	TAXHLS	SLRSD	IAVYC	ASGEQ	VAAPFDY	WGQG	TLVTVSS	13	411g (SEQ ID NO: 102)				
16c	TADSTDT	TAXHLS	SLRSD	IAVYC	ATEP	PLSGR	GGEDY	WGQG	TLVTVSS	TADSTDT	TAXHLS	SLRSD	IAVYC	ATEP	PLSGR	GGEDY	WGQG	TLVTVSS	16	16c (SEQ ID NO: 103)		
17b	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	17b (SEQ ID NO: 104)
23e	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	23e (SEQ ID NO: 105)
47e	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	47e (SEQ ID NO: 106)
412d	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	412d (SEQ ID NO: 107)
m16	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	m16 (SEQ ID NO: 108)
Sb1	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	Sb1 (SEQ ID NO: 109)
C12	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	C12 (SEQ ID NO: 110)
X5	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	X5 (SEQ ID NO: 111)
E51	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	TADKST	TVYLH	LRSLR	SDIAVYC	AGVY	EGE	ADG	YDNGEL	WGQG	TLVTVSS	21	E51 (SEQ ID NO: 112)

* Randomized

— Acidic residue

Figure 24

REPLACEMENT SHEET 27/25

Virus	Clade	Antibodies									
		m36	scFv m9	C34	m36SAbp	m36CH3	m36b0Fc	m36h1Fc	m36c2Fc	m36h3Fc	
92UG037.8	A	147 ± 20*	223 ± 7	297 ± 27	111 ± 44	- [†]	-	-	-	-	-
Ba1	B	8 ± 0.7	73 ± 7	270 ± 27	13 ± 3	-	-	-	-	-	-
JRFL	B	25 ± 5	77 ± 17	270 ± 54	61 ± 6	-	-	-	-	-	-
JR-CSF	B	25 ± 11	73 ± 3	351 ± 81	24 ± 4	-	-	-	-	-	-
IIIB	B	8 ± 0.7	20 ± 2	119 ± 14	12 ± 3	167 ± 10	26 ± 0.9	26 ± 2	57 ± 2	-	-
89.6	B	173 ± 13	8 ± 0.7	12 ± 6	94 ± 22	102 ± 32	>150	7 ± 0.5	5 ± 1	11 ± 6	-
R2	B	21 ± 7	37 ± 3	12 ± 4	24 ± 8	-	364 ± 51	>348	>348	>348	-
NL4-3	B	<8	29 ± 1	939 ± 135	<8	75 ± 12	>150	<1	<1	<1	-
GXC-44	C	16 ± 1	63 ± 10	351 ± 27	12 ± 4	-	25 ± 4	26 ± 10	28 ± 7	-	-
ZZ6	D	667 ± 60	22 ± 5	195 ± 35	667 ± 111	-	-	-	-	-	-
GXE	E	-	-	838 ± 27	-	-	-	-	-	-	-

* Antibody concentration (nM) resulting in 50% inhibition of virus infection (IC₅₀).
[†] No significant neutralization at the highest antibody concentration (667, 667, 1000, 667, 167, 150, 364, 348, and 348 nM for m36, scFv m9, C34, m36SAbp, m36CH3, m36b0Fc, m36h1Fc, m36c2Fc, and m36h3Fc, respectively).

Figure 25

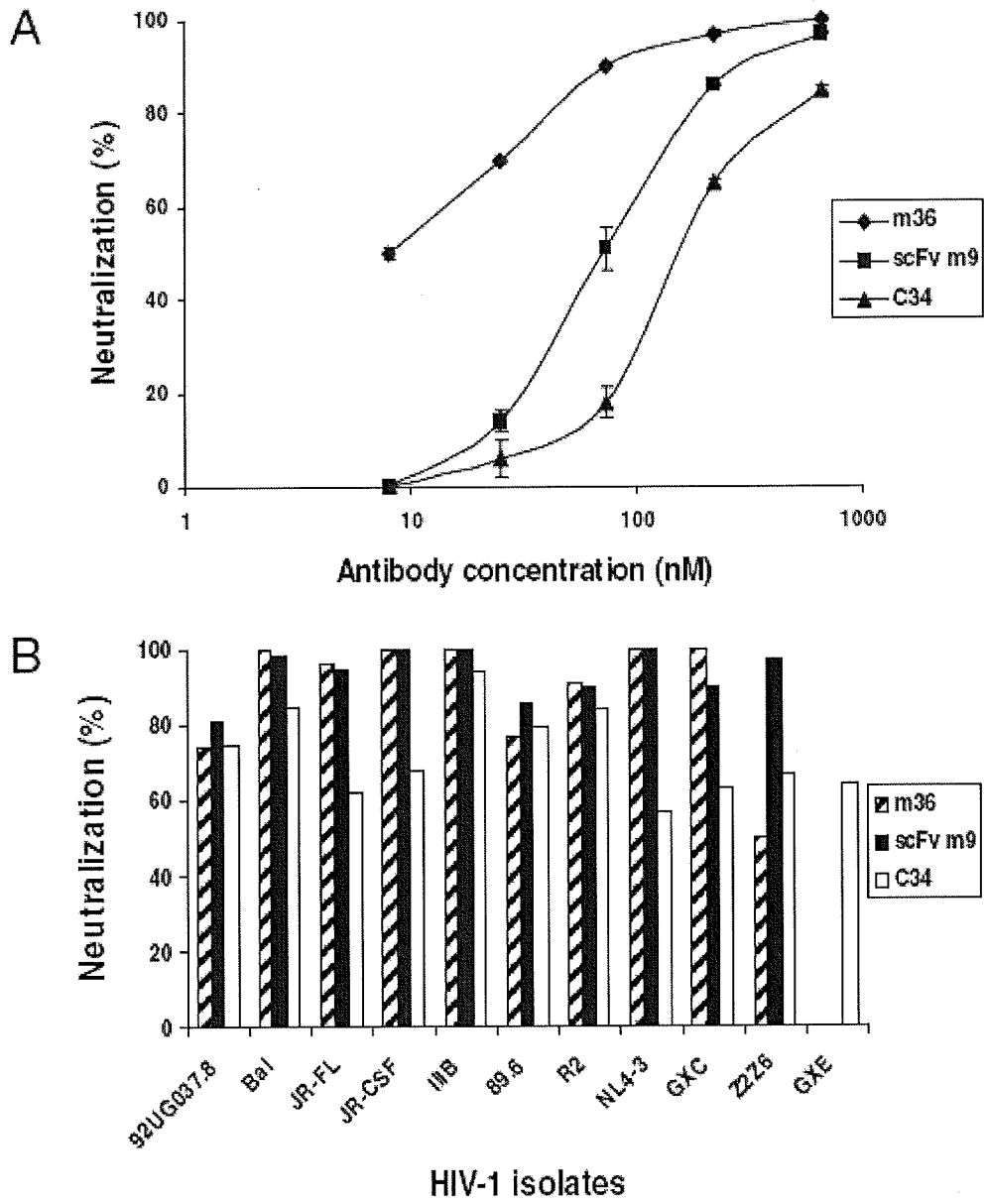


Figure 26

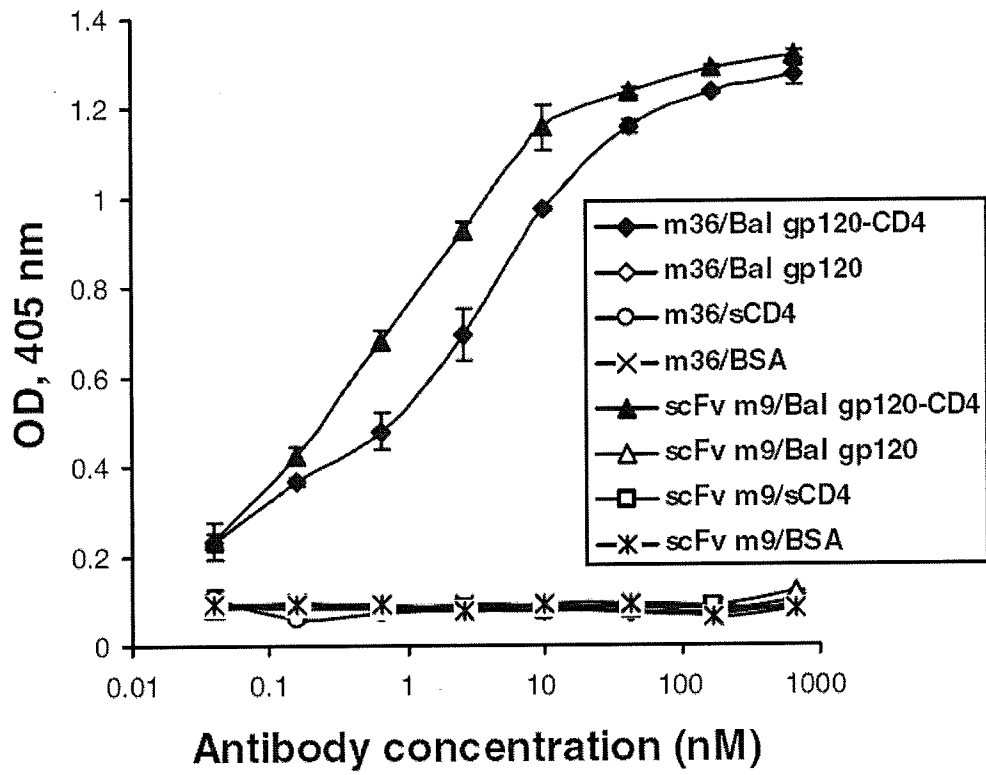


Figure 27

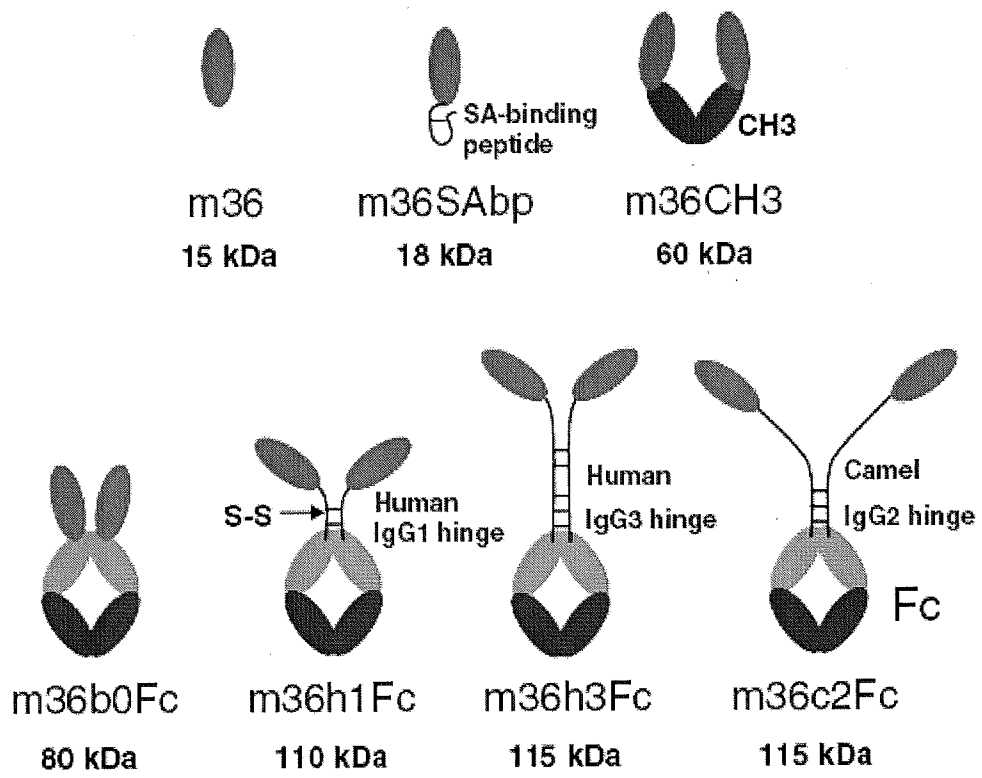


Figure 28

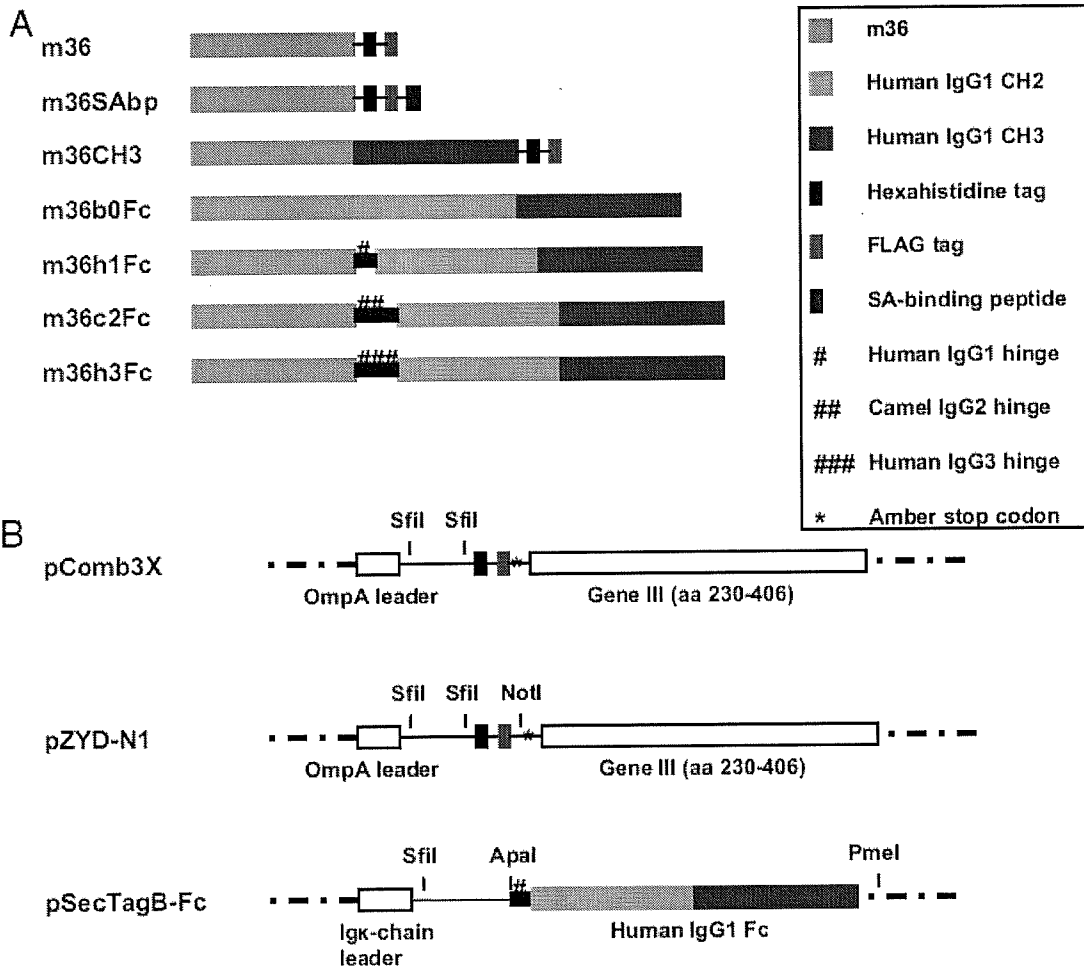


Figure 29

REPLACEMENT SHEET 32/35

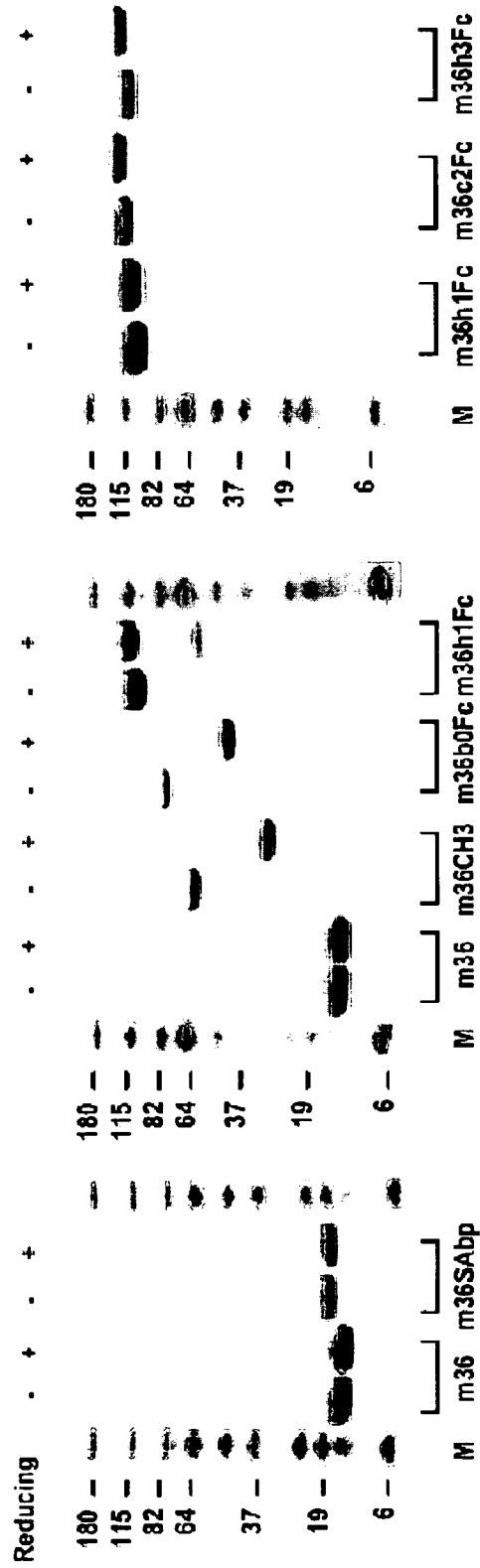


Figure 30

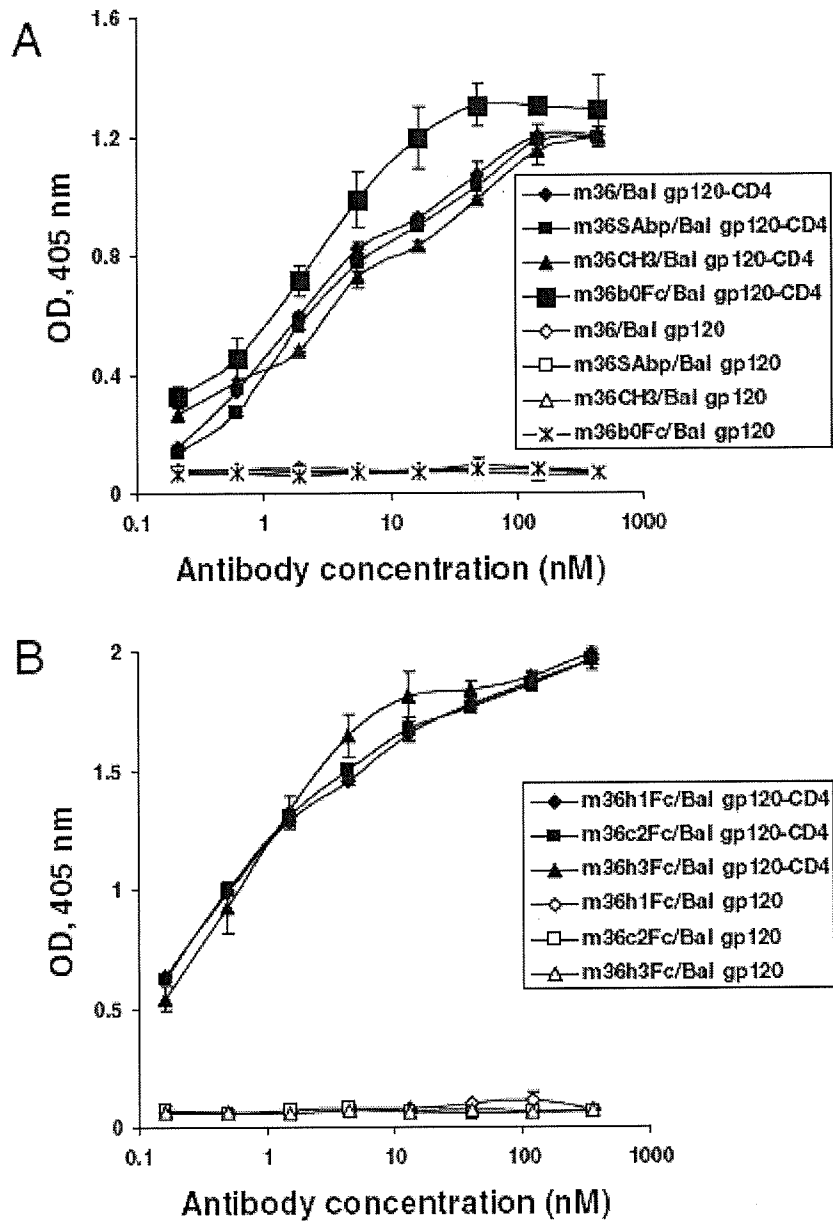


Figure 31

REPLACEMENT Sheet 34/35

Virus	Clade	Antibodies									
		m36	scFv m9	C34	m36SAbp	m36CH3	m36b0Fc	m36h1Fc	m36c2Fc	m36h3Fc	
92UG037.8	A	>667*	>667	>1000	>667	-	-	-	-	-	
Bal	B	73 ± 7	297 ± 37	>1000	144 ± 6	-	-	-	-	-	
JRFL	B	220 ± 40	500 ± 20	>1000	222 ± 17	-	-	-	-	-	
JR-CSF	B	73 ± 7	220 ± 13	>1000	222 ± 67	-	-	-	-	-	
IIIB	B	57 ± 1	67 ± 3	945 ± 81	56 ± 6	>167	100 ± 21	287 ± 9	330 ± 17		
89.6	B	>667	>667	>1000	>667	>150	235 ± 36	321 ± 3	330 ± 17		
R2	B	667 ± 93	667 ± 70	>1000	556 ± 50	-	>364	>348	>348		
NL4-3	B	<8	70 ± 7	>1000	24 ± 0.6	>150	12 ± 2	4 ± 0.8	13 ± 0.9		
GXC-44	C	73 ± 20	>667	>1000	211 ± 28	-	>364	>348	>348		
ZZZ6	D	>667	173 ± 3	>1000	>667	-	-	-	-		
GXE	E	-	-	>1000	-	-	-	-	-		

*Antibody concentration (nM) resulting in 90% inhibition of virus infection (IC₉₀).

†No significant neutralization at the highest antibody concentration (667, 667, 1000, 667, 167, 150, 364, 348, and 348 nM for m36, scFv m9, C34, m36SAbp, m36CH3, m36b0Fc, m36 h1Fc, m36c2Fc, and m36 h3Fc, respectively).

Figure 32

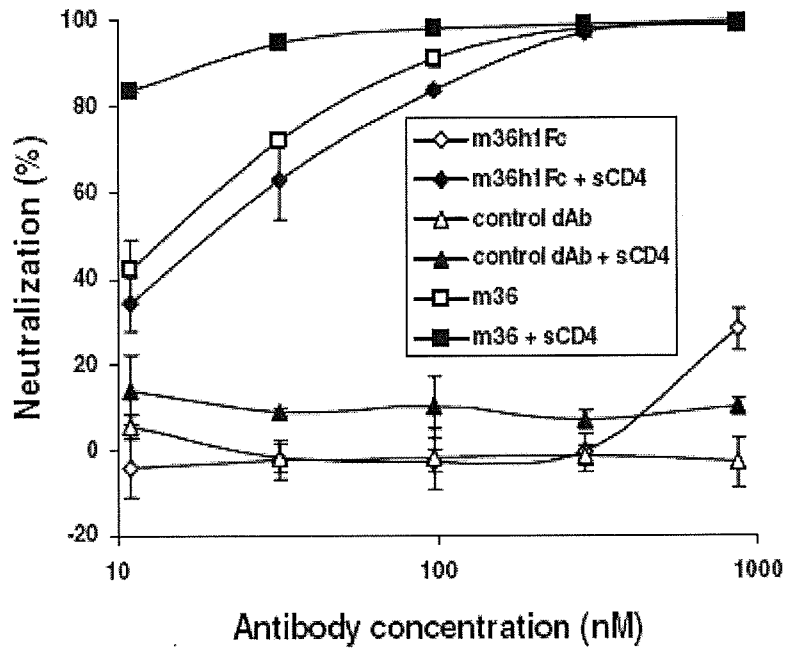


Figure 33