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(54) **Title**: ANIMAL MODEL FOR THE EVALUATION OF THE EFFICACY OF AN HIV VACCINE

(57) **Abstract**: The present invention relates to the use of a Severe Combined T-B-Immune Deficient (SCID) mouse engrafted with human immunocompetent cells (Hu-SCID-mouse) as an animal model for the evaluation of the effectiveness of an HIV vaccine. Furthermore, the present invention relates to a method for the evaluation of an HIV vaccine, wherein a Hu-SCID-mouse of the invention is inoculated with the HIV vaccine and thereafter challenged with HI-virus. The invention also relates to novel HIV vaccine compositions, which can be evaluated using the animal model.

Animal model for the evaluation of the efficacy of an HIV vaccine

The present application relates to the new attitude of an HIV preventive vaccine development, the particular details of its production and composition description and the method of the evaluation of the efficacy of an HIV vaccine in the laboratory animal model – sever combined T- B-immune deficient mice (SCID mice). Moreover, the present invention relates to an animal model for the evaluation of the efficacy of an HIV vaccine.

Library approach and mass spectrometry quantitative HIV envelop proteins variability analysis as well as in vivo confirmation of a correlation between HIV gp120-gp160 infection-active envelop protein complexes presentation with variability of human cells CD4 and co-receptors responsible for HIV infection obtaining and development in humans are in the background of the present invention.

The current application describes the method of immunizations and challenging of the immune deficient SCID mice capable for engraftment with human immunocompetent cells (Hu-SCID-mouse) as an animal model for the evaluation of the effectiveness of an HIV vaccine.

Summary of the Invention

The very first mathematic modeling calculations and mass spectrometry analysis of the variability of HIV envelop proteins collected using antibodies libraries and reverse panning techniques showed a huge gap between possible sequence variability up to 10^6 for a single patient and a number of gp120 major representatives responsible for HIV infection catching and further AIDS disease progression. The difference in these "calculated" and "found out" sequence numbers was similar to an in vitro disparity between HIV RNA titre measured in copies per ml in culture media and tissue cell infectivity dose ($TCID_{50}$) which is usually 10^6-10^8 lower than HIV RNA titre for laboratory strains.

A simple explanation of these differences was found. The number of gp120 sequence variability and/or a number gp160 trimer isoforms which provides infectiously active HI-virus particles survival selection and advantages for further multiplication depends on the number of human cells receptors such as CD4, CCR5 and CXCR4 variations available for virus invasion. As the variability of human peripheral blood mononuclear (PBMC) and other

cells membrane protein's sequences and isoforms exists but is limited by eukaryotic genome stability the number of passed cell-invading selection HIV gp160 infectiously-capable envelop proteins variations is limited to several decades or one-two hundred variants maximum too.

The assays of this theory were confirmed in HIV-1 laboratory strain's and their "playbacks" – the same laboratory strains human PBMC in vitro cultivation isolate's vaccine compositions. Results were obtained as in vivo immunizations efficiency evaluation in SCID mice engrafted with human immunocompetent cells (PBMC, DC) and challenged with HIV laboratory strain and their "playback" PBMC in vitro derivatives according to protocols described in detailed description of the invention.

In a preferred embodiment, the HIV vaccine is an immunogenic composition consist of HIV-1 envelop peptides/proteins cocktail in the presence of an adjuvant, wherein the proteins or peptides are of recombinant polyclonal origin and which immune boost adjuvant enhances vaccine efficiency through subcutaneous inoculation.

In another aspect, the invention relates to a method for the evaluation of an HIV vaccine, wherein a hu-SCID-mouse is inoculated with the HIV vaccine and thereafter challenged with HI-virus, wherein the efficacy is determined by determining the protection to HIV challenge.

Animal models for studying HIV infection were known in the art. These models include monkeys, and also SCID mice optionally engrafted with human leukocytes. However, in the past, the animal models used for studying preclinical assays of HIV vaccine forms were run only in a very limited number of animal models, mostly in two models only - Macaque Rhesus and Chimpanzees. In macaque rhesus human HIV infection does not work, so vaccines were tested exclusively for eliciting HIV-specific antibodies response with extremely sensitive ELISPOT kit's assays. In chimps that are bearing HIV infection but without developing any symptoms of immune deficiency disease (AIDS) finally tests results were resumed to the same ELISPOT data.

Therefore, there is a need for an efficient animal model for testing HIV vaccines, and for novel, efficient HIV vaccine compositions.

In one aspect, the present invention relates to the use of a Severe Combined T-B-Immune Deficient (SCID) mouse engrafted with human immunocompetent cells (Hu-SCID-mouse) as an animal model for the evaluation of the effectiveness of an HIV vaccine.

In a preferred embodiment, the immunocompetent cells are able to develop a human-type immune reaction for HIV. Preferably, the immune reaction is detectable in the blood of the animal, in particular in blood serum.

In a further preferred embodiment, the human immunocompetent cells are PBMC, dendritic cells or a mixture of PBMC and dendritic cells.

In a preferred embodiment, the human immunocompetent cells are PBMC, in particular PBMC pre-cultivated in vitro, more particularly PBMC pre-cultivated in vitro for a short period of time, even more preferred for about 1 day to about 6 weeks.

In a further preferred embodiment, the dendritic cells have been obtained by cultering human PBMC in the presence of cytokines capable of inducing the formation of dendritic cells in vitro. Preferably, the cytokines are also capable of cell differentiation blockage.

In a preferred embodiment, the human immunocompetent cells are derived from one human donor.

In a preferred embodiment, the mouse has been engrafted with 5-15x10^6 cells, in particular immunocompetent cells.

In a preferred embodiment, the Hu-SCID mouse is the particular breed of animals deficient in their own endogenous immune system and introduced with human immunocompetent cells or any other human cells, in particular human immunocompetent cells.

In a preferred embodiment, the Hu-SCID mouse is the particular breed of animals deficient in their own endogenous immune system and can bear the engraftment of human immunocompetent cells, PBMC, dendrite cells (DC) or a mixture of PBMC and dendrite cells able to develop a human-type immune reaction for HIV.

In another aspect, the invention relates to a method for the evaluation of an HIV vaccine, wherein a Hu-SCID-mouse of the invention is inoculated with the HIV vaccine and thereafter challenged with HI-virus.

In a preferred embodiment, the HIV vaccine is an HIV-1 envelop peptides/proteins cocktail, preferably wherein the proteins or peptides are of recombinant origin.

In a preferred embodiment, the evaluation of the vaccine is determined by determining the

efficacy of the vaccine.

In a preferred embodiment, the efficacy is determined by determining the protection to HIV challenge.

In a preferred embodiment, the Hu-SCID-mouse is inoculated with the HIV vaccine 1 to 4 weeks after the last engrafting of the human immunocompetent cells.

In a preferred embodiment, the human immunocompetent cells, PBMC or DC, or a mixture of PBMC and dendrite cells, intended for one mouse for one experiment are delivered from one human donor and have been engrafted as $5\text{-}15x10^6$ cell for one inoculation for a single animal.

An infectious doses of $5x10^2$ - 10^4 $TCID_{50}$ or higher, preferably higher than 10^4 $TCID_{50}$ for PBMC-cultivated and laboratory HIV strains are used for challenging of one Hu-SCID animal.

Preferably, infectious doses of at least 10^4 $TCID_{50}$, more preferably of at least 10^5 $TCID_{50}$, even more preferred of at least 10^6 $TCID_{50}$ are used. Preferably, infectious doses of up to 10^7 $TCID_{50}$ are used.

In a preferred embodiment, the efficacy of the HIV vaccine is determined by detection of the viral load, in particular the absence/presence of the viral load, and / or the specificity an / or the intensity of an immune response in a body fluid sample of the mouse. In particular, the efficacy of the HIV vaccine is determined by detection of the viral load absence/presence and/or the specificity of an immune response in a body fluid sample of the mouse.

In a preferred embodiment, the body fluid sample is blood serum. As HI virus infection in humans is being detected in blood samples the other tests like intraperitoneal liquid/cells viral load PCR assays or ELISA immune response assays can be due to residue ex vivo effect and should not be taken as vaccine efficiency results.

In a preferred embodiment, the inoculation with a vaccine is performed in the presence of an adjuvant.

The acceptability of control Hu-SCID mice to HIV challenging as well as their resistance to the challenging after vaccinations are preferably detected as viral load in the bloodstream measured as a number of HIV RNA copies in 1 ml serum by means of Real Time PCR.

The dynamics of HIV laboratory strains and PBMC- DC- "playback" strains replication in vitro controls is preferably carried out by means of Real Time PCR and p24 ELISA.

The HIV specificity of an immune response in a body fluid sample of the mouse is preferably carried out by means of ELISA with envelop p120-p160 antigens.

In a preferred embodiment, HIV-specific immune response for PBMC-Hu-SCID or DC-PBMC-Hu-SCID mice is detectable within the period of several weeks after the last vaccination.

In a preferred embodiment, the evaluation of the vaccine is carried out by means of RT-PCR, Real Time PCR or ELISA.

In a preferred embodiment, at least one HIV laboratory strain playback is used to infect and/or expose the Hu-SCID mice with/to HIV.

A method for producing an HIV vaccine, is described in WO2009/046984. Regarding the description and disclosure of the method and method steps, it is explicitly referred to the respective sections in WO2009/046984.

In particular the present invention also relates to a method for producing an HIV vaccine and are recombinant proteins/peptides cocktail composition obtained thereby, wherein the method comprises the steps:

a) creation of a library comprising HIV-1 specific antibodies,
b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,
c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,
e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
h) purification of the glycosylated env HIV-1 peptides, and

i) production of a vaccine composition,

characterized in that for cloning and/or expressing glycosylated env HIV-1 peptides in step f) and/or g), at least one primer selected from the group consisting of:

(i) V1 forward for subtypes A, G B, C, F1, and H:
5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1),

(ii) V2 forward for all subtypes:
5'- AAA ACT GCT CTT WCA XXX XXX XXX- 3' (SEQ ID No. 3),

(iii) V3 forward for A, B, G subtypes:
5'- TAV AAA TTA ATT GTA XXX XXX XXX X- 3' (SEQ ID No. 5),

(iv) V3 forward for subtype D:
5'- TAV CAA TTA ATT GCA XXX XXX XXX X- 3' (SEQ ID No. 7),

(v) V4 forward for all subtypes:
5'- GAA TTT TTC TAT TGY AAXXX XXX XXX- 3' (SEQ ID No. 9),

(vi) V5 forward for A, B, D, G subtypes:
5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11), and

(vii) V5 forward for subtype C:
5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13),

And at least one primer selected from the group consisting of:

(viii) gp41(160) Reverse for A, B,D subtypes, including sequence encoding His-Tag:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46),

(ix) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(x) gp41(160) Reverse for subtype A, rare variant:

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA
GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(xi) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA
GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

(xii) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA
GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

(xiii) gp120 Reverse Const5 for all subtypes , including sequence encoding His-Tag:
5'- ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT CTT TTT
TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 52),

and

(xiiii) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

are used.

In a preferred embodiment, the primers used are suitable for subtype identification. For example primer (xi) gp41(160) Reverse for C subtype and primer (vii) V5 forward for subtype C may be used for subtype C identification.

In a preferred embodiment, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 or more variants of primer (i), (ii), (iii), (iv), (v), (vi) and/or (vii) are used, wherein the variants differ in the variables "X".

In a preferred embodiment, the primers (i) to (vii), and (x) to (xii) and one of (xiii) and (xiiii) and one of (viii) and (ix) are used.

In a further embodiment, the present invention relates to a method for producing an HIV vaccine composition, comprising the steps of:

a) creation of a library comprising HIV-1 specific antibodies,
b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,

c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,

d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,

e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,

f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),

g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,

h) purification of the glycosylated env HIV-1 peptides, and

i) production of a vaccine composition,

characterized in that for cloning and/or expressing glycosylated env HIV-1 peptides in step f) and/or g), at least one primer selected from the group consisting of:

(i) Forward gp120 Const1 for A subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CRC TRC AGA AAA CTT GTG GGT YAC - 3'
(SEQ ID No. 52), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(ii) Forward gp120 Const1 for B subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3'
(SEQ ID No. 69), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iii) Forward gp120 Const1 for C subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3'
(SEQ ID No. 70), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end, and

(iv) Forward gp120 Const1 for G subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3'
(SEQ ID No. 71), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

And at least one primer selected from the group consisting of:

(v) V1 reverse for all subtypes:
5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25),

(vi) V2 reverse for subtypes A, D, C:
5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28),

(vii) V2 reverse for subtype B:
5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29),

(viii) V2 reverse for subtype G:
5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30),

(ix) V2 reverse for subtype F1:
5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31),

(x) V3 reverse for subtypes A, D, (C):
5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40),

(xi) V3 reverse for subtype B:
5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41),

(xii) V4 reverse for subtype G:
5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3' (SEQ ID No. 19),

(xiii) V4 reverse for subtypes A, B, C, D:
5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No. 20),

(xiv) V5 reverse for all subtypes:
5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3', (SEQ ID No. 15),

(xv) gp41(160) Reverse for A, B, D subtypes, including sequence encoding His-Tag:

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46),

(xvi) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(xvii) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(xviii) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

and

(xix) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

are used.

In a preferred embodiment, the primers used are suitable for subtype identification. For example primer (i) Forward gp120 Const1 for A subtype and primer (vi) V2 reverse for subtypes A, D, C may be used for subtype A identification.

In a preferred embodiment, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 or more variants of primer (v), (vi), (vii), (viii), (ix), (x), (xi), (xii), (xiii) and/or (xiv) are used, wherein the variants differ in the variables, in particular the variables "X".

In a preferred embodiment, the primers (i) to (xiv), and (xvii) to (xix) and one of (xv) and (xvi) are used.

According to the present invention, "X" in the nucleotide sequences is understood as, independently from each other, a DNA nucleotide selected from A, C, T and G.

In a preferred embodiment, the library is a human antibodies library.

In a further preferred embodiment, the library is a phagemid library or native antibodies library, or HIV envelop-specific peptides/proteins library bound to a solid support.

The antibodies may be in IgG, in particular an IgG1, IgG2, IgG3, IgG4, or IgM, IgA1, IgA2, IgAsec, IgD or IgE format. In a further preferred embodiment, the antibodies of the library are in IgGformat.

In a further preferred embodiment scFv antibodies, in particular scFv antibodies in a phagemid library may be used, as shown in the examples.

HIV envelop-specific peptides/proteins library may be presented as proteins and/or peptides selected for HIV specifity and capable to bind HIV gp120-gp160 proteins in enzyme-immune reactions, flow cytometry, fluorescent microscopy or Western blotting tests. Such envelop-specific peptides/proteins may be based on scaffolds like the DARPIN or lipocalin scaffold.

According to the present invention, "antibody" is understood as encompassing antibodies, antibody fragments and antibody mimetics. Antibody fragments encompass for example Fab, F(ab')2, scFv (single chain Fv), diabodies, single domain VHH, VH or VL single domains. A particularly preferred antibody is the scFv antibody fragment. Antibody mimetics encompass for example DARPINs and Lipocalins.

In a preferred embodiment, the sequence variables "X" in the primers are specified by

(a) identifying the peptide sequences or parts thereof according to method step (e) above, and

(b) identifying the nucleotide sequences encoding the peptide sequences or parts thereof of step (a).

In particular, step (b) is performed by taking into account the standard triplet code. This is for example described in Example 4.1.

In a further embodiment, the present invention relates to a composition comprising at least one of the following nucleic acids:

(i) V1 forward for subtypes A, G, B, C, F1, H:
5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1),

(ii) V2 forward for all subtypes:
5'- AAA ACT GCT CTT WCA XXX XXX XXX- 3' (SEQ ID No. 3),

(iii) V3 forward for A, B, G subtypes:
5'- TAV AAA TTA ATT GTA XXX XXX XXX X- 3' (SEQ ID No. 5),

(iv) V3 forward For subtype D:
5'- TAV CAA TTA ATT GCA XXX XXX XXX X- 3' (SEQ ID No. 7),

(v) V4 forward for all subtypes:
5'- GAA TTT TTC TAT TGY AAXXX XXX XXX- 3' (SEQ ID No. 9),

(vi) V5 forward for A, B, D, G subtypes:
5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11),

(vii) V5 forward for subtype C:
5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13),

(viii) gp41(160) Reverse for A,B,D subtypes, including sequence encoding His-Tag:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46) or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(ix) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(x) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

(xi) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

and

(xii) gp120 Reverse Const5 for all subtypes , including sequence encoding His-Tag:
5'- ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT CTT TTT TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 52), or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

In a preferred embodiment, the composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 of above nucleic acids.

In a further embodiment, the present invention relates to a composition comprising at least one of following nucleic acids:

(i) Forward gp120 Const1 for A subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CRC TRC AGA AAA CTT GTG GGT YAC - 3'
(SEQ ID No. 52), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end

(ii) Forward gp120 Const1 for B subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3'
(SEQ ID No. 69), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iii) Forward gp120 Const1 for C subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3'
(SEQ ID No. 70), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iv) Forward gp120 Const1 for G subtype (with *Xba*I site on 5' end):
5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3'
(SEQ ID No. 71), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(v) V1 reverse for all subtypes:
5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25),

(vi) V2 reverse for subtypes A, D, C:
5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28),

(vii) V2 reverse for subtype B:
5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29),

(viii) V2 reverse for subtype G:
5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30),

(ix) V2 reverse for subtype F1:
5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31),

(x) V3 reverse for subtypes A, D, (C):
5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40),

(xi) V3 reverse for subtype B:
5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41),

(xii) V4 reverse for subtype G:
5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3' (SEQ ID No. 19),

(xiii) V4 reverse for subtypes A, B, C, D:
5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No. 20),

(xiv) V5 reverse for all subtypes:
5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3', (SEQ ID No. 15),

(xv) gp41(160) Reverse for A,B,D subtypes, including sequence encoding His-Tag:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46), or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(xvi) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66)

(xvii) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

and

(xviii) gp41(160) Reverse for G subtype:

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68).

In a preferred embodiment, the composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 of above nucleic acids.

The primers and primer compositions are used for cloning the HIV-1 peptides, which are then part of the HIV-1 vaccine cocktail. Thus, the primers define the 5' and 3' ends of the amplified nucleic acids, respectively. It is of course understood, that sequences in the primers encoding His-Tags or sequences introducing an extra restriction endonuclease site are added in addition to the sequences encoding env peptide or protein fragments.

In a further preferred embodiment, the present invention relates to a kit, comprising at least one composition of the present invention and optionally further compounds suitable for PCR. Suitable further compounds are for example a thermostable polymerase and buffers.

The invention further relates to the use of the compositions comprising nucleic acids defined by the above sequences as primers for the production of a HIV vaccine composition.

In a further embodiment, the present invention relates to a method for producing an HIV vaccine composition, comprising the steps of:

a) creation of a library comprising HIV-1 specific antibodies,
b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,
c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,
e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
h) purification of the glycosylated env HIV-1 peptides, and
i) production of a vaccine composition,

wherein the glycosylated env HIV-1 peptides in step g) are characterized by:

(i) the gp120 peptides have a length of 441-541 amino acids and/or a glycosylated protein molecular weight of about 90 - 115kDa, and

(ii) the gp160 peptides have a length of 816-923 aminoacids, and/or a glycosylated protein molecular weight of about 145-175kDa, and

(iii) at least 50, preferably at least 70, more preferably at least 100, even more preferably at least 150 different HIV-1 envelop peptides are expressed.

In a preferred embodiment, the vaccine composition of step i) comprises

(a) at least one peptide characterized by the N-terminal sequence L C V T L (SEQ ID No. 72)

(b) at least one peptide characterized by the N-terminal sequence N C S X, wherein X is Y or F (SEQ ID No. 73)

(c) at least one peptide characterized by the N-terminal sequence X I N C, wherein X is K or Q or E (SEQ ID No. 74),

(d) at least one peptide characterized by the N-terminal sequence X I N C, wherein X is T or P or A, (SEQ ID No. 75),

(e) at least one peptide characterized by the N-terminal sequence E F F Y C (SEQ ID No. 76),

(f) at least one peptide characterized by the N-terminal sequence T R D G (SEQ ID No. 77),

and

(g) at least one peptide characterized by the N-terminal sequence L D X X E N L W V L D, wherein X is T or A, independently from each other (SEQ ID No. 78).

The invention further relates to a HIV vaccine composition, obtainable by a method of the invention.

The invention further relates to a HIV vaccine composition comprising at least 50, preferably at least 70, more preferably at least 100, even more preferably at least 150 different HIV-1 envelop proteins/peptides, characterized in that:

(i) the gp120 peptides have a length of 441-541 amino acids and/or a glycosylated protein molecular weight of about 90 - 115kDa, and

(ii) the gp160 peptides have a length of 816-923 amino acids, and/or a glycosylated protein molecular weight of about 145-175kDa.

In a preferred embodiment, the vaccine composition further comprises

(a) at least one peptide characterized by the N-terminal sequence L C V T L (SEQ ID No. 72)

(b) at least one peptide characterized by the N-terminal sequence N C S X, wherein X is Y or F (SEQ ID No. 73)

(c) at least one peptide characterized by the N-terminal sequence X I N C, wherein X is K or Q or E (SEQ ID No. 74),

(d) at least one peptide characterized by the N-terminal sequence X I N C, wherein X is T or P or A, (SEQ ID No. 75),

(e) at least one peptide characterized by the N-terminal sequence E F F Y C (SEQ ID No. 76),

(f) at least one peptide characterized by the N-terminal sequence T R D G (SEQ ID No. 77),

and

(g) at least one peptide characterized by the N-terminal sequence L D X X E N L W V L D, wherein X is T or A, independently from each other (SEQ ID No. 78).

In a preferred embodiment, the HIV-1 envelop peptides are produced recombinantly.

The peptides may be expressed in an expression system suitable for expressing eukaryotic peptides and proteins, like e.g. in yeast strains, insect cells or eukaryotic cellular parasite systems. In particular, the peptides may be expressed in L. tarentolae, as exemplified in example 4.2 and/or in WO 2009/046984.

In a preferred embodiment, gp120 peptides are produced recombinantly and/or are encoded by nucleic acids having a length of 1323 to 1623 bp.

In a preferred embodiment, gp160 peptides are produced recombinantly and/or are encoded by snucleic acid having a length of 2448 to 2769 bp

In a further preferred embodiment of the invention, the nucleic acids encoding the peptides of the HIV vaccine composition optionally comprise sequences encoding a tag, in particular a His-Tag, and/or sequences encoding a signal peptide suitable for recombinant expression and/or sequences enabling expression in a eukaryotic expression systems.

In particular, the nucleic acids encoding the peptides of the HIV vaccine composition are inserted in a suitable vector for eukaryotic expression. A vector may comprise one or more nucleic acids encoding one or more peptides of the HIV vaccine composition.

The original PCR products for expressing the peptides of the HIV vaccine composition are obtained using primers as described above and tailored in suitable (Leishmania tarentolae) vector in identified between for-rev ends loop, from V1 to V5, respectively.

The immunogenic component of immunogenic composition is expressed in and extracted from suitable eukaryotic host in vivo system, in particular in Leishmania tarentolae (*L. tarentolae*).

In a preferred embodiment, the HIV vaccine composition is effective as determined by detection of the viral load absence/presence and/or the specificity of an immune response in a body fluid sample of the mouse model of the present invention.

The immunogenic component consists of a number of recombinant HIV-1 envelop peptides/proteins not less than several decades of variants of them with length for gp120 representatives from 1323 to 1623 bp (441-541 aminoacids) and glycosylated protein molecular weight 90-115kDa, and with length for gp160 representatives from 2448 to 2769 bp (816-923 aminoacids, respectively) and glycosylated protein molecular weight 145-175kDa.

A "tag" according to the present invention is a peptide sequence which is suitable for isolation, and which is covalently attached to a peptide or protein sequence of interest. In a preferred embdoiment, the tag is a His-Tag. A His-tag is a peptide sequence consisting of six Histidine-residues. The His-Tag binds to metal matrices. Other suitable tags are chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST) and FLAG-tag.

According to the examples, a His-Tag was used, for purifying the expressed peptides. However, also other tags, in particular affinity tags may be used for purifying the peptides.

Also, several primers of the invention comprise a *Xba*I site. This site suitable for cloning amplified PCR products into suitable expression vectors. It is understood that also other restriction sites are suitable for cloning PCR products into expression vectors.

The creation of a humane recombinant IgG phagemid library containing HIV-1 specific scFv antibody fragments and the enrichment for HIV-specific scFv antibody fragments in the phagemid library by panning with native or recombinant HIV-1 peptides is described in examples 2.1, 2.2 and in WO2009/046984.

Methods for multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins is described in WO 2009/046984 and in the present invention. In particular, HIV-1 may be amplified in PBMC culture cells.

Collecting HIV-1 peptides by reverse panning of the multiplied HIV-1 material using the enriched HIV-1 phagemid library described above bound to a support is described in example 2.3 and in WO 2009/046984. The identification and characterization of the HIV-peptides thus obtained is described e.g. in WO 2009/046984 and in examples 3.1 and 3.2 of the present invention.

The identification and characterization of the HIV-1 peptides by mass spectrometry, in particular by MS-MS, more preferred by LC-MS-MS is for example described in example 3.

Expressing glycosylated env HIV-1 peptides using the results of above step in an expression system, is described in WO 2009/046984 or in examples 4.1, 4.2 and 4.3 of the present invention.

The purification of the glycosylated env HIV-1 peptides is described in WO 2009/046984 and in Example 4.3 of the present invention.

The production of a vaccine composition is described in WO2009/046984.

In a preferred embodiment of the method of the present invention, the individuals, from which the HIV material is obtained are infected by the same or a different HIV subtype.

In a preferred embodiment of the method of the present invention, the individuals, from which the viral material is obtained are antiretroviral therapy naïve patients or patients that have been subjected to antiretroviral therapy.

In a preferred embodiment of the method of the present invention, the phagemid library is prepared by the steps of

a) preparing DNA-fragments derived from nucleic acids encoding the variable region of a light chain and a heavy chain, respectively, of immunoglobulines expressed in B-Lymphocytes obtained from a number of individuals infected with HIV,

b) linking the DNA-fragments encoding the immunoglobuline light and heavy chain, to allow expression of a polypeptide, comprising the variable regions of a light chain and heavy chain, respectively, of immunoglobulines, to create a multitude of different specificities,

c) cloning of linked fragments in phagemid vector and transforming bacterial strain for expression on the bacteriophage's surface, preferably wherein amplification is carried out with any of the primer combination listed in tables 1-7 of WO2009/046984, more preferably wherein obtained scFv phagemid recombinant antibodies are specific to resistant HIV variants carried out in HAART- or any other antiretroviral therapy experienced patients.

In a preferred embodiment of the method of the present invention, step a) further comprises an enrichment of the phagemid library presenting antibody's scFv fragments in panning procedure binding HIV-specific antibodies with recombinant gp120-, gp41- and native HIV-polypeptides isolated from different donors.

In a preferred embodiment of the method of the present invention, LC mass spectrometry is applied for a quantitative analysis, identification and sequencing of HIV-1 gp120 and HIV-1 gp41 and its standard and variable fragments.

In a preferred embodiment preparing a HIV preventive vaccine composition is accomplished by addition and/or conjugation of optional immunogenic stimulants, adjuvants or carriers, such as sterically stabilized liposomes (SSL).

In a preferred embodiment, the present invention relates to a HIV vaccine composition of the present invention further comprising immunogenic stimulants, adjuvants or carriers, such as sterically stabilized liposomes (SSL).

The present invention also relates to the use of a HIV vaccine composition of the present invention for immunization of non-infected individuals against catching and development of HIV infection and AIDS disease.

Figure Legends:

Fig. 1 shows samples of preparative PAAG electrophoresis with or without marked bands for cutting for trypsinolysis with further mass spectrometry analysis:

A – PokA-79-PBMC and U455-PBMC samples (FIL-1 and FIL-2, respectively), Coumassi staining;

B – H9/IIIB and PokA-79 laboratory strains samples, Silver staining.

Fig. 2 shows SCID mice endogenous immune deficiency testing compared to BalbC response for immunization with isolated U455 laboratory strain proteins.

Fig. 3 shows SCID-PBMC and BalbC immune response for immunization with cocktails of recombinant p120-1 and p120-2 proteins.

Fig. 4 shows PokA-79 viral load rates on U937 and MT-4 background, 3 challenges 7 days b/w period.

Fig. 5 shows PokA-79 viral load rates on U937 and MT-4 background, 2 challenges 3 days b/w period.

Fig. 6 shows PokA-79 - MT4 and U937-background's viral load rates for different periods between challenging in SCID mice.

Fig. 7 shows PokA-79 strain in vitro replication dynamics, lymphocyte MT-4, monocyte U937 cultures, PBMC and DC background.

Fig. 8 shows PokA-79 strain in vitro replication dynamics, p24 expression, lymphocyte MT-4 and monocyte U937 background.

Fig. 9 shows the morphology of cell culture and HIV-1 strain infection manifestation on the 3rd day of in vitro cultivation. Syncithyum formations are marked with arrows.
A) MT-4 lymphocytes
B) PokA-79-MT4

Fig. 10 shows the morphology of U937 monocytes culture and and HIV-1 strain infection manifestation on the 5th day of in vitro cultivation. Syncithyum formations are marked with arrows.
A) U937 intact monocytes, B) PokA-79-U937 and C) U455-U937

Fig. 11 shows the morphology of PBMC and HIV-1 strain infection manifestation on the 5th day of PBMC passage in vitro cultivation. Syncithyum formations are marked with arrows.
A) Healthy donor PBMC
B) PokA-79-PBMC 6th passage (6 weeks)
C) U455-PBMC 5th passage (5 weeks)

Fig. 12 shows the morphology of DC and PokA-79-DC strain cultivation on the 3rd day of DC passage – the is no visible difference between donor dendrite cells and chronically infected with PokA-79 DC at 5th passage.
A) Healthy donor DC
B) PokA-79-DC 5th passage (5 weeks)

Fig. 13 shows Laboratory strains U455 and H9/IIIB viral load rates on MT-4 and U937 cell cultures background, 4 challenges with 7 days b/w period.

Fig. 14 shows U455 and H9/IIIB in vitro replication dynamics on lymphocytes MT-4 and monocyte U937 backgrounds.

Fig. 15 shows U455 and H9/IIIB in vitro replication dynamics, p24 expression, lymphocyte MT-4 and monocyte U937 cultures.

Fig. 16 shows PokA-79 viral load rates on cell culture U937 and MT4 background depending on the number of challenging.

Fig. 17 shows SCID-PBMC and SCID-cell culture background viral Load rates for challenging with PokA-PBMC and PokA-79 strains, respectively.

Fig. 18 shows negative challenging results for dendrite cells (DC) and Fresh PBMC PokA-79-U937 and U455-U937 lab. strains (PBMC 1st week) compared to 6-8th PBMC passage and U937 background.

Fig. 19 shows PokA-79 in vitro passages replication dynamics, DC and PBMC background, 1st week and 5-6th weeks passages.

Fig. 20 shows U455 and PokA-79 in vitro replication dynamics, dendrite cells (DC) or PBMC background, 5th and 6th passages.

Fig. 21 shows:
A) Scaffold software HIV Envelop protein's identification and trustworthy analysis,
B) HIV Envelop protein's spectra,
C) HIV Envelop protein's triptic peptides mapping,
D) Scaffold software HIV Envelop protein's identification and trustworthy analysis,
E) HIV Envelop protein's triptic peptides mapping.

Fig. 22 shows the HIV Envelop protein's triptic peptides mapping.

Fig. 23 A) to 23 M) show t:he HIV Envelop protein's spectra.

Fig. 24 shows primers disposition for PCR products of immunogenic composition's tailoring mapped for several sequences in alignment.

Fig. 25A) shows immune responses for gp160-1 and gp160-2 in challenged with PokA-79-PBMC strain SCID-PBMC Mice.

Fig. 25B) shows recombinant gp160-1 and gp160-2 immunizations effect for SCID-PBMC challenging with PokA-79-PBMC 6th passage strain.

Fig. 25C shows recombinant gp160-PokA-PBMC and gp160-U455-PBMC immunization effect for challenging with PokA79-PBMC and U455-PBMC 6-8 weeks strains.

Fig. 26 shows A) the scheme of constant and variable regions in HIV-1 gp120 and primers arrangemen, and B) Gp160 schematic map and primers arrangement.

THE STATE OF THE ART:

The present invention relates to the animal model for HIV vaccine effectiveness evaluation and novel HIV vaccine compositions. In particular the presented method comprises endogenous immune system deficient CSID-humanized mouse model application for testing preventive HIV vaccine's immunization effect for in vivo HIV challenging prevention. SCID humanized peripheral blood lymphocytes (SCID-PBMC) or SCID humanized dendrite cells-engrafted mice (SCID-DC-PBMC) were used for immunizations and following challenging with HIV laboratory strains which envelop protein's pool was obtained according to reverse panning and mass spectrometry analysis method. One donor PBMC or unmixed one donor DC-PBMC derivations were inoculated into one animal.

Severe Combined T- B- Immune Deficient (SCID) and thymus-free nude mice were never used in practice for in vivo evaluation of HIV vaccines effectiveness in spite it was known since early 90-ies that such mice can be successfully engrafted with human peripheral blood (PBMC), dendrite (DC) and stem cells believed to be capable for anti-HIV immune response formation [1, 13, 16] and in vivo HIV infection development [2, 3, 9, 12, 22]. An idea to combine these two features and to use hu-SCID mice for vaccination assays was discussed

[9]. Preclinical assays of DNA- and peptide-based HIV vaccine forms were run in a very limited number of animal models, mostly in two models only, i.e. in Rhesus Macaques and Chimpanzees (Chimps). In Rhesus Macaques human HIV infection does not work, so vaccines were tested exclusively for eliciting HIV-specific antibody responses detecting with extremely sensitive ELISPOT assays. In Chimps that are bearing HIV infection but without developing any symptoms of acquired immune deficiency syndrome (AIDS) finally test results were resumed to the same ELISPOT data. Therefore the existing monkey models are not at all satisfying since HIV infection does not at all develop in these animals like in humans. In general vaccines are distinguished from other therapeutic/preventive medical agents with their luck of the first stage of research in vitro in positive effect testing.

There are two main vaccine generations from beginning research in a vaccine development to finish with large scale medical purpose production. The first generation is represented by natural pathogen or its derivations deactivated with chemical compounds treatment and further neutralization of possible toxicity, fermentative pathogen's lysis into peptides and proteins, sterilization via autoclaving or any other similar method for vaccine active component's conservation. This way of vaccines creation was running for XXth century successfully for all bacterial pathogens like tuberculosis, plague, diphtheria, and also for large, stable DNA viruses diseases as smallpox, stable single- and double-strand RNA viruses diseases like morbilli, hepatitis A and B, human encephalitis, rabbies and other more rare tropical disease's vaccines. The first class of vaccines normally provides the formation of pathogen-specific, elongated and strong immune responses sufficient for infection development prevention. However the backwards of this vaccine's generation always were the lack of safety in administration due to high risk of survived pathogen's activity causing the development of real disease instead of immunization against it, and also anaphylactic reactions of immune system to foreign proteins and further immune vulnerability and disorders.

The second generation of vaccines development started with contributions of genetic engineering and molecular biology as recombinant proteins/peptides-based vaccines that are replacing the first class since the late 80-ies. Recombinant vaccines were quickly accepted since they provide a higher degree of safety in administration and production, since they are free of disease-evoking pathogens [14, 20]. In case they were targeted to conservative and stable pathogens after several years of efforts in development they matched highly specific immunity and close to complete protection against infectious disease. Recombinant proteins or peptide's mixtures produced in cheap and easy-to-maintain prokaryotic expression systems (E.coli transformed strains) are able to make exactly the mimic representation of prokaryote pathogen surface "checkpoints" crucial for antibodies recognition and vaccination against a

number of bacterial infectious diseases. Bacterial cell surface normally contains a standard number of very conservative proteins, totally about one thousand. The similar situation with standard surface antigens exposition and their number is observed for many large DNA and RNA pathogenic viruses of different families like coronaviridae, adenoviridae, poxviridae, etc. Some small RNA viruses also have very stable genotype spread in its host practically all over the world – for example picornaviridae hepatitis A virus. For each known recombinant vaccine in history years were spent to solve the difficulty of distinguishing these protein's "checkpoints" responsible for immunity against the current pathogen from several hundred of possible candidates. Then immunogenic peptides cocktail preparation and pre-clinic efficacy assays took some more years of efforts. Within next several years of clinical trials doctors studied and demonstrated the advantages of recombinant vaccine over native pathogen's derivatives administration. This process normally took 15-50 years for every individual recombinant vaccine development.

If we try to transfer an HIV vaccine development attempts to this scheme of two stages described above it becomes obvious immediately that HIV vaccines do not fit there. There are several main reasons for it. First of all, the development of anti-HIV vaccine based on native pathogen described in several patents from early 90-ies [5, 11] was not possible due to impossibility to produce and collect the viral substrate in somehow reasonable amounts. In our research we certainly showed that it was not possible to concentrate virus envelop proteins with standard sucrose gradient untracentrifugation methods with over 200000g gravitation speed even in amounts sufficient for analytical purpose [7].

Therefore the first stage was absent for HIV vaccine, the second stage - recombinant vaccines development attempts - was tried [8, 14]. The virus genome sequencing prior to attempts to create recombinant versions revealed so great variability of about one third viral genome that it was poor chances to find two identical full-length variants even from the same sample of one patient or group of patients mixed together [15].

As a result now modern spectrum of HIV vaccines issued for clinical trials is represented with DNA vectors form – in terms of vaccines development evolution it is an incomplete recombinant second stage which did not yet reach a protein composition necessary for vaccination effect. So DNA HIV vaccine preclinical assays were run in Rhesus Macaques and Chimpanzees.

Meanwhile the animal model for studying human HIV infection and its treatment with antiviral agents was originally created in the late 80-ies by Derek Mosier [13] and improved by several laboratories in the world for different practical applications in HIV research [2, 6,

9, 10]. SCID mice engrafted with human leukocytes (PBL) provided human-like immune responses for immunization with different antigens including HIV peptides and proteins [1, 2, 4, 6]. Animals were supplied with 20-50x10^6 PBL intraperitoneally in the age of 2-5 weeks [9, 10], for the next two months they were examined for presence of human lymphocytes in peritoneal cavity, spleen, lymph nodes, liver and for human-like cytokine's profiles in bloodstream. After two-three months lymphocytes depletion, anemia, in some cases - leukemia development, were observed in grown up animals [9, 16]. To avoid such side effects (graft versus host disease - GVHD) it was offered to engraft immune deficient mice with dendrite cells produced via treatment of human leukocytes with human IL-2, IL-12, INF-γ before engrafting [6, 9, 10]. Some researchers engrafted newborn CSID mice with parts of human organs and tissues – spleen, thymus, dendrite cells [19]. One human donor organ's tissue engrafting for a single mouse was successfully made by Stoddart group [18].

Different methods were adapted for modeling HIV infections in SCID and NOD-SCID mice, 95 percent of these experiments were based on laboratory HIV strains cultivated in vitro at cell cultures. CD4, CCR5 and CXCR4 receptors expressing cultures such as transfected HeLa-CD4, MT2, MT4, MAGI-CCR5, U937 and so on were used as an engrafted substrate for viral infection [10, 21]. Grafts of human tissues (Hu-SCID Thy/Liv), isolated human leukocytes (PBL) and their infected with laboratory strain variants were administered intraperitoneally and provided HIV infection [2, 11, 22]. Infection rates were measured with RT-PCR or Real-Time PCR from intraperitoneal lavage, liver, spleen and thymus area [9]. But more often SCID mice are engrafted with dendrite cells cultivated in vitro with interleukin factors from human peripheral blood leukocytes (PBL). Such humanized chimerical mice are reported to be able to bear HIV infection for long periods of time (1-6 months) [3, 9] and were used for testing and demonstration of antiviral activity for different classes of antiretroviral agents [9, 17, 21]. It was demonstrated that engrafted with dendrite cells animals normally are protected from development of graft vs host disease and live for months after the xenograft embedding [22].

A number of researchers especially from those who was convinced in clinical trials HIV vaccines they developed themselves do not work proclaimed it is not possible to invent HIV immunization vaccine which will protect humankind from viral infection catching and development [17]. Some experiments with dendrite cells cultivated in vitro in presence of HIV laboratory strain cell culture or cultivating media and then engrafted into SCID or NOD-SCID mice showed these animals do not catch and do not maintain HIV infections from challenging with the same laboratory strain [9]. The same experiment with dendrite cells engraftment into SCID mice and blockage of infection with natural HIV isolates cultivated on healthy donors PBLs was also described once [9].

Within the present invention, however, it is understood that the other immune deficient laboratory animals such as nude mice, immune deficient rats, immune deficient cats, etc. can be used.

Severe combined T-B-immune deficient mice (SCID mice) and methods for their production are known in the art. Furthermore, SCID mice are also commercially available. However the production of hu-SCID mice with presence of blood serum-detectable immune response to HIV envelop proteins and HIV challenging blood serum- measured viral load as a number HIV RNA copies per ml of serum of non-immunized mice with the same PBMC background was never described. In spite of high HIV-specific immunogeniety and declared HIV challenging ability nobody ever published results that SCID mice were used for testing DNA HIV vaccines efficacy [2, 6, 9, 10]. Only one of all developed hu-SCID mice models – grown up SCID off-springs with engrafted in life first 1-3 weeks human thymus and liver (hu-SCID Thy/Liv)- proved to be able to bear both HIV-specific immune response and the viral challenging in the bloodstream of the same animals [2, 11, 22]. However such humanized mice production is greatly labor-consuming and highly expensive. The cost of one human thymus and liver-humanized SCID mouse exceeds the cost of one small primate, such as Macaque Rhesus monkey, being used for laboratory experiments.

It is advantageous that Hu-SCID mice can provide a quick generations replacement due to the short lifespan, and also a valid statistical analysis because little 15-25 gram body weight animals need approximately 50 times less immunization material for testing each than Macaques with several kilos body weight. A real possibility to provide mice with enough immunocompetent human cells material for engraftment is also worth for consideration.

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrates that Hu-SCID mice represent an efficient animal model for the evaluation of an HIV vaccine.

Within the present invention, however, it is understood that also nude mice, immune deficient rats, immune deficient cats and other immunodeficient animals can be used.

Severe combined T-B-immune deficient mice (SCID mice) and methods for their production are known in the art. Furthermore, SCID mice are also commercially available.

Techniques for engrafting SCID mice with human immunocompetent cells are also known in the art and are, additionally, described in the attached example.

As used in the present invention the term "immunocompetent cells" comprises any cell of the specific immune system, such as lymphocytes and in particular T and B lymphocytes, which have been exposed to a particular antigen of interest, i.e. the HIV-vaccine to be evaluated, in vitro or in vivo. Examples for T-lymphocytes (T cells, thymus cells) are T-helper cells, cytotoxic T cells, NKT-cells, γδ-T cells, and memory T cells. T cells may be CD4 and/or CD8 positive and/or negative respectively, depending on their maturation status. Examples for B lymphocytes (B cells, bone cells), include plasma B-cells, B1- and B2-cells, marginal zone B cells and memory B cells. Immunocompetent cells may be obtained e.g. from individuals, such as peripheral blood mononuclear cells (PBMC), or from cell lines.

Preferably, the immunocompetent cells may be selected from the group consisting of PBMC, dendritic cells, B-cells, and T-cells. More preferably, the human immunocompetent cells are PBMC or dendritic cells.

Introduction of cells of human origin into the SCID mice may be achieved by using any conventional route, such as via the intraperitoneal or parenteral route. The intraperitoneal route is normally preferred due to the ease of administration and allowing administration of a volume of fluid. The cells may be introduced in form of a suspension in any suitable medium, allowing cell survival, such as phosphate buffered saline PBS or Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI) or Glasgow's Minimal Essential Medium (GMEM).

The immunocompetent cells may be stimulated with immune stimulators prior to being introduced into the SCID mice. Immune stimulators for such purpose may be the immune stimulators mentioned above. Preferred stimulators include but are not limited to interleukin factors such as IL-2 (interleukin-2), IL-4 (interleukin-4), IL-12 (interleukin-12), and other factors such as INF-γ and GCSF (granulocyte colony stimulating factor). Further or different factors or combinations of such factors may also be used. The immune stimulators promote growth and/or development of e.g. T, B, and hematopoietic cells.

The dendritic cells may have been obtained by cultering human PBMC in the presence of cytokines capable of inducing the formation of dendritic cells in vitro. Methods for the production of dendritic cells in vivo are known in the art (see e.g. [25]).

The cell count for each introduction of cells of human origin may vary from about $1x10^3$ to

$1x10^8$ cells per introduction, preferably from about $1x10^3$ to $1x10^7$ cells per introduction, more preferably from about $1x10^4$ to $1x10^7$ cells per introduction, even more preferred from about $1x10^5$ to $1x10^7$ cells per introduction, and even more preferred about $1\text{-}15x10^6$ cells per introduction (i.e. for a single engraftment only). The introduction of cells of human origin may be repeated prior to step (b), such as two to three times once weekly, or twice weekly, or providing a pause prior to a third or further introductions.

In case PBMCs are introduced into SCID mice followed by immunization these mice are referred to as "PBMC-Hu-SCID mice". In case dendritic cells (DCs) are introduced into SCID mice followed by immunization and subsequent introduction of cells of the immunogenic background such as PBMCs, these mice are referred to as "DC-PBMC-Hu-SCID mice".

Preferably, the immunocompetent cells intended for one mouse are from one human donor. In case that various mice are used, it is preferred that for one assay, the imunocompetent cells are derived from one human donor and are then engrafted into the different mice.

As discussed above, the present invention relates to a method for the evaluation of an HIV vaccine, wherein a Hu-SCID-mouse as defined above is inoculated with the HIV vaccine and thereafter challenged with HI-virus.

The vaccine may be applied/introduced in form of a suspension or mixture in any suitable medium and the exposure (vaccination) may be carried out in the presence of one or more adjuvants such as one or more pharmacological or immunological agents that modify the effect of the HIV-vaccine, such as immunopotentiators - also known as boosters of the immune system - which are effective in stimulating cell-mediated immunity. A preferred adjuvant for this purpose is Freund's adjuvant.

For an in vivo vaccination the vaccine to be tested may be administered directly to the mice on the day following the last introduction of immunocompetent cells or of the cells of the immunogenic background, or it may be administered some time (e.g. a couple of days or even weeks, preferably after two weeks) after the last introduction of said cells. Immunization may be carried out once or in a number of subsequent steps, e.g. it may be repeated after some time, e.g. after one to three weeks, preferably after two weeks two to four times, preferably three times.

The Hu-SCID-mouse may be inoculated with the HIV vaccine 1 to 3 weeks after engrafting of the human immunocompetent cells.

Administering the vaccine to the mice may be performed using any route considered suitable, e.g. via the parenteral route or via the intraperitoneal route such as intravenous injection, subcutaneous injection, or intramuscular injection. The preferred route for administering the vaccine to the mice is via subcutaneous injection.

The vaccine to be tested may be administered to the Hu-SCID mice once, twice or more often (three to six times). Preferably, the Hu-SCID mice are vaccinated for a total of four times within two to four weeks, preferably within three weeks.

In case that the vaccine is a peptide or protein, the amount of vaccine peptides or proteins administered to the Hu-SCID mice may be between 1 and 100 μg, preferably between 10 and 80 μg, more preferably between 15 and 50 μg, even more preferably between 20 and 40 μg and most preferably about 25 μg of vaccine peptides or proteins in each step for each mouse. Administering may be performed in one or more individual steps, interrupted by lag phases lasting one day, days or weeks.

The infection of the hu-SCID mouse be performed by using an amount of HIV strain infectious doses in cell culture, e.g. from about $5x10^2$ to $5x10^6$ $TCID_{50}$ (50% Tissue Culture Infective Dose), preferably from about $5x10^2$ to $5x10^4$ $TCID_{50}$. For example, for infecting Hu-SCID mice having freshly isolated PBMCs or DCs as background, from about $5x10^3$-10^4 $TCID_{50}$ of HIV-1 laboratory strain infectious doses in cell culture are administered to each individual mouse, or from about $5x10^2$ - $5x10^3$ $TCID_{50}$ infectious doses. Suitable cell cultures for this purpose include but are not limited to MT-4 or U937 culture cells. Preferably Hu-SCID mice can be challenged with $5x10^3$-10^4 $TCID_{50}$ of HIV-1 laboratory strain infectious doses in MT-4 culture cells, or with from about $5x10^2$ - $5x10^3$ $TCID_{50}$ infectious doses in U937 culture cells.

It will be understood that different ranges of cell numbers and $TCID_{50}$ may be used for infecting/exposing the Hu-SCID mice with/to HIV. Also, the HIV strains used for this purpose are not limited to a specific strain. Rather, all known HIV strains including HIV-1 strains and HIV-2 strains may be used as long as it is made sure that unprotected immunocompetent cells/mice (i.e. cells/mice not vaccinated with the HIV-vaccine to be tested) will be successfully infected with HIV. Preferably the HIV strain is an HIV-1 strain. More preferably the HIV strain is selected from the group consisting of U455, 9H/IIIB, and PokA-79.

In particular, an infectious dose of $5x10^2$ - 10^4 $TCID_{50}$ or higher for challenging of one Hu-

SCID animal may be used. Preferably, infectious doses of at least 10^4 $TCID_{50}$, more preferably of at least 10^5 $TCID_{50}$, even more preferred of at least 10^6 $TCID_{50}$ are used. Preferably, infectious doses of up to 10^7 $TCID_{50}$ are used.

"HIV laboratory strain playback" according to the present invention is understood as an HIV laboratory strain obtained by culturing a HIV laboratory strain in PBMC cells of a healthy donor for several weeks passages, in particular for at least about 2 weeks passages, and up to about 10 weeks passages.

In a preferred embodiment, at least one HIV laboratory strain playback is used to infect and/or expose the Hu-SCID mice with/to HIV.

As an alternative to challenging Hu-SCID mice with an amount of HIV strain infectious doses in cell culture, simultaneous challenging of Hu-SCID mice with a mixture of infected and uninfected cells may be performed. Such a mixture of infected and uninfected cells may comprise from about $1x10^4$ to $10x10^8$, preferably from about $1x10^5$ to $1x10^8$ and most preferably about $10x10^6$ of pure (uninfected) cells and from about $1x10^4$ to $10x10^7$, preferably from about $1x10^6$ to $1x10^7$ and most preferably about $5x10^6$ cells infected with an HIV strain. In a preferred embodiment SCID mice are challenged with e.g. $5\text{-}10x10^6$ MT-4 or U937 pure culture cells and with $5x10^2\text{-}10^6$ $TCID_{50}$ of HIV-1 laboratory strain infectious doses in cell culture. Preferably the mice are challenged with $5x10^3\text{-}10^4$ $TCID_{50}$ of HIV-1 laboratory strain infectious doses in MT-4 culture, or with $5x10^2$ - $5x10^3$ $TCID_{50}$ infectious doses in U937 culture.

The challenges with HIV may be repeated for a number of times, e.g. for 2-5 times, preferably for 2 to 3 times with different time periods in between. A 3 day period between challenging is preferred for the achievement of the high viral load numbers in a lymphocyte background culture and a 7 day period is preferred for a high viral load in a macrophage background culture.

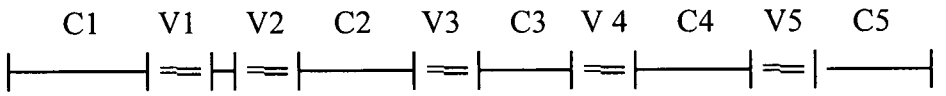
For measuring the efficacy of a vaccine, all methods generally known in the art for that purpose may be used. In particular, the efficacy of the HIV vaccine may be determined by determining the viral load in a body fluid sample of the mouse. Said body fluid sample may be blood serum.

For determining the viral load every method known in the art can be used. This includes Real Time PCR, RT-PCR and ELISA.

The figures, scientific work and examples provided herein are intended to illustrate, but not to limit the invention.

1. HIV envelop proteins variability modeling analysis.

In the very beginning of the current immunogenic composition development we tried to estimate a number of possible variations in main envelop protein gp120 for cohorts of patients applying new mathematics modeling based on differential analysis and molecular biochemistry parameters [23, 24]. The length of gp120 nucleotide sequence is variable and depends on deletions and insertions presence, their number and size. There is a scheme of gp120 mapping, where C1-C5 – conservative regions; V1-V5 – variable loops or variable regions.



According to formula: $\ln N = \sum_{i,j} \alpha_{i,j} \ln C_{n_i}^{j}$,

where i — is number of variable nucleotide positions in env gp 120 sequence, n_i — number of matching sites in each of i variable regions (in our case i=5, as variable regions of gp120 are V_1, V_2, V_3, V_4, V_5) , j — number of particles, that can be placed. In our case there are four particles — AT, GC and TA, CG.

According to experimental data total number of possible positions, in which nucleotides (A,T,G,C) can be allocated, is $N_{box} = N_{var} + N_{del}$. Number of components — nucleotides, that will be allocated in boxes is $N_p = 4$.

We can estimate variations number by calculating number of combinations by which can be realized states of these $N_p = 4$ components in total number of boxes N_{box}:

$$C_{N_{box}}^{Np} = \frac{(N_{box})!}{(N_{box} - N_p)!(N_p)!} \quad [23]$$

Length of gp120 sequences used in our alignments is 1443 bp.

Alignment 1 – HIV-1 subtype A infected 30 individuals from Former Soviet Union territories and Africa. Nucleotide substitution positions $N_{var} = 546$, there are deletions in 10 positions.

Constant positions 887. Total number of positions = 1443. For calculation of possible variants number we must summarize number of nucleotide substitution positions and number of deletions. Altogether: $N_{box} = 546 + 10 = 556$.

$$C_{556}^{4} = \frac{556!}{552! \bullet 4!} = 3939049415 \approx 3{,}9 \cdot 10^{9}$$

Alignment 2 – HIV-1 subtype A infected 10 individuals from Former Soviet Union territories. Nucleotide substitution positions $N_{var} = 331$, there are deletions in 30 positions. Constant positions 1082. Total number of positions = 1443. For calculation of possible variants number we must summarize number of nucleotide substitution positions and number of deletions. Altogether: $N_{box} = 331 + 30 = 361$.

$$C_{361}^{4} = \frac{361!}{357! \bullet 4!} = 695946630 \approx 6{,}9 \cdot 10^{8}$$

Researchers tried to overcome such genetic diversity for many years fishing out, cloning and reproducing fragments of the most conservative proteins or their domains. However it occurred that internal viral peptides like nef, pol and gag have no connection with virion's infectious activity and immunogenicity against it, and envelop proteins conservative fragments are small enough and are always covered with "loops" parts from outside of invading new host cells virions. These "loops" envelop peptides fragments were not only highly variable but extremely quickly mutation-altering regions at least up to now, until sequence information was obtained from standard infected individual's lymphocyte's DNA sequencing.

It is known from published and our practical experimental data that HIV infectious dose (TCID) and HIV viral load (virus titre or a number of RNA copies per 1 ml of body fluid sample) are very much different numbers. Precisely, TCID value is the thousands times lower number than the viral load. We believe this difference represents the real percentage of infectiously active virions that matches to a number of CD4, CCR5 and CXCR4 receptors variations available for virus recognition and cell entrance in a single human organism subjected to HIV infection danger. An eukaryotic genome is very conservative and stable characteristics. The whole HIV great genetic variability calculated above cannot be repeated in any human genome for CD4 and two more receptors including the number of these proteins isoforms. Therefore the number of HIV infectiously active variants with certain env sequences is limited by the number of tissue-specific variations of CD4 receptor and co-receptors. The identification of these viral env variants is the answer for question about preventive HIV vaccine creation.

2. Viral Envelop Proteins Material Collection with Reverse Panning and Native Human Antibodies Libraries Affine Separation Techniques

We suppose mass spectrometry is the best possible method which can provide quantitative and sequence analysis of HIV envelop proteins for the antiviral vaccine development. We suppose the method was never applied for gp120 and gp160 study due to inability to collect any sufficient biomaterial amount for envelop proteins identification. We have used two stages of envelop material collection from laboratory strains culture media, patients isolates and from laboratory strains put back into "isolates" conditions for cultivation during at least 5 weeks:

1. Virus ultracentrifugation concentration;
2. Reverse panning and native human antibodies libraries affine separation.

2.1. Virus ultracentrifugation concentration

Three methods of virus ultracentrifugation concentration were used. According to the first method, HIV proteins were harvested from supernatants by PEG precipitation, resuspended in TNE buffer (0.01 M Tris-HCl pH 7.2, 0.1M NaCl, and 1 mMEDTA in Milli-Q water), and clarified by centrifugation for 30 min at 4,000 rpm. About half of total viral sample volume in TNE buffer was stratified over 20% sucrose solution pored to the bottom of ultracentrifuge tubes and ultraspinned at 160000g during 45 minutes through sucrose gradient with density 1,16-1,18 g/sm^3. The virus-containing pellets were pooled, diluted approximately 1:3 with TNE buffer.

According to the second method, supernatant was run spinning at 1000g for 15 minutes, then the obtained supernatant is run spinning at 16000g for next 15 minutes, then stratified over 20% sucrose solution pored to the bottom of ultracentrifuge tubes and ultraspinned at 160000g during 45 minutes through sucrose gradient with density 1,16-1,18 g/sm^3. The pellet is dissolved in small volume of culture media.

The third ultrafiltration method is reasonable for not loosing surface envelop glycoproteins in sucrose gradient and keep them without any denaturating effect. Supernatants were run through ultrafiltration with 30 kDa membrane (Pelicon device Biomax) at 4 C for 2 hours. Filtered hydrolyzed protein mixture was lyophilized. Viral RNA concentration in collected pellets was measured by Real time PCR using Amplisense HIV-monitor-FRT (Amplisense) on Rotor-Gene 6000 thermal cycler (Corbett Research). Protein concentration in the virus-containing fractions was determined by BCA assay. Protein material pellets were stored at -80°C after dilution and measurements.

The first and the third approaches – PEG precipitation and ultrafiltration - previous to ultracentrifugation and PAAG protein bands separation showed reasonable results in further mass spectrometry analysis.

2.2. Reverse panning libraries affine separation

The combination of reverse panning technique described in [7] and native antibodies libraries affine chromatography was used for HIV envelop proteins further concentration.

For phagemid library presenting recombinant phage production M13KO7 helper phage is added to overnight TG1 E. coli culture for 1 hour pre-incubation and 12 hours incubation in presence of 100 μg/ml ampicillin and 50 μg/ml kanamycin at 37 ^{0}C (the typical phage yield is 10^{10} to 10^{11} ampicillin-transducing units per ml). The culture is spinned at 1000g for 10 min., supernatant was collected and cooled. Then 1/5 v/v of PEG8000/NaCl (20%PEG/ 2,5MNaCl) solution is added to supernatant and incubated 1 hour at ice, then precipitation performed with spinning 10000g at 4^{0}C for 20 minutes. The pellet is dissolved in LB or 10mM TrisHCl pH 8.0 and filtered through 0.45μm. Recombinant phage can be stored at 4^{0}C overnight when 0.01% timerosal is added.

Phage presented mAb libraries were immobilized on streptavidine-coated ferroxide magnetic nanoparticles. Particles were washed with sterile water and equilibrated with 0.1M carbonate buffer, pH 9.5 at a flow rate of 1 ml/min. M13 phages in concentration $2.5*10^{10}$ in 10 ml 0.1M carbonate buffer pH 9.5 were immobilized on streptavidine by re-circulating phage coupling solution for 16 h at a flow rate of 1 ml/min at 4 ◦C using peristaltic pump. After coupling the phages particles were washed with 0.1M carbonate buffer pH 9.5 until all non-bound phages were washed away. The remaining active streptavidine groups were blocked by re-circulation of 25 ml 0.1M ethanolamine in 0.1M carbonate buffer pH 9.5 for 3 h at a flow rate of 1 ml/min . The suspension was washed with sterile water and phosphate-buffered saline (PBS) and stored at 4 ◦C until further use. 8. To estimate immobilization yield, 100μl of suspension was sonicated and total protein concentration was determined by micro BCA assay. To converse protein concentration into phage concentration, calibration with control phage particles was performed.

HIV-1 peptides mixture was proceeded through reverse panning with current technique:

HIV-1 peptides mixture hydrolyzed in 0.05 M Tris-HCl, pH 8.0 buffer was pored into 50 ml glass tubes together with phage-embedded streptavidine particles and stirred for 2 hours in orbital shaker with speed 120r/min. Then the suspension was diluted with 5 volumes of the same buffer. AC magnetic field conductor was applied to collect the particles. After supernatant was discarded HIV peptides-bound phage was eluted with 0.1M glycine pH 2.2 gradient. Obtained fractions were incubated in glycine elution buffer with presence 0.001M PMSF for 5 hours at RT until phage-antigen complexes are re-adjusted completely.

2.3. Native human antibodies libraries column preparation and pool of HIV envelop proteins capturing.

Native human antibodies libraries were extracted from a cohort of HIV-1 infected individuals blood serum with ammonium sulfate precipitation method and then and Protein G affinity chromatography. For that 150-200 of patients blood serum was spinned for 20- 30 min at 10000 g at 4°C, the pellet was discarded. The supernatant was cooled to 4°C and stirred slowly in presence of solid ammonium sulfate added to saturation (31.5g ammonium sulfate per 100 ml of serum = 50% saturation at 25°C). The sediment was clarified at 4000g for 15min at 4°C. Supernatant was discarded and the precipitate was dissolved in 20% of the original volume in PBS. Then supernatant in 20mM PBS pH 7.4 is pored into 1 ml HiTrap protein G column with highly cross-linked 6% agarose from 20 ml syringe. The elution of Ab library from protein G is carried out in 0.1M glycine buffer with pH 2.8 at a flow rate of 0.5ml\min. The antibody purity 99% was determined by SDS-PAGE.

Polyclonal antibodies were dialized against coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3 overnight at 4°C. The protein mixture (0.5ml, c=20 mg\ml) was added to 1 ml of NHS-activated Sepharose 4 Fast Flow, previously washed with 10–15 medium volumes of cold 1 mM HCI immediate before use, and incubated overnight at 4°C. After the coupling is completed, any non-reacted groups on the medium should be blocked. Thus, the medium can be kept in 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 or 0.1 M Tris-HCl, pH 8.5 for a few hours. To wash the medium after coupling, use a method which alternates two different buffers (high and low pH respectively). Buffers used are 0.1 M Tris-HCl buffer pH 8–9 and 0.1 M acetate buffer, 0.5 M NaCl pH 4–5. A suitable procedure could be 3 x 1 medium volumes Tris buffer followed by 3 x 1 medium volumes acetate buffer. This cycle is repeated 3–6 times. To prevent microbial contamination antibodies library column can be stored in 20% ethanol.

For capturing the pool of HIV envelop proteins the virus-containing ultracentrifugation precipitate was applied to Ab library column, equilibrated with PBS. One milliliter virus containing sample was diluted up to 10 ml with binding buffer (PBS) and applied to the column at a flow rate of 0.5 ml/min, by recirculation during 2 hours (all buffers were filtered through a 0.22um). Gradient elution of targeted protein with increased sodium chloride concentration was applied. Two peaks were collected: one – at 23% sodium chloride (0.3M NaCl, major fraction) and second - at 50% sodium chloride (0,6M NaCl, minor fraction). Fractions of 1ml were collected. The column was re-equilibrated with PBS buffer. Fractions were assayed by measuring absorbance at 214 nm and by Phage Western blot for the detection of viral peptides as it was shown in [7].

The properly glycosylated gp120 molecule consists of 60 kDa polypeptide core (Example 4), and extensive carbohydrate by N-linked glycosylation increases the apparent molecular

weight to 90-110 kDa in average and up to 120 kDa. This difference is due to five hyper-variable domains (loops) that contain extensive amino acid substitutions, insertions and deletions for up to 25% overall molecular weight variability. Gp160 polypetide core is 95-100 kDa (Example 4), and glycosylation increases the molecular weight to 120-160 kDa.

Samples of collected HIV envelop proteins pools were run through reparative SDS-PAGE (Fig. 1), bands of different molecular weight were cut and applied for trypsinolysis.

3. HIV envelop proteins variability quantitative mass spectrometry analysis

3.1. Triptic Hydrolysis of Polyacrylamide Gel Protein Stripes with Trypsin

Mass spectrometry identification of HIV envelop proteins, especially gp120 in its variability requires 1) the sufficient amount of protein material provided in reverse panning technique cycles; 2) the precise envelop sequence mapping for search and identification of env triptic peptides in samples.

For triptic hydrolysis protein stripes of interest masses from 1D-SDS-PAGE gels stained with Coumassi (Fig. 1A) were cut out and incubated in deionized water at room temperature 15 min, supernatant was discarded. 50-100 μl acetonitryl were added to rinsed gel strips and incubated 20 min at room temperature, acetonitryl excess was evaporated with passive drying. 10 μg/ml modified trypsin (Promega) solution in 0.05M NH_4HCO_3 was added to dehydrated gel stripes and incubated on ice at 4°C for 60 minutes. Trypsinolysis reaction was run overnight at 37 °C. Tryptic peptides extraction from gel was made at room temperature in presence of 0.5% three-fluoric acetic acid in water. Extraction supernatant was used for the following mass spectrometry analysis.

3.2. One-Dimensional Liquid Chromatography-Mass Spectrometry (LC-MS-MS Analysis)

The tryptic peptides from 1D-SDS-PAGE gels were analyzed with ion electrospray quadrupole mass analyzer trap method using HCT-Ultra instrument (Agilent Technologies, USA, cat. # G4240-62001). HCT-Ultra instrument is supplied with integrated liquid chromatography system Agilent Chip Cube tm (Agilent Technologies, USA) consisting of a preliminary enrichment and deionization column (C18), an analytic column (C18), a nanospray needle and quadrupole ion electrospray mass analyzer trap. This instrument is joined to nanochromatography system and sample's fractions collector Agilent 1200 in on line manner.

The mixture of triptic peptides (8 µl in every sample) was delivered into the column for superconcentration and deionization (5 µm Zorbax 300 SB-C18 precolumn, 40 nl, Agilent Technologies) using capillary high pressure pump with stream speed 3 µl/min. in 3% acetonitryl - 0.1% formic acid buffer. Chromatography peptides separation in system described above was run using capillary high pressure pump with stream speed 0.3 µl/min. (before splitter) in column with diameter 75µm x 50µm and length 43mm, the real elution speed achieved 200 nl/min.

For separation of desalinated triptic peptides the gradient of buffer A (5% acetonitryl, 0.1% formic acid) and buffer B (90% acetonitryl, 0.1% formic acid) was run according to the following program:

Time, minutes	Buffer B concentration, %
2	0
5	15
20	20
55	50
60	90
65	90
66	0

Peptides masses measurements were taken in the quadrupole ion electrospray mass analyzer trap in diapason 300-2200 m/z with trap mass optimization equal to 900; positively charged ions were detected. Ions- predecedors accumulation time in ion trap was 150 ms, the average number of ions in trap (ICC parameter) was 500000, the number of mass-spectrum average was equal 2. Peak's selection for further MS/MS analysis was done in DDA regime, 3 ions-predecedors with maximum intensity were selected and then defragmented during helium co-hits CID regime. Ions with charge number equal 2 or higher and intensity higher than the threshold are taken for tandem experiments. The obtained mass-prints were sent into MASCOT search system.

Peptide's identification confirmation was verified with two software complexes Scaffold 01-07-00 (http://www.proteomesoftware.com) and PEAKS (Canada). The database protein's identification search was run through Los Alamos and NCBI proteomics databases, Peptides with identification expectancy more than 50% were listed in the final schedule. Some results of Scaffold identification are presented in Example 1. However only results obtained with PEAKS analysis (Example 2) allowed quantity-comprehensive HIV envelop triptic peptides identification and gp160 mapping for recombinant cocktail tailoring. PEAKS proteomics

makes possible loops V1-V5 ends sequence probability mapping for "playback" HIV strains and can serve the same for HIV isolates analysis.
It is worth to mention that LC-MS-MS mass spectrometry method could not identify HIV gp120 and gp41 triptic peptides in samples which were prepared from the stage of ultracentrifugation virus concentration and without further reverse panning and/or Ab libraries HIV peptides/proteins collection stage.

4. Immunogenic cocktail composition tailoring.

4.1. HIV envelop mapping and original PCR products size analysis.

The same sequence mapping is the basic description of recombinant peptides/proteins cocktail composition being used for immunization of laboratory animals before challenging. There are envelop mapping and sequence analysis calculations that were optional for these two stages of vaccine producing such as 1) gp120 major variants MS-MS identification and 2) recombinant glycosylated env proteins immunogenic composition's production.

gp120 start — V1 loop end about 393 bp
gp120 start — V2 loop end about 534 bp
gp120 start — V3 loop end about 940 bp
gp120 start — V4 loop end about 1193 bp
gp120 start — V5 loop end about 1342 bp
V1 loop start — gp120 end about 1184 bp
V1 loop start — gp41 end about 2240 bp
V2 loop start — gp120 end about 1096 bp
V2 loop start — gp41 end about 2152 bp
V3 loop start — gp120 end about 679 bp
V3 loop start — gp41 end about 1735 bp
V4 loop start — gp120 end about 416 bp
V4 loop start — gp41 end about 1472 bp
V5 loop start — gp120 end about 202 bp
V5 loop start — gp41 end about 1258 bp

The scheme of constant and variable regions in HIV-1 gp120 and primers arrangement is shown in Figure 26.

For gp120 aminoacid sequence length's possible variations the situation is as follows:
Mechanic calculation:

The minimal length of loops V1-V5 sequences is 279 bp or 93 aa
The maximal length of loops V1-V5 sequences is 711 bp or 237 aa
Constant fragments sum length is 1008 bp or 336 aa
Then the **whole gp120** sequence length is vary **from 1287 to 1719 bp**
or from **429 to 573 aa**

gp41 sequence length does not vary much: **996 – 1068 bp or 332 – 356 aa.** In average it is 1056 bp (352 aa) for A,C,G and 1035 bp (345 aa) for subtype B.
Then with average gp41 length 1032 bp the total **gp160** sequence for subtype A is from **2319 bp to 2751 bp** or from **773 aa to 917 aa.**

However, mechanic calculations are very much not precise in terms of gp120 sequence and its length analysis due to the evolution selection of real existing protein isoforms.
First, one cannot find only short or only long loops in one gp120 sequence, therefore real minimal and maximal lengths differences are two times smaller.
Second, constant fragments are also variable in length, though it is not that significantly and not in every sequence. Insertions and deletions appear in constant fragments in random regime:

Const 1 length is from 294 to 339 bp (309 in average)
Const 2 length is from 261 to 303 bp (297 in average)
Const 3 length is from 153 to 186 bp (159 in average)
Const 4 length is from 120 to 126 bp (123 in average)
Const 1 length is from 105 to 141 bp (120 in average)

In order to avoid such mistakes in protein molecular weight analysis we suggest it is reasonable to use an alignment calculation. Gaps are mechanically removed from sequences in alignment in Clustal X. Then the software calculates a number of bp in every sequence, and range between them becomes obvious.
Alignment calculation:
The whole gp120 sequence is vary from **1310 to 1595 bp,** or **437 – 532 aa;**
In sequence alignment **gp41** sequence length is vary between 1035-1068 bp normally, in average it is **1051 bp or 350 aa** for all HIV-1 subtypes;
The whole gp160 sequence therefore is vary from **2361 to 2646 bp,** or **787 – 882 aa.**

For peptides and proteins being synthesized in Leishmania tarentolae and further being excreted outside cells into the growth media the signal peptide of L.mexicana 15 bp or 5 aminoacids is added to C-end of protein (this peptide is responsible for transitory expression in growth media). 18 bp 6-His fragment is added to N-terminus for possibility to extract

recombinant proteins from growth media using ion-exchange liquid chromatography. So, final recombinant products of HIV env proteins are elongated for 33 bp.

The whole gp120 sequence is vary from **1343 to 1628 bp,** or **448 – 543 aa;**

The whole gp160 sequence therefore is vary from **2394 to 2769 bp,** or **798 – 923 aa.**

For ***translating primers into aminoacids sequence*** standard triplet code is used; aminoacids primer sets can be found easily in presented in Example 3 sequences.

The following primers may be used according to the invention:

V1 forward for subtypes A,G B,C,F1,H

DNA 5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1)

Protein L C V T L X X X (SEQ ID No. 2)

(X – unknown)

V2 forward for all subtypes:

DNA 5'- AA AAC TGC TCT TWC AXX XXX XXX X- 3' (SEQ ID No. 3)

Protein N C S Y/F X X X (SEQ ID No. 4)

(W = A or T)

V3 forward for A, B, G subtypes:

DNA 5'- TA VAA ATT AAT TGT AXX XXX XXX XXX- 3' (SEQ ID No. 5)

Protein K/Q/E I N C X X X X (SEQ ID No. 6)

V3 forward for subtype D:

DNA 5'- TA VCA ATT AAT TGC AXX XXX XXX XXX- 3' (SEQ ID No. 7)

Protein T/P/A I N C X X X X (SEQ ID No. 8)

(V = A or C or G)

V4 forward for all subtypes:

DNA 5'- GAA TTT TTC TAT TGY AAX XXX XXX XX- 3' (SEQ ID No. 9)

Protein E F F Y C X X X (SEQ ID No. 10)

(Y = C or T)

V5 forward

For A,B,D,G subtypes:

DNA 5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11)

Protein T R D G X X X (SEQ ID No. 12)

V5 forward

For subtype C:

DNA 5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13)

Protein T R D G X X X (SEQ ID No. 14)

V5 reverse for all subtypes:

5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3' (SEQ ID No. 15)

Complementary DNA strand

3'- AGG AGG ASG TCC AGA CTT XXX XXX XXX X- 5' (SEQ ID No. 16)

(S = G or C)

V5 reverse complement strand

DNA 5'-X XXX XXX XXX TTC AGA CCT GSA GGA GGA- 3' (SEQ ID No. 17)

Protein X X X F R P G/A G G (SEQ ID No. 18)

V4 reverse for subtype G:

5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3' (SEQ ID No. 19)

(Y = C or T)

V4 reverse for subtypes A,B,C,D:

5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No. 20)

V4 reverse comlement strand for subtype G:

DNA 5'- XXX XXX XXX TGT ARR ATA AAA CAA ATT G- 3' (SEQ ID No. 21)

Protein X X X C K/R I K Q I (SEQ ID No. 22)

(R = A or G)

V4 reverse comlement strand for subtypes A,B,C,D:

DNA 5'- XXX XXX XXX TGC ARA ATA AAR CAA ATT A (SEQ ID No. 23)

Protein X X X C K/R I K Q I (SEQ ID No. 24)

V1 reverse for all subtypes:

5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25)

V1 reverse complement strand for all subtypes:

DNA 5'- XX XXX XXX XRA GAA ATR AAA AAC TGC- 3' (SEQ ID No. 26)

Protein X X X E I K N C (SEQ ID No. 27)

V2 reverse for subtypes A,D,C:

5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28)

For subtype B:

5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29)

For G subtype:

5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30)

For subtype F1:

5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31)

V2 reverse complement strand for subtypes A,D,C:

DNA 5'- XXX XXX XXA ATA AAT TGY AAT ACC T- 3' (SEQ ID No. 32)

Protein X X X I N C N T (SEQ ID No. 33)

V2 reverse complement strand for subtype B:

DNA 5'- X XXX XXX XXT TGT AAY ACC TCA G- 3' (SEQ ID No. 34)

Protein X X X C N T S (SEQ ID No. 35)

V2 reverse complement strand for G subtype:

DNA 5'- XXX XXX XXA ATA AAT TGT AAT GTC T- 3' (SEQ ID No. 36)

Protein X X X I N C N V (SEQ ID No. 37)

V2 reverse complement strand for subtype F1:

DNA 5'- X XXX XXX XXT TGY AAT ACC TCA A- 3' (SEQ ID No. 38)

Protein X X X C N T S (SEQ ID No. 39)

V3 reverse

For subtypes A, D, (C):

5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40)

For subtype B:

5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41)

V3 reverse complement strand for subtypes A, D, (C):

DNA 5'-XXX XXX XXX TGG AAT VAA ACT TT (SEQ ID No. 42)

Protein X X X W N K/Q/E T (SEQ ID No. 43)

V3 reverse complement strand for subtype B:

DNA 5'-XXX XXX XXX TGG AAY AAC ACT TT (SEQ ID No. 44)

Protein X X X W N N T (SEQ ID No. 45)

gp41(160) Reverse for A,B,D subtypes *Not*I restriction site is marked light grey, 6His tag is marked dark grey)

5' - ATA **GCG GCC GCC TAG TGG TGG TGA TGG TGG TG**T AG YAA AGC YCT TTC NAA GCC CTG TC -3' (SEQ ID No. 46)

gp41(160) Reverse complement strand for A,B,D subtypes

5' - GA CAG GGC TTN GAA AGR GCT TTR CTA CAC CAC CAT CAC CAC CAC TAG GCG GCC GC TAT (SEQ ID No. 47)

Protein Q G X E R A L L H H H H H H stop (SEQ ID No. 48)

gp41(160) Reverse for subtype A (rare variant)

ATA **GCG GCC GCC TAG TGG TGG TGA TGG TGG TG**T AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66)

gp41(160) Reverse for C subtype

ATA **GCG GCC GCC TAG TGG TGG TGA TGG TGG TG**T WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67)

gp41(160) Reverse for G subtype

ATA **GCG GCC GCC TAG TGG TGG TGA TGG TGG TG**T AGC AAA GCY CTT TCN AAG CCT TGT C ((SEQ ID No. 68)

gp120 Reverse Const5 for all subtypes (*Not*I restriction site is marked light grey, 6His tag is marked dark grey)

5'- ATA **GCG GCC GCC TAG TGG TGG TGA TGG TGG TG**T CTT TTT TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 49)

gp120 Reverse complement strand Const5 for all subtypes

5'AGR AGA GTG GTG SAR AGA GAA AAA AGA CAC CAC CAT CAC CAC CAC TAG GCG GCC GCT AT (SEQ ID No. 50)

R R V V E/Q R E K R H H H H H H stop (SEQ ID No. 51)

Forward gp120 Const1 for A subtype with *Xba*I site on 5' end (marked grey)

DNA 5'- A ATT **CTA GA**C RCT RCA GAA AAC TTG TGG GTY AC - 3' (SEQ ID No. 52)

Protein L D T/A T/A E N L W V LDXXENLWV (SEQ ID No. 53)

Forward gp120 Const1 for B subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3' (SEQ ID No. 69)

Forward gp120 Const1 for C subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3' (SEQ ID No. 70)

Forward gp120 Const1 for G subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3' (SEQ ID No. 71)

In a preferred embodiment, following primers are used:

(i) Forward gp120 Const1 for A subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CRC TRC AGA AAA CTT GTG GGT YAC - 3' (SEQ ID No. 52), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end

(ii) Forward gp120 Const1 for B subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3'

(SEQ ID No. 69), , or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end

(iii) Forward gp120 Const1 for C subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3'

(SEQ ID No. 70), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iv) Forward gp120 Const1 for G subtype (with *Xba*I site on 5' end)

5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3'

(SEQ ID No. 71), , or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(v) V1 reverse for all subtypes:

5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25)

(vi) V2 reverse for subtypes A,D,C:

5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28)

(viii) V2 reverse for subtype B:

5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29)

(ix) V2 reverse for subtype G:

5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30)

(x) V2 reverse for subtype F1:

5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31)

(xi) V3 reverse for subtypes A, D, (C):

5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40

(xii) V3 reverse for subtype B:

5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41)

(xiii) V4 reverse for subtype G:

5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3' (SEQ ID No. 19)

(xiv) V4 reverse for subtypes A,B,C,D:
5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No. 20)

(xv) V5 reverse for all subtypes:
5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3', (SEQ ID No. 15) and

(xvi) gp41(160) Reverse for A,B,D subtypes, including sequence encoding His-Tag:

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46)

(xvii) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(xviii) gp41(160) Reverse for subtype A (rare variant)

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66)

(xix) gp41(160) Reverse for C subtype

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67), and

(xx) gp41(160) Reverse for G subtype

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68).

In a further preferred embodiment, following primers are used:

(i) V1 forward for subtypes A,G B,C,F1,H:

5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1)

(ii) V2 forward for all subtypes:
5'- AAA ACT GCT CTT WCA XXX XXX XXX- 3' (SEQ ID No. 3)

(iii) V3 forward for A, B, G subtypes:
5'- TAV AAA TTA ATT GTA XXX XXX XXX X- 3' (SEQ ID No. 5)

(iv) V3 forward For subtype D:
5'- TAV CAA TTA ATT GCA XXX XXX XXX X- 3' (SEQ ID No. 7)

(v) V4 forward for all subtypes:
5'- GAA TTT TTC TAT TGY AAXXX XXX XXX- 3' (SEQ ID No. 9)

(vi) V5 forward
For A,B,D,G subtypes:
5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11)

(vii) V5 forward For subtype C:
5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13)

(viii) gp41(160) Reverse for A,B,D subtypes, including sequence encoding His-Tag:

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46)

(ix) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(x) gp41(160) Reverse for subtype A (rare variant)

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66)

(xi) gp41(160) Reverse for C subtype

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67)

(xii) gp41(160) Reverse for G subtype

ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68)

(xiii) gp120 Reverse Const5 for all subtypes, including sequence encoding His-Tag

5'- ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT CTT TTT TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 52),

and

(xiiii) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag.

The complete HIV envelop proteins mappings for primers design are presented in the Example 3.

4.2. Glycosylated envelop proteins pool expression and separation.

An inducible expression of HIV envelop proteins from amplified genetic material is carrying out in Leishmania tarentolae system as it was previously described in [7] with the following improvements. The cloning of mapped amplified genes, transformation and further antibiotics selection, is being run as a pool cloning, i.e. vectors with amplified genes of envelop proteins from biomaterial with approximately equal lengths and variable sequences are used as a mixture for transformation with elctroporation method. Cuvette electro squid impulse instrument BTX-830 with HV module (Harvard Instruments), 450V twice in 3 milliseconds is being run for the task. Transformed L. tarentolae variants are exposed to G418 selection in liquid media in horizontal positioned flasks at 26°C and light stirring. Monoclones are not being selected on solid media not to lose their natural variability of envelop proteins.

Large scale tetracycline-inducible expression is carried out into cultivation media. It is run in hollofiber system in order to provide the possibility for eukaryotic proteins effective isolation in reasonable quantities from the media freed from large concentrations of proteins and

peptides, animal-originated proteins as well.

4.3. Glycosylated recombinant proteins molecular weight calculations

For aminoacid's sequence molecular weight determination the Free Protein and Peptide's Molecular Weight Calculator software was used: http://www.encorbio.com/protocols/Prot-MW.htm. Several examples are presented below and in Example 4 calculations.

160 #1 (B.FR.83.HXB2_LAI_IIIB_BRU_K034) There are 856 Amino acids, and MW is 97241.93 Daltons, or 97.24 KiloDaltons; Charge is 30 assuming N and C termini are not blocked
120 #1 There are 511 Amino acids, and MW is 57695.64 Daltons, or 57.70 KiloDaltons
41#1 There are 345 Amino acids, and MW is 39564.30 Daltons, or 39.56 KiloDaltons

160 #43(K.CM.96.MP535_AJ249239): There are 842 Amino acids, and MW is 95252.58 Daltons, or 95.25 KiloDaltons, Charge is 22 assuming N and C termini are not blocked.
120 #43: There are 497 Amino acids, and MW is 55884.46 Daltons, or 55.88 KiloDaltons
41 #43: There are 345 Amino acids, and MW is 39386.14 Daltons, or 39.39 KiloDaltons

160 #6 (our A1.RU.03.03RU20_06_13_AY500393) There are 805 Amino acids, and MW is 90669.32 Daltons, or 90.67 KiloDaltons: Charge is 24 assuming N and C termini are not blocked
120 #6 There are 493 Amino acids, and MW is 55328.29 Daltons, or 55.33 KiloDaltons
41#1 There are 346 Amino acids, and MW is 39333.71 Daltons, or 39.33 KiloDaltons

160 #26 (C.ZA.01.01ZATM45_AY228557) There are 878 Amino acids, and MW is 99122.18 Daltons, or 99.12 KiloDaltons; Charge is 15.5 assuming N and C termini are not blocked
120 #26 (C.ZA.01.01ZATM45_AY228557) There are 526 Amino acids, and MW is 59182.32 Daltons, or 59.18 KiloDaltons
41 #26 (C.ZA.01.01ZATM45_AY228557) There are 352 Amino acids, and MW is 39957.88 Daltons, or 39.96 KiloDaltons

5. Immunogenic composition efficiency analysis

5.1. Techniques for immunocompetent PBMC, DC and cell cultures obtaining for background SCID mice engraftment

Techniques for engrafting SCID mice with human immunocompetent cells are known in the art. Normally the immunocompetent cells are selected from bloodstream-originated group of PBMC, dendrite cells, B-lymphocytes and T-lymphocytes. However it occurred that non of known and described methods are able to provide Hu-SCID mice for HIV vaccine efficiency testing. As discussed above, for the evaluation of an HIV vaccine infection-preventive potential the Hu-SCID-mouse should be able to show two reactions. First, mouse should emit a human-type HIV-specific immune response after being inoculated with the HIV vaccine. Second, the same animal without vaccination should be able to catch and to develop HI-virus challenging in means of viral load detectable in its bloodstream exactly like HIV infection presence is being determined in humans.

Maintaining and experimental work with immune deficient animals are complicated with a number of special conditions and restrictions, however these expensive in vivo systems are worth the troubles they require for the survivorship. Thus, when new medicines are being tested for toxicity in vivo, SCID mice are known to deliver dosage regimes the most close to human's, while normal laboratory mice, rats and dogs ID_{50} are 10-2 times higher and nude mice – 2-5 times lower than human ones. However to achieve informative results the researchers have to consider this number of conditions and adopt them for animals handling to avoid obtaining some false-positive data. The first little example of this approach is Fig. 2 presenting how SCID's immune status was checked before experiments with vaccines. SCID and BalbC mice were immunized with concentrated in 20% sucrose gradient ultracentrifugation U455 proteins mixture and ELISA results were compared with BalbC mice immune response. One of six SCIDs accidentally provided low immunity this time (Fig. 2). In case SCID mice show some response for immunization (and it might happen for some percent of baby mice to be born with it) it is necessary to remove these animals from experiment assessment because viral infection in these mice might be blocked with this even low immunity and not with vaccination. For reproducing animals without such defect 3-5 cycles of brother-sister inbreeding is recommended.

For measuring the efficacy of a vaccine, all methods generally known in the art for that purpose may be used. In particular, the efficacy of the HIV vaccine may be determined by determining the viral load in a body fluid sample of the mouse. Said body fluid sample may be blood serum.

For determining the viral load in animal's bloodstream every method known in the art can be used. This includes Real Time PCR, RT-PCR and p24 ELISA.

Our two-years long in vivo experiments with Hu-SCID mice proved that, contradictory to well-admitted opinion, neither fresh PBMC (PBL) along, nor dendrite cells, nor fresh patient's HIV isolates can provide HIV challenging as a viral load in animals blood serum

(see Figs. 7, 18). However PBMC (PBL) and dendrite cells introduced into SCID mice can elicit HIV immune response which strength and specifity depend on immunocompetent cells characteristics, regimes of cells engraftment and also on immunogenic composition efficient quality (Figs. 2-3). Contrariwise, T-, B-based immortalized or tumor cell cultures being used for in vitro cultivation of HIV laboratory strains are able to deliver detectable viral loads in Hu-SCID mice bloodstream (Figs. 4-6, 13, 16) but they cannot provide the HIV-specific immune response after administration of a vaccine. The task for presented research was to make these two functions available in the same Hu-SCID mouse.

Introduction of cells of human origin into the SCID mice may be achieved by using any conventional route, such as via the intraperitoneal route. The intraperitoneal route is normally preferred due to the ease of administration and allowing administration of a volume of fluid. The cells may be introduced in form of a suspension in any suitable medium, allowing cell survival, such as phosphate buffered saline (PBS) or Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI) or Glasgow's Minimal Essential Medium (GMEM).

Preferably the immunocompetent cells are cultivated in vitro prior to being introduced into the SCID mice because their engraftment efficiency can be enhanced with cytokines stimulators. Preferred stimulators include but are not limited to interleukin factors such as IL-2 (interleukin-2), IL-4 (interleukin-4), IL-12 (interleukin-12), and other factors such as phitohemagglutinin, INF-γ and GCSF (granulocyte colony stimulating factor). The immune stimulators promote growth and/or development of e.g. T, B, and hematopoietic cells.

Healthy donor peripheral blood mononuclears (PBMC) are isolated by standard Ficoll gradient blood separation method with the following adjustments. Blood collected in 10 ml vacutainers in presence of EDTA and 30 ME/ml heparin is being left at room temperature for 1.5 – 2.5 hours in vertical position until serum with lymphocyte's fraction is in upper layer, and erythrocytes, platelets with fibrinous blood proteins are in lower layer. Then blood serum is collected into 50 ml sterile tubes and centrifuged at 1200g for 20-25 min. The pellet – mostly big PBMC cells - is collected and put into culture flasks for cultivation, the supernatant – human blood serum (HBS) – is stored at -20°C in 15 ml tubes for further PBMC in vitro cultivation 1-2% supplementation. The lower layer donor blood is diluted 4-5 times with DPBS (Dulbecco's modified phosphate buffer saline) and pored over equal volume of 1.077 g/ml Ficoll into 15 ml sterile tubes. 980g gradient centrifugation for 30 min. left small differentiated lymphocytes fraction and a few mononuclears in interphase. Interphase cells from the circle are collected and rinsed in RPMI-1640 twice with spinning for 10 min. at 980g.

It is possible to make SCID mice engraftment with freshly isolated PBMC. But it is more reasonable to make 3-days in vitro cultivation before. If necessary one donor PBMC can be cultivated in vitro for 2-4 weeks.

The obtained cell suspension is diluted in 15-20% FBS-HBS-supplied culture medium to $2x10^6$ cells in 1 ml and incubated in Petri dishes or 6-well culture plates at 37°C and 5% CO_2. In case cells have to be divided into lymphocytes and monocytes sub-populations cell suspension is being layered onto dishes or culture flasks plastic previously treated with human blood serum (HBS). After 1-2 hours incubation at 37°C non-adhesion fraction (lymphocytes) are transferred to a new culture dishes, and ancored monocytes (DC-producers) are supplied with new medium for cultivation. Standard scheme is used for cells proliferation activation. Mitogenic lectin – phitohemagglutinin (PHG) in sub-mitogenic concentration 1-2 μg/ml is being added during the 1st day of incubation. 24 hours later 10 μg/ml exogenous cytokine interleukine-2 is added to culture medium. This activation scheme is effective and allows diminishing activator's working concentration. PHG is getting into interaction with T-cell receptors complex and induces cell cycle G_0 - G_1 phase transfer. Cells in G1 phase cells are more sensitive to IL-2 effect.

22 collections of blood of "one donor" were carried out in 2009. 13 blood collections of "one donor" and 4 collections of blood from different donors were carried out in 2010. 4 blood collections of "one donor" were made in 2011.

Every blood samples collection from the same donor delivers from 80 to 200 million freshly isolated PBMC. The cell count for each introduction of cells of human origin may vary from $1x10^6$ to $5x10^7$ cells per introduction, preferably from $1x10^7$ to $1.5x10^7$ cells per introduction. The introduction of cells of human origin may be repeated weekly, or twice weekly, for running one experiment every Hu-SCID mouse receives from 4 to 10 immunocompetent cells introduction depending on the task of a current assay: immunization effect boost, challenging or consequetive combination of both for vaccination to prevent challenging development.

In case PBMCs are introduced into SCID mice followed by immunization these mice are referred to as "PBMC-Hu-SCID mice". In case dendrite cells (DCs) are introduced into SCID mice followed by immunization and subsequent introduction of cells of the immunogenic background such as PBMCs, these mice are referred to as "DC-PBMC-Hu-SCID mice".

The dendrite cells may have been obtained by cultivation of human PBMC in the presence of cytokines capable of inducing the formation of dendrite cells in vitro. Methods for the production of dendrite cells in vivo are known in the art (see e.g. [25]).

Thus, 3-days in vitro pre-cultivated PBMC, freshly isolated PBMC and DC can be used for SCID mice "humanization" as immune response formation background. 4-5 weeks old SCID mice were inoculated with re-suspended in PBS (phosphate buffer saline) or DMEM without phenol red (Dulbecco's modified Eagle's media) 5-15x10^6 PBMC intraperitoneally once a week two times. Two weeks later the 3rd PBMC inoculation was performed and next day mice were immunized subcutaneously with peptides mixture obtained as it was described previously in [7] and in the current application. Each animal received 25 mkg peptides in cocktail mixed in 200 μl incomplete Freund adjuvant for 1 hour at +4C. Immunizations were repeated three times after two weeks, SCID mice were provided with fresh portions of PBMC weekly. The special requirement was the supplying one donor PBMC 5-15x10^6 cells for each inoculation for one mouse (one donor's isolation was enough for a group of 5-10 animals). Immune response was tested in ELISA assays. Two weeks after the last immunization SCID mice pre-treated with PBMC weekly had U455, p120-1 and p120-2 immunity rates the intensity comparable with response of normal BalbC mice (Figs. 2-3).

The other method of immune response formation in SCID mice we used included dendrite cells cultivation and initial mice supply. Donor PBMC were isolated with described above method and cultivated in vitro for 6 weeks in presence of differentiation blocking cytokine's factors – IL-2 (interleukin-2), IL-4 (interleukin-4) and GCSF (granulocyte colony stimulating factor) until cells are multiplied in number several times. 4-5 weeks SCID mice were inoculated with re-suspended in PBS or DMEM without phenol red 15x10^6 dendrite cells. Then after two weeks animals were immunized subcutaneously with p120-1 and p120-2 peptides mixture. Each animal received 25 mkg peptides in cocktail mixed in 200 mkl incomplete Freund adjuvant for 1 hour at +4C. Immunization was repeated three times after two weeks, SCID mice were supplied with fresh portions of PBMC once in 2-3 weeks. PBMC for next inoculations were isolated from the blood of the same donor whose blood was used as a source for dendrite cells cultivation for the current group of animals used in experiment. Response for immunization was of much weak intensity than pre-treated with PBMC SCID mice showed, but it was more elongated, and it didn't disappear completely after two weeks passed since the last immunization.

The conditions important for HIV-specific short-time immune boost are summarized in Table 1. There are relatively small number of PBMC - 5-15x10^6 cells for one animal for a single inoculation (1), the presence of PBMC (dendrite) cells substrate one-two weeks before every immunization (2), the implementation of one donor material for one mouse (group of mice) preventing them from GVHD development and early death (3). It is detectable also that intensity (specifity and values) of response after immunization/vaccination is 2-4 times higher in case one donor PBMC material is being used; immune response activity is comparable to that non-immune deficient mice elicit as murine antibodies production for the

same immunization/vaccination (3). Several immunizations, preferably three, should be done during one vaccination course (4).

The quality of biomaterial used for vaccination is crucial for success of vaccinations. Immunogenic cocktails of recombinant HIV envelop peptides/proteins with sufficient not less than 20 mkg target peptide's concentrations for one animal should be used for successful vaccination (5). In case low-immunogenic and low-specific compositions such as HIV DNA vaccines are used Hu-SCID model will not provide positive results (6). It is preferable to use adjuvants for immune boost compositions in course of immunization/vaccination (Table 1). The limited period of detectable HIV-specific immune response (7-8) 3-4 weeks for PBMC and 5-6 weeks for DC-PBMC mice is due to: 1) animals were immunized subcutaneously with simple peptide's cocktails without immune boost composition; 2) small-animal models lifespan is months, not years.

The composition/cocktail for successful immunizations against HIV challenging normally contains a great number of envelop sequences from decades for HIV laboratory strains to a library of hundreds and thousands for HIV-infected patient's isolates cloned as a pool in suitable expression system vector (See "Envelop variability analysis" pp. 11-13).

Table 1: HIV-specific immune boost conditions in PBMC-SCID and DC-PBMC-SCID mice.

##	Applied Treatment (Conditions) for Hu-SCID Mice Immunity for HIV env Peptides/Proteins	Immune Response for p120 when Treatment is:	
		Applied	Not Applied
1	Modest PBMC, DC/PBMC number, 5-15x10^6 for one animal's each dose	+	-
2	Substrate (PBMC, DC) cells providing 1-2 weeks before immunizations	+	-
3	One donor PBMC material for each mouse during immunizations course	+	-
4	Three or more immunizations for the course with 2 weeks period before the next one for each PBMC-SCID, DC-PBMC-SCID mouse	+	- +
5	Vaccination/immunization with immunogenic HIV-env-specific components such as cocktails/mixtures of recombinant viral envelop peptides/proteins	+	_

6	Vaccination/immunization with HIV genes-containing viral vectors and non-viral plasmid DNA	-	-
7	Detectable env-specific immune boost time period for PBMC-Hu-SCID mice after the last immunization	2-4 weeks	-
8	Detectable env-specific immune boost time period for DC-PBMC-Hu-SCID mice after the last immunization	3-4 weeks	-

Immunization may be carried out once or in a number of subsequent steps, e.g. it may be repeated after some time, e.g. after one to three weeks, preferably after two weeks two to four times, preferably three times.

The Hu-SCID-mouse may be vaccinated with the HIV immunogenic composition 2 weeks after the first engrafting of the human immunocompetent cells. The preferred route for administering the vaccine to the mice is via subcutaneous injection though intranasal route is also possible and could be preferred for bigger immune deficient animals. The vaccine to be tested may be administered to the Hu-SCID mice three or more times (three to six times). As the vaccine is a peptide or protein mixture, the amount of vaccine peptides or proteins administered to the Hu-SCID mouse is between 15 and 50 μg in each step for each mouse. Immunizations of the animals are performed once in 2 weeks and repeated 3 or more times.

Further experiments were targeted to test HIV laboratory strains challenging maintenance in Hu-SCID mice. The conditions, important for virus challenging formation in vivo are summarized in Table 3. There are two possible methods of infection modeling in SCID mice different in virus replication substrate:

1) donor PBMC or DC as background;
2) in vitro cultivation cell culture as background.

The first several experiments challenging of the hu-SCID mice with an amount of HIV laboratory strain infectious doses in human PBMC culture, e.g. from about $5x10^4$ to $5x10^6$ $TCID_{50}$ (50% Tissue Culture Infective Dose) for one animal ***have been ended with negative results***. In order to make out what is getting wrong in regimes of challenging it was reasonable to run a set of cell culture background experiments HIV laboratory strain as a positive control.

According to the cell culture background method 5-8 weeks old SCID mice were inoculated intraperitoneally with $5\text{-}10x10^6$ MT-4 or U937 pure culture cells rinsed twice in PBS from cultivation serum with 10 min spinning at 800rpm and re-suspended in PBS. One week later pure cell culture inoculation was repeated. The next day after the second MT-4 or U937 cells inoculation animals were challenged intraperitoneally with $5x10^3\text{-}10^4$ $TCID_{50}$ one of HIV-1 laboratory strains infectious doses in cell culture MT-4 or $5x10^2$ - $5x10^3$ $TCID_{50}$ infectious

doses in U937. Challenges were repeated 2-3 times with different time periods between, SCID mice were provided with fresh portions of MT-4 or U937 weekly. The other way of challenging allows simultaneous inoculation of Hu-SCID mice with $10x10^6$ pure MT-4 or U937 culture and $5x10^6$ MT-4 or U937 infected with one of HIV-1 laboratory strains mixed in one syringe every 3-7 days, respectively. In both methods applied blood samples were collected from the animals 1-4 weeks after the last challenging and tested for viral infection rates by Real Time PCR, they achieved $10^3 - 10^8$ HIV RNA copies per ml in bloodstream (Figs 4, 5, 6).

HIV laboratory strains can have aggressive or slow multiplication kinetics; quickly developing strains are better and more demonstrative for in vivo experiments. We have used only highly aggressive strains of A and B subtypes with fast replication dynamics and high rates of viral concentration in vitro – $10^{10} - 10^{12}$ copies/ml HIV RNA within 1-4 days of cultivation (Table 2). But cell culture virus replication kinetics has the same value for in vivo viral load and percentage of successful challenging. Analysis of viral load in SCID-PBMC mice bloodstream after 2-4 times challenging with the same laboratory strain cultivated on different cell cultures shows dependency of HIV RNA copies rate from the regime of inoculation (Fig. 4-6). When inoculations of infected with laboratory virus cells were made 3 days after the previous ones rates of viral load (HIV RNA copies per one ml of mice blood number) were always several times higher for variant which was cultivated on MT-4 culture background (Fig.4) than for the variant cultivated on monocyte's U937 background. When inoculations were made 7 days after the previous ones HIV RNA copies numbers were always several times higher for cultivated on U937 background variant (Fig. 5) than for the variant cultivated on lymphocyte's MT-4 background. In one experiment viral load in SCID mice bloodstream was also 3-10 times higher for MT-4 cultivation with 3 days period between challenging than for U937 cultivation and 3-15 times higher for U937 cultivation with 7 days period between challenging than for MT-4 cultivation (Fig. 6). The same in vivo HIV infection development dynamics has been observed for U455 and H9/IIIB laboratory strains (Fig. 13).

In vivo HIV-1 laboratory strains cultivation dynamics on lymphocyte's and monocyte's background repeats precisely in vitro dynamics of virus strains multiplication. So, in vitro replication titre for U455, PokA-79 and H9/IIIB measured both as Real Time HIV RNA copies data and p24 expression ELISA results were reaching maximums in 24-48 hours for lymphocyte MT-4 and MT-2 cell cultures (Table 2, Figs 7-8, 14-15). Contrariwise, in vitro replication titre for U455, PokA-79 and H9/IIIB measured both as RNA copies and p24 expression were growing to maximums within 72-96 hours for monocyte U937 cell culture (Table 2, Figs 7-8, 14-15). As it is necessary to use log phase infected cell culture for in vivo challenging success we can conclude 3 days period between challenging is the best for the

achievement of the highest viral load numbers in lymphocyte culture background and 7 days period – for the highest viral load in monocyte culture background.

As one can see from microscopy images on Figs. 9-12 the morphology and functional activity of PBMC (Fig. 11) is settled between monocyte cells presented as U937 culture (Fig. 10) and lymphocytes cells such as MT-4 culture (Fig. 9) but dynamics of their metabolism and multiplication/differentiation is rather matches monocyte cells dynamics. They are more similar to U937 then to dendrite cells (Fig. 12). Therefore the study of in vitro HIV strains titres dynamics and $TCID_{50}$ parameters for the used for vaccine development's HIV strain or HIV isolate is very important for getting successful results.

According to data received from in vitro dynamics/kinetics study HIV laboratory strains having passage on lymphocytes cell culture background, such as MT-4 or MT-2 in the preferred embodiment, have log phase in virus RNA copies titre on the 2nd day of fresh cells infection cultivation and should be challenged to Hu-SCID animals 24-36 hours after the passage (Table 2). HIV laboratory strains having passage on monocytes cell culture background, such as U937 in the preferred embodiment, have log phase in virus RNA copies titre on the 3rd-5th day of fresh cells infection cultivation and should be challenged to Hu-SCID animals 48-60 hours after the passage (Table 2). Positive challenging data are received for these regimes of infectious material intraperitoneal administration (Figs 4-6, 13, 16). The in vitro passages dynamics for PBMC-HIV "playback" strains is similar to monocyte-type cell cultures (Table 5). Thus, Positive challenging results can be obtained for 3rd day of passage cultivation PBMC-PokA79, PBMC-U455, etc. infectious material intraperitoneal administrations (Figs 17-18).

Table 2: Titre and dynamics of HIV laboratory strains viral replication on lymphocyte-type and monocyte-type cultivation cell cultures, p24 and RNA copies/ml

HIV Lab. Strain	Cell Culture	Time of Incubation Period, hours											
		0		24		48		72		96		120	
		p24, P/N	RNA, c/ml	p24, P/N	RNA, c/ml	p24, P/N	RNA, c/ml	p24, P/N	RNA, c/ml	p24, P/N	RNA, c/ml	p24, P/N	RNA, c/ml
U455	MT-2	2.3	$4.8x10^{8}$	18.1	$3.3x10^{11}$	22.5	$5.2x10^{11}$	14.4	$3.4x10^{11}$	14.1	$9.5x10^{10}$	8.8	$9.2x10^{8}$
	MT-4	2.7	$2.4x10^{8}$	17.6	$8.9x10^{11}$	23.3	$7.4x10^{11}$	22.2	$8.1x10^{11}$	21.3	$3.6x10^{11}$	17.4	$8.4x10^{9}$
	U937	2.4	$1.6x10^{7}$	18.3	$3.9x10^{9}$	20.7	$2.9x10^{10}$	20.4	$7.7x10^{10}$	19.1	$5.6x10^{10}$	17.4	$2.5x10^{8}$
H9/IIIB	MT-2	3.0	$5.9x10^{7}$	20.7	$5.7x10^{12}$	22.1	$3.8x10^{12}$	22.3	NM*	22.4	NM*	13.0	NM*
	MT-4	2.4	$3.5x10^{8}$	21.8	$4.5x10^{12}$	20.9	$2.7x10^{12}$	NM*	NM*	NM*	NM*	NM*	NM*
	U937	2.7	$6.1x10^{7}$	19.3	$2.9x10^{10}$	22.4	$7.8x10^{9}$	25.7	$8.5x10^{10}$	25.4	$5.1x10^{11}$	18.5	$1.7x10^{9}$
PokA-79	MT-2	2.0	$4.2x10^{8}$	16.2	$8.2x10^{12}$	20.1	$2.3x10^{13}$	18.1	$2.7x10^{12}$	16.3	$1.9x10^{10}$	8.0	NM*
	MT-4	2.9	$5.4x10^{8}$	17.3	$8.3x10^{10}$	20.0	$9.2x10^{10}$	26.0	$6.4x10^{9}$	26.1	$7.3x10^{9}$	16.0	$7.9x10^{8}$
	U937	2.8	$8.6x10^{7}$	23.0	$1.8x10^{9}$	25.2	$5.5x10^{9}$	28.2	$6.6x10^{9}$	29.1	$9.8x10^{9}$	27.5	$2.5x10^{9}$

NM* - not measured

WO 2012/025167 PCT/EP2011/002737

The same consequences are important for modeling HIV challenging in Hu-SCID mice on PBMC-DC background:
1) Virus strain/isolate multiplication kinetics and animal's challenging timing;
2) The difference between laboratory strain infective activity for cell culture and PBMC substrates.

According to the PBMC-DC background challenging method 4-5 weeks old SCID mice were inoculated intraperitoneally with freshly isolated PBMC, or pre-cultivated for 3 days PBMC, or pre-cultivated for 7 days DC, 5-10x10^6 one week before the next step. Cells were rinsed twice from human/fetal bovine serum in PBS (phosphate buffer saline) with 10 min spinning at 1200rpm and then re-suspended in PBS or DMEM without phenol red before the inoculation. The next day after the second PBMC inoculation animals were inoculated intraperitoneally with log phase 10-15x10^6 cells one of HIV-1 laboratory strains "playbacks" on PBMC or DC passages (Figs 17-18). Viral RNA titre in cell material used for challenging should be not less than 10^7 copies per ml in infective material, it matches to 5x10^4-10^5 $TCID_{50}$ per ml for positive results in challenging (Table 4). Every challenging in vitro material samples are stored for control Real Time PCR measurements (Table 5). Challenges were repeated 2-3 times with different time periods between, SCID mice were supplied with fresh portions of one donor PBMC 5-10x10^6 cells for each inoculation for one Hu-SCID mouse weekly (one donor's isolation was enough for a group of 5-10 animals).

The term "HIV laboratory strain playback" was created in order to distinguish HIV isolates obtained from HIV-infected individuals and run into cultivation on healthy donor's PBMC in vitro for several days or weeks from characterized HIV laboratory strains that were passed the same healthy donor's PBMC in vitro cultivation for several weekly passages. Thus the term "HIV laboratory strain playback" according to the present invention is understood as as HIV laboratory strain obtained by culturing a HIV laboratory strain on PBMC cells of a healthy donor for several weeks or passages, in particular for at least 5 weeks passages. Preferred laboratory strains according to the present invention are PokA-79, U455 and H9/IIIB. The variability of envelop proteins sequences for "playbacks" is similar to one of envelop sequences for isolates freshly obtained from HIV infected individuals blood samples.

HIV lab. strain "playbacks" for the current application were obtained as follows: PokA-79, U455 and H9/IIIB strains passaged in vitro on MT-4 cell culture background in log phase were added as 1:20 to pre-cultivated with FHG and cytokine factors at least for 3 days healthy donor's PBMC (in ration 250000 HIV-MT4 infected cells to 5000000 PBMC). Initial in vitro PokA-79-MT4, U455-MT4, IIIB-MT4 titres were 10^{11} – 10^{12} copies/ml HIV RNA. Virus sorption on PBMC was left for 4 hours incubation in vertical position of culture flasks. Then lymphocyte MT-4 cell culture was discarded from more adhesive (laying on culture flasks plastic surface) PBMC and fresh medium supplied with 1-10% FCS/HS was added for "Playback" passage.

Fresh medium was supplied every 4th day of incubation, new healthy donor pre-cultivated PBMC were added every 7th day. Weekly fresh PBMC supply is named an in vitro passage of HIV strain "playback".

The current application is summarizing in vivo virus modeling data with laboratory strains U455, 9H/IIIB (Figs. 13-15) and PokA-79 (Figs. 4-9) for PBMC background challenging. So infectious source was multiplied and prepared in log phase for inoculation either in MT-4(MT-2), U937 culture or PBMC cells background. Immunity challenging blockage formation requires PBMC in vivo substrate, therefore we run both cell culture and PBMC for control challenging rates tests. The conditions for in vivo virus cultivation are listed in Table 3. These conditions include the presence of 5-15 million "fresh" or infection-free cell culture or PBMC intraperitoneally before challenging (1-2), minimal infectious dose supply for each animal (3), continuous challenging detection period for one donor PBMC material for each animal's treatment (4-7) and matching typical for cell's background in vivo virus cultivation dynamics (8).

Table 3: HIV challenging formation conditions in PBMC-, cell culture background Hu-SCID mice.

##	Applied Treatment (Conditions) for PBMC-Hu-SCID Mice and cell culture background HIV Infection Rates in Bloodstream	Detectable in Bloodstream HIV RNA Copies/ml Load When Treatment is:	
		Applied	Not Applied
1	Modest PBMC, DC/PBMC number, 5-15x10^6 for one animal	+	-
2	Substrate (PBMC, cell culture) cells provided within one week before challenging	+	-
3	2-5x10^6 infected cells ($TCID_{50}$) for challenging of one SCID animal	+	-
4	One donor PBMC material for each animal	2-4 weeks	2-4 weeks
5	Two or more infected cells inoculations for every animal with 2 weeks period between inoculations, viral RNA copies/ml	10^4-10^8	0-10^3
6	Detectable viral load time period for Hu-SCID mice after the challenging	1-2 weeks	-
7	Detectable viral load time period for Hu-SCID mice after the challenging	2-4 weeks	-
8	Virus cultivation dynamics typical for cell's background in vivo	+	-

For the practice application it is worth to have in mind that long-time observation measurements in Hu-SCID blood serum samples are not reasonable in positive controls for vaccine efficiency in vivo assessment. In case challenging is repeated 3-4 times animals often slay within a week

after the last administration from total fatigue and exhaust caused by high viral load in their bloodstream like presented on Fig. 13.

It will be understood that different ranges of cell numbers and $TCID_{50}$ may be used for challenging/engraftment the Hu-SCID mice with HIV. Also, the HIV strains used for this purpose are not limited to a specific strain. Theoretically, all known HIV strains including HIV-1 strains and HIV-2 strains may be used as long as it is made sure that unprotected immunocompetent cells/mice (i.e. cells/mice not vaccinated with the HIV-vaccine to be tested) will be successfully infected with HIV. Preferably the HIV strain for Hu-SCID mice in vivo challenging is selected from the group of highly aggressive breeds with quickly and highly rising in vitro RNA copies/ml titre, such as U455, 9H/IIIB, and PokA-79 (Table 2, Figs 7-8, 14-15). However the problem for successful challenging is the matching between HIV strain envelop proteins variability and immunocompetent cell's CD4 and co-receptors variants presentation.

Therefore at first we carried out in vivo minimal infectious dose titration of HIV-1 laboratory strains on lymphocytes MT-4 and monocytes U937 cell culture backgrounds. It occurred that blood serum-detectable challenging can be guaranteed if $2x10^6$ or more infected cells in culture log phase with HIV RNA from $5x10^7$ copies/ml or higher are being used for challenging of one Hu-SCID animal. These numbers are equal to $5x10^4$ $TCID_{50}$ or higher infection activity measured for in vitro 2-weeks TCID titration per ml for HI-virus laboratory strain on MT-4 or U937 cultures background.

In a preferred embodiment SCID mice are challenged intraperitoneally with e.g. $5\text{-}10x10^6$ PBMC or DC cells as background and with $5x10^3\text{-}10^5$ $TCID_{50}$ of PBMC-HIV-1 or DC-HIV-1 "playback" strains infectious doses in the same cell culture. The positive results of challenging are presented on Figs 17-18.

The activity of in vivo challenging (bloodstream viral load) and in vitro infection titre are very different for HIV laboratory strains multiplying on different backgrounds. First, five in vivo experiments with PokA-79, U455 and H9/IIIB HIV-1 laboratory strains challenging of 34 Hu-SCID mice engrafted with PBMC background carried out in 2009 brought negative results. Second, we tried well-described dendrite cells Hu-SCIDs challenging with the same laboratory strains. Four challenging experiments with PokA-79, U455 and H9/IIIB HIV-1 lab. strains (one group with PokA-79, U455 in vitro DC passages) were carried out on 23 Hu-SCID mice engrafted with DC background in 2010; all results were negative as well (Fig. 18). The third attempt with PBMC-"playbacks" challenging was performed as a conclusion, it occurred to be successful.

In vivo challenging rates (Figs 16-17, Table 4) and in vitro multiplication dynamics (Figs 14-15, 19-20, Table 5) were analyzed for PokA-79 "playback" strain cultivated on human peripheral blood mononuclear cells (PBMC) and cell cultures background. In vivo PokA-79-PBMC

challenging rates on PBMC background were not detectable as HIV RNA copies in animals bloodstream after one single infection's inoculation, achieved 10^3 viral RNA copies per ml after two inoculations and 10^3-10^4 viral RNA copies per ml after three infected material inoculations (Table 4, 3rd column, Fig. 17). PokA-79 strain challenging rates for lymphocyte MT-4 in vivo infection modeling were 10^2-10^3 RNA copies per ml after the first inoculation, 10^3-10^5 - after two inoculations and 10^4-10^6 HIV RNA copies per ml in bloodstream after three infected material challenges (Table 4, 1st column, Fig. 16). PokA-79 challenging rates for monocyte U937 in vivo infection modeling were 10^3 RNA copies per ml after the first inoculation, 10^3-10^5 - after two inoculations and 10^5-10^7 HIV RNA copies per ml in bloodstream after three infected material challenges (Table 4, 2nd column, Fig. 16). These rates on PBMC background in average were 10^2-10^4 times lower for two and three challenging than the same results obtained for cell culture background (Fig. 17, Table 4). The infection regimes for PBMC background were similar to monocyte-based modeling, so challenging viral RNA rates were detectable in SCID-PBMC mice bloodstream after two-three inoculations when period between challenges was 7 days (Table 4 data, Figs 6, 17). If 3 days period was settled between challenges in SCID-PBMC mice like it is being done for higher rates in lymphocyte-background SCID HIV challenging modeling the results in animals bloodstream were not detectable in 45 percent of taken samples.

Table 4: PokA-79 strain HIV RNA copies/ml titre in SCID-hu mice bloodstream on PBMC and cell cultures background in vivo depending on number of challenging.

Animal Groups	PokA-79 Challenging Viral Load, RNA copies/ml								
	MT-4			U937			Human PBMC 6th week		
	I	II	III	I	II	III	I	II	III
1	$9.9x10^2$	$6.9x10^5$	$1.6x10^5$	$6.0x10^3$	$9.9x10^3$	$5.2x10^6$	-	$4x10^2$	$5x10^3$
2	$2.0x10^3$	$3.9x10^4$	$9.8x10^6$	$5.7x10^3$	$3.0x10^5$	$1.2x10^7$	-	-	$9.4 x10^3$
3	$3.9x10^3$	$3.9x10^4$	$2.7x10^4$	$2.2x10^3$	$3.6x10^4$	$4.8x10^5$	-	$5x10^2$	$3.2 x10^4$
4	-	$8.4x10^3$	$2.6x10^5$	-	$3.0x10^4$	$8.6x10^5$	-	-	$2.8x10^4$

So, according to in vivo data of the current embodiment, the challenges with HIV may be repeated for a number of times, e.g. for 2-3 times with different time periods in between (Figs 16-17). A 3 day period between challenging is preferred for the achievement of the high viral load in a lymphocyte culture background and a 7 day period is preferred for the high viral load in a monocyte culture or PBMC background.

We supposed the lower rates for PBMC-virus "playbacks" challenging background to be in correlation with their in vitro dynamics. In vitro cultivation titres of virus strain on PBMC

background were always lower 10^2-10^5 times than on competent for it's cultivation cell cultures background; it was true for both HIV RNA copies per ml Real Time detection and for p24 expression ELISA calibration methods. So, lymphocyte-based PokA-79 cultivation on MT-2 and MT-4 cell lines brings HIV RNA titres to 10^{11}- 10^{13}, monocyte-based cultivation on U937 cells – to 10^9 copies/ml RNA (Table 5, Figs 14-15). Cultivation on human PBMC for 5-8 weeks brings titre to 10^7- 10^9 RNA copies/ml (Table 5 line 1, Fig. 7-8, the numbers of infected and fresh cells in culture mixtures were standardized to equal ones). In vitro dynamics of HIV laboratory strain's multiplication for human PBMC background and on DC background (Table 5 lines 2 and 4) was looking like gradual virus dilutions and eradication, curve of HIV RNA copies is dropping down (Fig. 19). It became evident why virus challenging never worked for laboratory strains PBMC and DC background (so called "1st week passages", Fig. 18).

Table 5: PokA-79 laboratory strain and "playbacks" viral replication on PBMC, DC and cell cultures background in vitro.

Culture background	Time of Incubation Period, hours							
	0	24	48	72	96	120	144	240
	PokA-79 RNA copies in 1 ml cell suspension in culture media							
PBMC 6th passage (week)	$8.4x10^5$	$6.2x10^6$	$3.1x10^7$	$2.7x10^7$	$5.6x10^7$	$9.3x10^7$	$1,4x10^8$	$2,7x10^8$
PBMC 1st passage (week)	$5.2x10^8$	$4.3x10^8$	$9.7x10^6$	$3.0x10^6$	$2.8x10^5$	$1.7x10^5$	$8.0x10^4$	-
DC 5th passage (week)	$5x10^4$	$4.5x10^5$	$6.1x10^5$	$2.7x10^5$	$5.4x10^5$	$2.5x10^5$	$4.8x10^5$	$7.0x10^4$
DC 1st passage (week)	$9.6x10^7$	$1.0x10^6$	$5.5x10^5$	$4.4x10^5$	$8.8x10^4$	$7.3x10^4$	$4.8x10^4$	-
MT-2	$7.0x10^7$	$7.5x10^{12}$	$5.4x10^{12}$	$7.9x10^{11}$	$7.2x10^{10}$	$8.2x10^8$	NM*	NM*
MT-4	$7.0x10^7$	$8.2x10^{12}$	$2.3x10^{13}$	$2.7x10^{12}$	$1.9x10^{10}$	$7.9x10^8$	NM*	NM*
U937	$8.6x10^7$	$1.8x10^8$	$5.5x10^9$	$6.6x10^9$	$9.8x10^9$	$2.5x10^9$	NM*	NM*

PBMC-HIV "playbacks" in vitro titres are similar to monocyte cell culture background: infection titres grow slowly and achieve maximums at 3rd-6th day of cultivation (Table 5 line 1, Fig. 20), whenever lymphocyte-based cultivation brings HIV RNA and p24 titres hit maximums at 1st-2nd day of cultivation and at 3rd day titres of HIV RNA copies start to go down (Table 5 lines 5-7, Figs 14-15). However monocyte-based in vitro cultivation viral RNA titres start to drop down after 5th day of cultivation unlike PBMC-based model titres that continue approximately the same levels for 6-10 days of cultivation (Table 5 line 1) and can be maintain with passages on fresh healthy donor pre-cultivated PBMCs weekly.

The most interesting and un-expectable dynamics of HIV strains "playbacks" was observed on dendrite cells (DC) background. In vitro passages on DC made weekly did not have raised virus

titres on 5^{th} and further generations, just the opposite, RNA copies continue to be kept on the same levels 10^4 -10^5 per ml (Table 5 line 3, Figs 19-20).

This phenomenon explains why it was not possible to obtain viral load in blood serum of challenged Hu-SCID mice on dendrite cells in vitro passages background (Fig. 20). HIV infectivity potential is determined by envelop gp120's ability to identify and bind CD4 receptor and in some cases co-receptors CXCR4 and CCR5. In case these widely represented in differentiated human tissues receptors are not found on some type of human cells current type of cells cannot be infected with HIV. It is exactly the case with DC that and do not represent as many receptors and other membrane proteins as differentiated tissue cells. In the other words they are median-positioned in development between stem and differentiated cells, therefore they are more "bald" on cell surface and capable of proliferation in vitro and in animal's organism. As it was measured for cell cultures background HIV laboratory strains challenging controls the minimal HIV RNA titre in laboratory strain cultivation material being used for challenging should be 10^7 copies/ml or higher, and infectious dose measured as $TCID_{50}$ – $5x10^3$ copies/ml or higher. Challenging do not lead to viral load in animals bloodstream from lower titres and $TCID_{50}$ concentrations in infective material. Consequently, it is expectable that challenging should not work in fresh DC and cultivated DC "playbacks". Thus, young researcher should not repeat the mistakes that were done for HIV in vivo models challenging.

Figures 9-12 demonstrate the microscopy visualization of the difference between infective potential of HIV strains on different backgrounds. Figures 9A, 10A, 11A and 12A show intact non-infected cell's cultures MT-4, U937, PBMC and DC, respectively. Figures 9B and 10B show lymphocyte MT-4 and monocyte U937 in vitro infection with PokA-79 laboratory strain which is visible as synthythyum formation and cells death on the 3^{rd} day (MT-4) and 5^{th} day of cultivation (U937, proliferation inhibition); synthythyums are marked with white arrows. Figures 11B-C present the outlook of PokA79-PBMC and U455-PBMC "playbacks" 6^{th} passage synthythyum formation on 5^{th} day of cultivation. Dendrite cells on figures 12A-B has no difference in morphology without and after infection and no synthythyum formation in infected cells due to low titre and $TCID_{50}$ values.

In vitro PokA-79 laboratory strain viral replication on PBMC and cell cultures background analysis explains in vivo inoculation regimes and challenging rates for SCID-PBMC HIV infection modeling but it does not reveal the reason why these HIV titre numbers are so very much different for PBMC and cell cultures. Our experience with mass spectrometry analysis of HIV-1 envelop protein variability run for laboratory strains PokA-79, U455 and H9/IIIB and for virus isolates from several cohorts of HIV-1 subtype A infected individuals RF territory-inhabitants [6] brought the only possible conclusion. Laboratory strains selected in cultivation on one or two cell cultures expressing CD4 and/or one of co-receptors CCR5 or CXCR4 in the way how it is normally represented on monoclonal cell culture surface have luck of gp120/gp160 envelop protein's variations. Cell cultures are characterized as monoclonal originated population

of human tissue material selected in cultivation in vitro in permanent conditions. Due to monoclonal and constant conditions cultivation cell cultures represent relatively small number of variations of CD4 (CCR5, CXCR4) receptors available for recognition and infection compared to human cells in the bloodstream, PBMC. Therefore the variability of gp120/gp160 for HIV laboratory strains is hundreds or thousands times restricted versus native virus envelop proteins. It means if laboratory strain was put into fresh PBMC environment in vivo or donor PBMC isolates in vitro it has to invade human PBMC instead of cell culture for it\s further multiplication. This viral strain survives with lower titre of infectivity and slower infection development kinetics (Table 5, Figs 7, 19) because it's existing gp120/gp160 modifications can recognize only small number of existing variations of CD4/co-receptors on PBMC surface.

$TCID_{50}$ (tissue cell infectivity dose) is one of the crucial parameters of in vivo HIV challenging because viral load (viral RNA copies number in blood serum or tissue) and virus infective potential are different values in calculation for the same virus particles number. $TCID_{50}$ is a parameter that characterizes alive and active for new cells invasion virus units. Even in log phase laboratory strain in vitro culture $TCID_{50}$ referred to the number of HIV viral particles is minimum 100-1000 times lower than total viral particles number, and in overgrown viral in vitro culture where cell deaths are closer to 95-100 percent the difference between $TCID_{50}$ and RealTime PCR data is 10^3-10^7 in viral particles representation. A simple correlation between viral activity measured in $TCID_{50}$ and HIV preventive vaccine effectiveness is like this: the higher is $TCID_{50}$, the lower are chances that HIV-specific immunity can block further challenging effectively. However in reality HIV infection doses transmitted from human to human during sexual contact or IDU (intravenous drug usage) are modest, incomparably lower than ones we run in animal's challenging. Therefore the possibility of HIV infection blockage with specific immune-boost vaccination can be applied into practice.

Infectious doses calculation (Mensch-Reed protocol) in $TCID_{50}$ units was performed via gradual dilutions method and measured within a period of three weeks in ELISA tests as p24 expression. The infectious dose is correspondent to amount of newly replicated and secreted into cultural media infective virus. All samples were taken from infected cell culture before concentration of material for every animal's challenging and evaluated as average for two similar experiments. $TCID_{50}$ data for three laboratory strains are presented in Table 6. 3rd and 4th columns contain

Table 6: HIV Strains Infectious Doses ($TCID_{50}$) used for Hu-SCID challenging on cell culture background.

Laboratory Strain	Infected Culture Background	$TCID_{50}$ in 1 ml 30 h	$TCID_{50}$ in 1 ml 56 h	$TCID_{50}$ for each Hu-SCID Animal
U 455	MT-4	2.9×10^7	-	5.8×10^6
	MT-2	7.2×10^7	-	-

	U937	6.9×10^2	5.4×10^7	1.08×10^6
H9/IIIB	MT-4	4.1×10^7	-	8.2×10^6
	MT-2	9.6×10^7	-	-
	U937	7.2×10^2	8.2×10^7	1.64×10^6
PokA-79	MT-4	$6{,}8 \times 10^6$	-	1.36×10^6
	MT-2	$2{,}9 \times 10^7$	-	-
	U937	7.1×10^2	3.3×10^7	6.6×10^5

$TCID_{50}$ data in 1 ml culture suspension for all three laboratory strains on MT-4, MT-2 and U937 cell culture background for 30 and 56 hours of incubation, respectively. 5th column shows $TCID_{50}$ which was applied to one single animal every inoculation calculated as measured for 30 hours log phase incubation multiplied to 10 ml infected cell culture suspension in average used for concentration of infectious material for one mouse challenging.

$TCID_{50}$ 30 hours (3rd column) for any of HIV-1 laboratory strains on MT-4 cell culture background varied within 2.9×10^7-9.6×10^7 units/ml culture media, $TCID_{50}$ 56 hours (4th column) – within 6.8×10^6 – 9.6×10^7 units/ml. $TCID_{50}$ 30 hours (3rd and 4th columns) on U937 cell culture background was 7×10^2 and for 56 hours – 3.3×10^7 – 8.2×10^7. The dynamics of $TCID_{50}$ increase was the same for U455, H9/IIIB and PokA-79 laboratory strains. These data show that there is a great difference in $TCID_{50}$ growth between 30 and 56 hours of incubation for lymphocyte cell culture background infection but not much difference in $TCID_{50}$ growth between 30 and 56 hours of incubation for monocyte U937 cell culture background. It means that maximum $TCID_{50}$ infectious doses for lymphocyte-based HIV laboratory strains Hu-SCID mice challenging could be achieved after 24-30 hours of culture in vitro incubation, monocyte U937 could be achieved after 48-72 hours of culture in vitro incubation before challenging. So we started to run 30 hours incubation period for MT-4-based challenging protocols and 56 hours for U937-based HIV strains and PBMC-based challenging protocols. These differentiated protocols allow the achievement of higher levels of RNA copies viral load in animal's bloodstream.

Hu-SCID mice can be challenged with 5×10^4-10^7 $TCID_{50}$ of HIV-1 laboratory strain infectious doses in lymphocyte and monocyte culture cells. Suitable cell cultures for this purpose include but are not limited to MT-4 or U937 culture cells. Preferably for successful bloodstream-detectable Hu-SCID mice challenging freshly isolated or pre-cultivated PBMCs as background and 5×10^4-10^6 $TCID_{50}$ of HIV-1 "playback" strain cultivated for 1.5 month on human PBMC are administered to each individual mouse. Suitable HIV-1 or HIV-2 strains for this purpose include but are not limited to U455, H9/IIIB and PokA-79 laboratory strains "playbacks".

Examples

Example 1. Identification of collected with antibodies libraries/reverse panning technique HIV peptides/proteins material, LC-MS-MS (mass spectrometry) data.

a) Scaffold software HIV Envelop protein's identification and trustworthy analysis is shown in Figure 21A)

b) HIV Envelop protein's spectra is shown in Figure 21B).

c) HIV Envelop protein's triptic peptides mapping is shown in Figure 21C).

d) Scaffold software HIV Envelop protein's identification and trustworthy analysis is shown in Figure 21D).

e) HIV Envelop protein's triptic peptides mapping is shown in Figure 21E).

Example 2. Identification of collected HIV peptides/proteins material, LC-MS-MS (mass spectrometry) data.

Table 7: The PEAKS analysis software:

Marked protein and peptide details							
Accession	ID	Mass	InChorus (Score %)	Coverage (%)	Query matched	Description	Marked
gi|62905448	5924	24174,23	29,44	3,2	1	envelope glycoprotein [Human immunodeficiency virus 1]	true
gi|90192283	567	43626,53	4,77	1,57	55	envelope glycoprotein [Human immunodeficiency virus 1]	true
gi|16798231	568	22471,13	3,38	2,94	36	envelope glycoprotein [Human immunodeficiency virus type 1]	true
gi|51848079	605	15251,62	2,95	10,22	3	envelope glycoprotein [Human immunodeficiency virus 1]	true
gi|51537736	664	17998,39	2,79	12,5	2	envelope glycoprotein [Human immunodeficiency virus 1]	true
gi|841085	675	39568,91	2,77	3,35	3	envelope glycoprotein, V1-V5 region	true
gi|1469281	677	95679,71	2,76	3,08	10	envelope glycoprotein [Human immunodeficiency virus type 1]	true
gi|15321690	1065	12589,34	2,7	16,81	12	envelope protein [Human immunodeficiency virus type 1]	true
gi|112378976	1118	19705,54	2,7	8,72	2	envelope glycoprotein [Human immunodeficiency virus 1]	true
gi|164374477	1135	28011,64	2,67	4,4	2	envelope glycoprotein [Human immunodeficiency virus 1]	true

The HIV Envelop protein's triptic peptides mapping is shown in Figure 22. The HIV Envelop protein's spectra are shown in Figures 23 A) to 23 M)

Example 3. Primers disposition for PCR products of immunogenic composition's tailoring mapped for several sequences in alignment is shown in Fig. 24.

Example 4: Glycosylated recombinant proteins molecular weight calculations.

http://www.encorbio.com/protocols/Prot-MW.htm

160 #1 (B.FR.83.HXB2_LAI_IIIB_BRU_K034) There are 856 Amino acids, and MW is 97241.93 Daltons, or 97.24 KiloDaltons; Charge is 30 assuming N and C termini are not blocked

MRVKEKY QHLWRWGWRW GTMLLGMLMI CSAT-EKLWV TVYYGVPVWK
EATTTLFCAS DAKAYDTEVH NVWATHACVP TDPNPQEVVL VNVTENFNMW
KNDMVEQMHE DIISLWDQSL KPCVKLTPLC VSLKCTDLKN DTNTNSSSGRMI
MEKGEIKNCS FNISTSIRGK VQKEYAFFYK LDIIPIDNDTTSY KLTSCNTSVI
TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI
RPVVSTQLLL NGSLAE-EEV VIRSVNFTDN AKTIIVQLNT SVEINCTRPN
NNTRKRIRIQ RGPGRAFVTI GKIGNMRQA HCNISRAKWN NTLKQIASKL
REQFGNNKTIIFKQS SGGDPEIVT HSFNCGGEFF YCNSTQLFNS TWFNSTWSTEGS
NNTEGSDTITLPCR IKQIINMWQK VGKAMYAPPI SGQIRCSSNI TGLLLTRDGG
NSNNESEIFRP GGGDMRDNWR SELYKYKVVK IEPLGVAPTK AKRRVVQRE
KRAV-G-IGA LFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQNNLLR
AIEAQQHLLQ LTVWGIKQLQ ARILAVERYL KDQQLLGIWG CSGKLICTTA
VPWNASWSNK SLEQIWNHTT WMEWDREINN YTSLIHSLIE ESQNQQEKNE
QELLELDKWA SLWNWFNITN WLWYIKLFIM IVGGLVGLRI VFAVLSIVNR
VRQGYSPLSF QTHLPTPRGP DRPEGIEEEG GERDRDRSIR LVNGSLALIW
DDLRSLCLFS YHRLRDLLLI VTRIVELLGR RGW EALKYWWNLL QYWSQELKNS
AVSLLNATAI AVAEGTDRVI EVVQGACRAI RHIPRRIRQG LERILL
(SEQ ID No. 54)

120 #1 There are 511 Amino acids, and MW is 57695.64 Daltons, or 57.70 KiloDaltons

MRVKEKY QHLWRWGWRW GTMLLGMLMI CSAT-EKLWV TVYYGVPVWK
EATTTLFCAS DAKAYDTEVH NVWATHACVP TDPNPQEVVL VNVTENFNMW
KNDMVEQMHE DIISLWDQSL KPCVKLTPLC VSLKCTDLKN DTNTNSSSGRMI
MEKGEIKNCS FNISTSIRGK VQKEYAFFYK LDIIPIDNDTTSY KLTSCNTSVI
TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI
RPVVSTQLLL NGSLAE-EEV VIRSVNFTDN AKTIIVQLNT SVEINCTRPN
NNTRKRIRIQ RGPGRAFVTI GKIGNMRQA HCNISRAKWN NTLKQIASKL
REQFGNNKTIIFKQS SGGDPEIVT HSFNCGGEFF YCNSTQLFNS TWFNSTWSTEGS
NNTEGSDTITLPCR IKQIINMWQK VGKAMYAPPI SGQIRCSSNI TGLLLTRDGG
NSNNESEIFRP GGGDMRDNWR SELYKYKVVK IEPLGVAPTK AKRRVVQRE KR

(SEQ ID No. 55)

41#1 There are 345 Amino acids, and MW is 39564.30 Daltons, or 39.56 KiloDaltons
AV-G-IGA LFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQNNLLR
AIEAQQHLLQ LTVWGIKQLQ ARILAVERYL KDQQLLGIWG CSGKLICTTA
VPWNASWSNK SLEQIWNHTT WMEWDREINN YTSLIHSLIE ESQNQQEKNE
QELLELDKWA SLWNWFNITN WLWYIKLFIM IVGGLVGLRI VFAVLSIVNR
VRQGYSPLSF QTHLPTPRGP DRPEGIEEEG GERDRDRSIR LVNGSLALIW
DDLRSLCLFS YHRLRDLLLI VTRIVELLGR RGW EALKYWWNLL QYWSQELKNS
AVSLLNATAI AVAEGTDRVI EVVQGACRAI RHIPRRIRQG LERILL
(SEQ ID No. 56)

160 #43(K.CM.96.MP535_AJ249239): There are 842 Amino acids, and MW is 95252.58 Daltons, or 95.25 KiloDaltons Charge is 22 assuming N and C termini are not blocked
MRVRGMQRNW QTLGNWG ILFLGILII CSNA-DKLWV TVYYGVPVWK
EATPTLFCAS DAKAYEKEVH NVWATHACVP TDPNPQEVEM ENVTENFNMW
KNNMVEQMHT DIISLWDESL KPCVELTPLC VTLNCTDYKG TNSTN NATSTV
VSPAEIKNCS FNITTEIKDK KKKESALFYR LDVLPLNGEG NNSSTEY RLINCNTSTI
TQTCPKVTFE PIPIHYCAPA GFAILKCKDK RFNGTGPCKN VSTVQCTHGI
KPVVSTQLLL NGSLAE-EEI IIRSENITDN TKNIIVQLNE TVQINCTRPN NNTRKSIHM
GPGKAFYTT GDIIGDIRQA HCNISGEKWN MTLSRVKEKL KEHFK NGTITFKPP
NPGGDPEILT HMFNCAGEFF YCNTTKLFNE TGENGTITLPCR IKQIINMWQK
VGKAIYAPPI AGSINCSSNI TGMILTRDGG NNTHN ETFRP GGGDMRDNWR
SELYKYKVVQ IEPLGIAPTR ARRRVVQRE KRAVGLGAVFFGFLGAAG
STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ LTVWGIKQLR
ARILAVERYL KDQQLLGIWG CSGKLICTTN VPWNSSWSNK SWEEIWNNMT
WMEWEKEIGN YSDTIYKLIE ESQTQQEKNE QDLLALDKWA SLWNWFDITK
WLWYIKIFIM IIGGLIGLRI AFAVLSVVNR VRQGYSPLSF QTLIPTSRGA
DRPEGIEEEG GEQDKNRSVR LVSGFLALAW DDLRNLCLFS YRQLRNLILI
VTRILERGLRGGW EALKYLWNLV QYWSQELKNS AISLLNTTAI AVAGGTDRII
EIGQRAFRAL LHIPRRIRQG LERALL
(SEQ ID No. 57)

120 #43: There are 497 Amino acids, and MW is 55884.46 Daltons, or 55.88 KiloDaltons
MRVRGMQRNW QTLGNWG ILFLGILII CSNA-DKLWV TVYYGVPVWK
EATPTLFCAS DAKAYEKEVH NVWATHACVP TDPNPQEVEM ENVTENFNMW
KNNMVEQMHT DIISLWDESL KPCVELTPLC VTLNCTDYKG TNSTN NATSTV
VSPAEIKNCS FNITTEIKDK KKKESALFYR LDVLPLNGEG NNSSTEY RLINCNTSTI

TQTCPKVTFE PIPIHYCAPA GFAILKCKDK RFNGTGPCKN VSTVQCTHGI
KPVVSTQLLL NGSLAE-EEI IIRSENITDN TKNIIVQLNE TVQINCTRPN NNTRKSIHM
GPGKAFYTT GDIIGDIRQA HCNISGEKWN MTLSRVKEKL KEHFK NGTITFKPP
NPGGDPEILT HMFNCAGEFF YCNTTKLFNE TGENGTITLPCR IKQIINMWQK
VGKAIYAPPI AGSINCSSNI TGMILTRDGG NNTHN ETFRP GGGDMRDNWR
SELYKYKVVQ IEPLGIAPTR ARRRVVQRE KR
(SEQ ID No. 58)

41 #43: There are 345 Amino acids, and MW is 39386.14 Daltons, or 39.39 KiloDaltons
AVGLGA VFFGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR
AIEAQQHLLQ LTVWGIKQLR ARILAVERYL KDQQLLGIWG CSGKLICTTN
VPWNSSWSNK SWEEIWNNMT WMEWEKEIGN YSDTIYKLIE ESQTQQEKNE
QDLLALDKWA SLWNWFDITK WLWYIKIFIM IIGGLIGLRI AFAVLSVVNR
VRQGYSPLSF QTLIPTSRGA DRPEGIEEEG GEQDKNRSVR LVSGFLALAW
DDLRNLCLFS YRQLRNLILI VTRILERGLRGGW EALKYLWNLV QYWSQELKNS
AISLLNTTAI AVAGGTDRII EIGQRAFRAL LHIPRRIRQG LERALL
(SEQ ID No. 59)

160 #6 (our A1.RU.03.03RU20_06_13_AY500393) There are 805 Amino acids, and MW is 90669.32 Daltons, or 90.67 KiloDaltons: Charge is 24 assuming N and C termini are not blocked
MKAKGMQRNY QHLWRWG--- -XMLFWXIIM CKAA-ENLWV TVYYGVPVWR
DAETTLFCAS DAKAYDKEVH NVWATHACVP TDPNPQEIAL ENVTEKFDMW
KNNMVEQMQT DIISLWDQSL KPCVKLTPLC VTLNCAEPNS TSSNNNS--- ---------- --
-VNSNSSD SVFEEMKNCT FNMTTELRDK RKTVHSLFYK LDIVSTGS-- --NGS----- ----
---GQY RLINCNTSAM TQACPKVTFE PIPIHYCAPA GFAILKCKDT NFTGTGPCKN
VSTVQCTHGI KPVVSTQLLL NGSLAE-KEV MXRSENITDN GKXIIVQLTE
PVNITCIRPG NNTRTSIRI- -GPGQTFYAT XDVIGDIRKA YCXVSRAAWX
STLQKISTQL R--K-YFN-- NKT-IXFKNS S-GGDLEVTT HSFNCGGEFF YCNTTDLFNS
TW----DGXG TXT------- XITXA----N GT--ITLPCR IKQIINMWQR
VGQAMYAPPIKGSIRCESNI TGLXLTRDGG GGT--NXX-- ----XETFRP
IGGNMRDNWR SELYKYKVVK IEPIGVAPTR AKRRVV-ERE KR AI-G-LGA
AFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQSNLLR AIEAQQHLLK
LTVWGIKQLQ ARVLAVERYL KDQQLLGIWG CSGKLICTTN VPWNSSWSNK
SQSEIWDNMT WMQWDKEVIN YTXIIYDLIE KSQNQQEKNE QDLLALDKWA
SLWXWFDISN WLWYIKIFII IVGSLIGLRI XFAVLYIINR ARQGYSPLSL
QTLTPHPEGP DRPGRIKEEG GEQGRDRSIR LVSGFLALAW DDLRSLCLFS

YHRLRDFIXI AARTVELLGR SSLKGLRLGW EGLKYLGNLL GYWGQELKSS
AINLIDTIAI
(SEQ ID No. 60)

120 #6 There are 493 Amino acids, and MW is 55328.29 Daltons, or 55.33 KiloDaltons
MKAKGMQRNY QHLWRWG--- -XMLFWXIIM CKAA-ENLWV TVYYGVPVWR
DAETTLFCAS DAKAYDKEVH NVWATHACVP TDPNPQEIAL ENVTEKFDMW
KNNMVEQMQT DIISLWDQSL KPCVKLTPLC VTLNCAEPNS TSSNNNS--- ---------- --
-VNSNSSD SVFEEMKNCT FNMTTELRDK RKTVHSLFYK LDIVSTGS-- --NGS----- ----
---GQY RLINCNTSAM TQACPKVTFE PIPIHYCAPA GFAILKCKDT NFTGTGPCKN
VSTVQCTHGI KPVVSTQLLL NGSLAE-KEV MXRSENITDN GKXIIVQLTE
PVNITCIRPG NNTRTSIRI- -GPGQTFYAT XDVIGDIRKA YCXVSRAAWX
STLQKISTQL R--K-YFN-- NKT-IXFKNS S-GGDLEVTT HSFNCGGEFF YCNTTDLFNS
TW----DGXG TXT------- XITXA----N GT--ITLPCR IKQIINMWQR
VGQAMYAPPIKGSIRCESNI TGLXLTRDGG GGT--NXX-- ----XETFRP
IGGNMRDNWR SELYKYKVVK IEPIGVAPTR AKRRVV-ERE KR
(SEQ ID No. 61)

41#1 There are 346 Amino acids, and MW is 39333.71 Daltons, or 39.33 KiloDaltons
AI-G-LGA AFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQSNLLR
AIEAQQHLLK LTVWGIKQLQ ARVLAVERYL KDQQLLGIWG CSGKLICTTN
VPWNSSWSNK SQSEIWDNMT WMQWDKEVIN YTXIIYDLIE KSQNQQEKNE
QDLLALDKWA SLWXWFDISN WLWYIKIFII IVGSLIGLRI XFAVLYIINR
ARQGYSPLSL QTLTPHPEGP DRPGRIKEEG GEQGRDRSIR LVSGFLALAW
DDLRSLCLFS YHRLRDFIXI AARTVELLGR SSLKGLRLGW EGLKYLGNLL
GYWGQELKSS AINLIDTIAI AVARWTDXVI EIGQRLCRAI RNIPRRIRQG XEKALQ
(SEQ ID No. 62)

160 #26 (C.ZA.01.01ZATM45_AY228557) There are 878 Amino acids, and MW is 99122.18 Daltons, or 99.12 KiloDaltons; Charge is 15.5 assuming N and C termini are not blocked
MRVRGIPRNW QQWWIWI--- -ILGFWMLLI CNVG-GNSWV TIYYGVPVWR
EAKTTLFCAS DAKAHETEVH NVWATHACVP TDPNPQEIEL ENVTENFNMW
KNDMVDQMHE DIISLWDQSL KPCVKLTPLC VTLNCTNATR PVTRTNTTAT
GTNNTVTNCS GSASTNNTCM ENIEGMKNCS FNITTELRDK KKKEYALFYR
LDIVPLNETR D-NSS----- ---------Y RLINCNTSTI TQACPKVSFD PIPIHYCAPA
GYAILKCNNK TFSGTGPCNN VSTVQCTHGI MPVVSTQLLL NGSLAE-EMV
IIRSDNMTNA ATTIIVHLKD PVEIVCTRPN NTTRREVGI- -GPGQTFYTT

GQIIGDIRQA HCNITGKEWN KTLRQVGAEL E--K-HFP-- NKT-IQFKPH S-
GGDLEITT HSFTCSGEFF YCNTSKLFNI SMS---NLT- ---Y------ NNTDNTD--N PTQ-
ITLPCR IKQIINMWQE VGRAIYAPPI AGNITCKSNI TGLLLTWDGG SGE--NNT-- -----
ETFRP GGGDMRDNWR SELYKYKVVE IKPLGIAPTE AKRRVV-ERE KR AI-G-IGA
VFLGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ
LTVWGIKQLQ ARVLAIERYL KDQQLLGIWG CSGKLICTTS VPWNSSWSNK
TLGEIWNNMT WMEWDKEISN YTHTIYQLLE ESQIQQEQNE KELLALDSWK
NLWNWFDISN WLWYIKIFIM IVGGLIGLRI VFAVLSIVNR VRQGYSPLSF
QTLTPSPREP DRLRGIEEEG GEQDKGRSIR LVQGFLALAW DDLRSLCLFS
YHRLRDFISI AARVVEVLGH SSLRGLQRGW EALKYLKSLV QYWGLELKKS
AVSLLDTLAI AVGEGTDRII ELIQGICRAI RNIPRRIRQG FEAALL
(SEQ ID No. 63)

120 #26 (C.ZA.01.01ZATM45_AY228557) There are 526 Amino acids, and MW is 59182.32 Daltons, or 59.18 KiloDaltons
MRVRGIPRNW QQWWIWI--- -ILGFWMLLI CNVG-GNSWV TIYYGVPVWR
EAKTTLFCAS DAKAHETEVH NVWATHACVP TDPNPQEIEL ENVTENFNMW
KNDMVDQMHE DIISLWDQSL KPCVKLTPLC VTLNCTNATR PVTRTNTTAT
GTNNTVTNCS GSASTNNTCM ENIEGMKNCS FNITTELRDK KKKEYALFYR
LDIVPLNETR D-NSS----- ---------Y RLINCNTSTI TQACPKVSFD PIPIHYCAPA
GYAILKCNNK TFSGTGPCNN VSTVQCTHGI MPVVSTQLLL NGSLAE-EMV
IIRSDNMTNA ATTIIVHLKD PVEIVCTRPN NTTRREVGI- -GPGQTFYTT
GQIIGDIRQA HCNITGKEWN KTLRQVGAEL E--K-HFP-- NKT-IQFKPH S-
GGDLEITT HSFTCSGEFF YCNTSKLFNI SMS---NLT- ---Y------ NNTDNTD--N PTQ-
ITLPCR IKQIINMWQE VGRAIYAPPI AGNITCKSNI TGLLLTWDGG SGE--NNT-- -----
ETFRP GGGDMRDNWR SELYKYKVVE IKPLGIAPTE AKRRVV-ERE KR
(SEQ ID No. 64)

41 #26 (C.ZA.01.01ZATM45_AY228557) There are 352 Amino acids, and MW is 39957.88 Daltons, or 39.96 KiloDaltons
AI-G-IGA VFLGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR
AIEAQQHLLQ LTVWGIKQLQ ARVLAIERYL KDQQLLGIWG CSGKLICTTS
VPWNSSWSNK TLGEIWNNMT WMEWDKEISN YTHTIYQLLE ESQIQQEQNE
KELLALDSWK NLWNWFDISN WLWYIKIFIM IVGGLIGLRI VFAVLSIVNR
VRQGYSPLSF QTLTPSPREP DRLRGIEEEG GEQDKGRSIR LVQGFLALAW
DDLRSLCLFS YHRLRDFISI AARVVEVLGH SSLRGLQRGW EALKYLKSLV
QYWGLELKKS AVSLLDTLAI AVGEGTDRII ELIQGICRAI RNIPRRIRQG FEAALL
(SEQ ID No. 65)

Example 5. Vaccination with PokA-79-PBMC strain envelop protein cocktails and challenging with PokA-79-PBMC in SCID-PBMC mice.

Example 5A: Immune responses for gp160-1 and gp160-2 in challenged with PokA-79-PBMC strain SCID-PBMC Mice. The result is shown in Figure 25 A).

Example 5B: Recombinant gp160-1 and gp160-2 immunizations effect for SCID-PBMC challenging with PokA-79-PBMC 6th passage strain. The result is shown in Figure 25 B).

Example 5C: Recombinant gp160-PokA-PBMC and gp160-U455-PBMC immunization effect for challenging with PokA79-PBMC and U455-PBMC 6-8 weeks strains. The result is shown in Figure 25 C).

Immunization effect tests were completed for HIV subtype A laboratory strain's PokA-79 and U455 "playback" cultivated in vitro on isolated healthy donor PBMC for 5-6 weeks. The application of principle of mass spectrometry analysis of represented in majority HIV-1 gp120/gp160 epitopes for vaccine development brought the material for SCID-PBMC immunizations. These immunizations occurred to be able to prevent or suppress further animal's challenging with the same laboratory HIV strain which aminoacid sequences recognitions were used for design of primers for major gp120/gp160 epitope's cloning and expression.

The groups of animals were treated as follows: 4-5 weeks old SCID mice were inoculated with 5-10x10^6 of pre-cultivated for 3 days in presence of FHG and IL-2 healthy donor PBMC intraperitoneally once a week. Two weeks later the 1st PBMC inoculation mice were divided into groups (3 animals in every group) and immunized subcutaneously with peptides mixture gp160-1 (PokA79-PBMC) or gp160-2 (U455-PBMC). Each animal received 25 mkg proteins in cocktail mixed in 200 mkl incomplete Freund adjuvant for 1 hour at +4C. Immunization was repeated three times with the between period of two weeks. Immune response was tested in ELISA assays (Fig. 25A). Three days after the last immunization and the scheduled PBMC inoculation animals were challenged intraperitoneally with log phase 5x10^6 PokA-79-PBMC (Fig. 25 B), PokA-79-PBMC and U455-PBMC "playbacks" (Fig. 25 C). Viral RNA titre in cell material used for challenging should be minimum 5x10^7 copies per ml, preferably not less than 10^{10} copies per ml; in vitro material controls were stored for Real Time PCR measurements. The blood of the same donor was used for PBMC isolation for mice engraftment and "playbacks" cultivation. Control Hu-SCID mice were challenged intraperitoneally with the same amount of PokA-79-PBMC and U455-PBMC "playbacks" in the same time with experimental groups using background PBMC engraftment but without

previous immunizations. Challenges were completed 2 times with three days periods between, Hu-SCID mice were provided with fresh portions of PBMC weekly. Animals were sacrificed one week after the second challenging and blood serum samples were taken for ELISA and Real Time PCR measurements.

There were three groups of SCID-PBMC immunized with gp160-1 and four groups immunized with gp160-2; serums of BalbC mice immunized with the same cocktails gp160-1 and gp160-2 were taken as controls for immunization results detection (Fig. 25 A, B). In mice serums diluted 4-14 times with quantitative calibration curve gp160-1 and gp160-2 antibodies titre were detectable for all SCID-PBMC animals, however control BalbC mice HIV env-specific antibodies titre were always higher (approximately 2-2.5 times in ELISA test) than SCID-PBMC animals titre. Gp160-1 and gp160-2 immune responses assessment were positive for immunized SCID-PBMC mice for at least 2 weeks after the last immunization (Fig. 25 A).

HIV titre in bloodstream measured with Real Time PCR was not detectable in 4 groups immunized with gp160-2. In one of 3 groups immunized with gp160-1 two animals provided challenging rate 400 and 500 HIV RNA copies/ml (Fig. 25 B). 3rd group's viral load "leakage" (Positive PokA-79 viral RNA titre in bloodstream, Fig. 25 B) correlates with the lowest ELISA gp160-1 antibodies titre evaluated for the same SCID-PBMC – gp160-1 animal's group (Fig. 25 A). SCID-PBMC mice challenged with PokA-79-PBMC "playback" without immunization pre-treatment were tested as positive controls (Fig. 25 B, C). Challenging did not work in 2 animals from different groups for both "playbacks", we suppose it can happen in case of alterations in immune deficiency status of SCID animals. However these results are normal deviation for group's statistic analysis. Both PokA-79-PBMC and U455-PBMC "playbacks" proteins cocktails immunizations led to diminishing number of positive challenging cases (Fig. 25 B) or to blockade of challenging in Hu-SCID animals engrafted with "playbacks" of the same HIV strain (Fig. 25 C).

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Claims

1. A method for producing an HIV vaccine composition, comprising the steps of:
   a) creation of a library comprising HIV-1 specific antibodies,
   b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,
   c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
   d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,
   e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
   f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
   g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
   h) purification of the glycosylated env HIV-1 peptides, and
   i) production of a vaccine composition,

   characterized in that for cloning and/or expressing glycosylated env HIV-1 peptides in step f) and/or g), at least one primer selected from the group consisting of:

   (i) V1 forward for subtypes A, G B, C, F1, H:
   5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1),

   (ii) V2 forward for all subtypes:
   5'- AAA ACT GCT CTT WCA XXX XXX XXX- 3' (SEQ ID No. 3),

   (iii) V3 forward for A, B, G subtypes:
   5'- TAV AAA TTA ATT GTA XXX XXX XXX X- 3' (SEQ ID No. 5),

   (iv) V3 forward For subtype D:
   5'- TAV CAA TTA ATT GCA XXX XXX XXX X- 3' (SEQ ID No. 7),

   (v) V4 forward for all subtypes:
   5'- GAA TTT TTC TAT TGY AAXXX XXX XXX- 3' (SEQ ID No. 9),

   (vi) V5 forward for A,B,D,G subtypes:

5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11), and

(vii) V5 forward for subtype C:
5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13),

and at least one primer selected from the group consisting of:

(viii) gp41(160) Reverse for A, B, D subtypes:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46),

(ix) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(x) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(xi) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

(xii) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

(xiii) gp120 Reverse Const5 for all subtypes:
5'- ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT CTT TTT TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 52),

and

(xiiii) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

are used.

2. The method according to claim 1, wherein 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 or more variants of primer (i), (ii), (iii), (iv), (v), (vi) and/or (vii) are used, wherein the variants differ in the variables "X".

3. The method according to claim 1 or 2, wherein the primers (i) to (vii), and (x) to (xii) and one of (xiii) and (xiiii) and one of (viii) and (ix) are used.

4. A method for producing an HIV vaccine composition, comprising the steps of:
   a) creation of a library comprising HIV-1 specific antibodies,
   b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,
   c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
   d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,
   e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
   f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
   g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
   h) purification of the glycosylated env HIV-1 peptides, and
   i) production of a vaccine composition,

   characterized in that for cloning and/or expressing glycosylated env HIV-1 peptides in step f) and/or g), at least one primer selected from the group consisting of:

   (i) Forward gp120 Const1 for A subtype:
   5'- AAT TCT AGA CRC TRC AGA AAA CTT GTG GGT YAC - 3'(SEQ ID No. 52), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

   (ii) Forward gp120 Const1 for B subtype:
   5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3' (SEQ ID No. 69), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iii) Forward gp120 Const1 for C subtype:
5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3' (SEQ ID No. 70), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end, and

(iv) Forward gp120 Const1 for G subtype:
5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3'(SEQ ID No. 71), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

and at least one primer selected from the group consisting of:

(v) V1 reverse for all subtypes:
5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25),

(vi) V2 reverse for subtypes A, D, C:
5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28),

(vii) V2 reverse for subtype B:
5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29),

(viii) V2 reverse for subtype G:
5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30),

(ix) V2 reverse for subtype F1:
5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31),

(x) V3 reverse for subtypes A, D, (C):
5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40),

(xi) V3 reverse for subtype B:
5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41),

(xii) V4 reverse for subtype G:
5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3' (SEQ ID No. 19),

(xiii) V4 reverse for subtypes A, B, C, D:

5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No. 20),

(xiv) V5 reverse for all subtypes:
5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3', (SEQ ID No. 15),

(xv) gp41(160) Reverse for A,B,D subtypes:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46),

(xvi) a primer derivative of (viii) lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(xvii) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(xviii) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

and

(xix) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

are used.

5. The method according to claim 4, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, or more variants of primer (v), (vi), (vii), (viii), (ix), (x), (xi), (xii), (xiii) and/or (xiv) are used, wherein the variants differ in the variables, in particular the variables "X".

6. The method according to claim 4 or 5, wherein the primers (i) to (xiv), and (xvii) to (xix) and one of (xv) and (xvi) are used.

7. A method for producing an HIV vaccine composition, comprising the steps of:

a) creation of a library comprising HIV-1 specific antibodies,
b) enrichment for HIV-1-specific antibodies in the library by panning with HIV-1 peptides, in particular native and/or recombinant HIV-1 peptides,
c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1-specific antibodies of step b) bound to a support,
e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
h) purification of the glycosylated env HIV-1 peptides, and
i) production of a vaccine composition,

wherein the glycosylated env HIV-1 peptides in step g) are characterized by:

(i) the gp120 peptides have a length of 441-541 amino acids and/or a glycosylated protein molecular weight of about 90 - 115kDa, and

(ii) the gp160 peptides have a length of 816-923 aminoacids, and/or a glycosylated protein molecular weight of about 145-175kDa, and

(iii) at least 50, preferably at least 70, more preferably at least 100, even more preferably at least 150 different HIV-1 envelop peptides are expressed.

8. A method according to claim 7, wherein the vaccine composition of step i) comprises
(a) at least one peptide characterized by the N-terminal sequence LCVTL (SEQ ID No. 72)
(b at least one peptide characterized by the N-terminal sequence NCSX, wherein X is Y or F (SEQ ID No. 73)
(c) at least one peptide characterized by the N-terminal sequenceXINC, wherein X is K or Q or E (SEQ ID No. 74),
(d) at least one peptide characterized by the N-terminal sequence XINC, wherein X is T or P or A (SEQ ID No. 75),
(e) at least one peptide characterized by the N-terminal sequence EFFYC (SEQ ID No. 76),

(f) at least one peptide characterized by the N-terminal sequence TRDG (SEQ ID No. 77),

and

(g) at least one peptide characterized by the N-terminal sequence LDXXENLWVLD, wherein X is T or A, independently from each other (SEQ ID No. 78).

9. A HIV vaccine composition, obtainable by a method according to any of claims 1 to 8.

10. A HIV vaccine composition comprising at least 50, preferably at least 70, more preferably at least 100, even more preferably at least 150 different HIV-1 envelop peptides, characterized in that:

(i) the gp120 peptides have a length of 441-541 amino acids and/or a glycosylated protein molecular weight of about 90 - 115kDa, and

(ii) the gp160 peptides have a length of 816-923 amino acids, and/or a glycosylated protein molecular weight of about 145-175kDa.

11. The vaccine composition of claim 10, further comprising:

(a) at least one peptide characterized by the N-terminal sequence LCVTL (SEQ ID No. 72)

(b) at least one peptide characterized by the N-terminal sequence NCSX, wherein X is Y or F (SEQ ID No. 73)

(c) at least one peptide characterized by the N-terminal sequence XINC, wherein X is K or Q or E (SEQ ID No. 74),

(d) at least one peptide characterized by the N-terminal sequence XINC, wherein X is T or P or A (SEQ ID No. 75),

(e) at least one peptide characterized by the N-terminal sequence EFFYC(SEQ ID No. 76),

(f) at least one peptide characterized by the N-terminal sequence TRDG (SEQ ID No. 77),

and

(g) at least one peptide characterized by the N-terminal sequence LDXXENLWVLD, wherein X is T or A, independently from each other (SEQ ID No. 78).

12. A composition comprising at least one of following nucleic acids:

(i) V1 forward for subtypes A, G, B, C, F1, H:

5'- CTC TGY GTY ACT TTA XXX XXX XXX- 3' (SEQ ID No. 1),

(ii) V2 forward for all subtypes:
5'- AAA ACT GCT CTT WCA XXX XXX XXX- 3' (SEQ ID No. 3),

(iii) V3 forward for A, B, G subtypes:
5'- TAV AAA TTA ATT GTA XXX XXX XXX X- 3' (SEQ ID No. 5),

(iv) V3 forward For subtype D:
5'- TAV CAA TTA ATT GCA XXX XXX XXX X- 3' (SEQ ID No. 7),

(v) V4 forward for all subtypes:
5'- GAA TTT TTC TAT TGY AAXXX XXX XXX- 3' (SEQ ID No. 9),

(vi) V5 forward for A, B, D, G subtypes:
5'- ACA AGA GAT GGT GGX XXX XXX X- 3' (SEQ ID No. 11),

(vii) V5 forward For subtype C:
5'- ACA CGT GAT GGA GGX XXX XXX X- 3' (SEQ ID No. 13),

(viii) gp41(160) Reverse for A,B,D subtypes
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46) or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(ix) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66),

(x) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

(xi) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68),

and

(xii) gp120 Reverse Const5 for all subtypes:
5'- ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT CTT TTT TCT CTY TSC ACC ACT CTY CT - 3' (SEQ ID No. 52), or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

13. A composition comprising at least one of following nucleic acids:

(i) Forward gp120 Const1 for A subtype:
5'- AAT TCT AGA CRC TRC AGA AAA CTT GTG GGT YAC - 3' (SEQ ID No. 52), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end

(ii) Forward gp120 Const1 for B subtype:
5'- AAT TCT AGA CGC TRC AGA AMA ATT GTG GGT CAC - 3'(SEQ ID No. 69), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iii) Forward gp120 Const1 for C subtype:
5'- AAT TCT AGA CGT RRT GGG RAA CTT GTG GGT CAC - 3' (SEQ ID No. 70), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(iv) Forward gp120 Const1 for G subtype:
5'- AAT TCT AGA CGC CTC ARA TAA CTT GTG GGT CAC AG - 3'(SEQ ID No. 71), or a derivative thereof lacking the *Xba*I site, and/or comprising a restriction site at the 5' end,

(v) V1 reverse for all subtypes:
5'- GCA GTT TTT YAT TTC TYX XXX XXX XXX- 3' (SEQ ID No. 25),

(vi) V2 reverse for subtypes A, D, C:
5'- AGG TAT TRC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 28),

(vii) V2 reverse for subtype B:
5'- CTG AGG TRT TAC AAX XXX XXX X- 3' (SEQ ID No. 29),

(viii) V2 reverse for subtype G:
5'- AGA CAT TAC AAT TTA TTX XXX XXX X- 3' (SEQ ID No. 30),

(ix) V2 reverse for subtype F1:
5'- TTG AGG TAT TRC AAX XXX XXX X- 3' (SEQ ID No. 31),

(x) V3 reverse for subtypes A, D, (C):
5'- AAA GTT TBA TTC CAX XXX XXX XX- 3' (SEQ ID No. 40),

(xi) V3 reverse for subtype B:
5'- AAA GTG TTR TTC CAX XXX XXX XX- 3' (SEQ ID No. 41),

(xii) V4 reverse for subtype G:
5'- CAA TTT GTT TTA TYY TAC A XX XXX XXX X- 3'(SEQ ID No. 19),

(xiii) V4 reverse for subtypes A, B, C, D:
5'- TAA TTT GYT TTA TTY TGC A XX XXX XXX X- 3' (SEQ ID No.20),

(xiv) V5 reverse for all subtypes:
5'- TCC TCC TSC AGG TCT GAA XXX XXX XXX X- 3', (SEQ ID No. 15),

(xv) gp41(160) Reverse for A,B,D subtypes:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AG YAA AGC YCT TTC NAA GCC CTG TC (SEQ ID No. 46), or a derivative thereof lacking the sequence encoding a His-Tag, and/or comprising a sequence encoding a tag,

(xvi) gp41(160) Reverse for subtype A, rare variant:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN GCG CCC TGT C (SEQ ID No. 66)

(xvii) gp41(160) Reverse for C subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT WGC AAA GCT GCT TCA AAG CCC TGT C (SEQ ID No. 67),

and

(xviii) gp41(160) Reverse for G subtype:
ATA GCG GCC GCC TAG TGG TGG TGA TGG TGG TGT AGC AAA GCY CTT TCN AAG CCT TGT C (SEQ ID No. 68).

14. The use of a Severe Combined T-B-Immune Deficient (SCID) mouse engrafted with human immunocompetent cells (Hu-SCID-mouse) as an animal model for the evaluation of the effectiveness of an HIV vaccine.

15. The use of claim 14, wherein the immunocompetent cells are able to develop a human-type immune reaction for HIV.

16. The use of claim 14, wherein the human immunocompetent cells are PBMC, in particular PBMC pre-cultivated in vitro, more particularly PBMC pre-cultivated in vitro for about 1 day to about 6 weeks.

17. The use of claim 14, wherein the human immunocompetent cells are dendritic cells.

18. The use of claim 14, wherein the immunocompetent cells are a mixture of PBMC and dendritic cells.

19. The use of claim 18, wherein the dendritic cells have been obtained by culturing human PBMC in the presence of cytokines capable of inducing the formation of dendritic cells in vitro.

20. The use of any of claims 14 to 19, wherein the human immunocompetent cells are derived from one human donor.

21. The use of any of claims 14 to 20, wherein the mouse has been engrafted with 5-15x10^6 cells, in particular immunocompetent cells.

22. The use of any of claims 14 to 21, wherein the Hu-SCID mouse is the particular breed of animals deficient in their own endogenous immune system and introduced with human immunocompetent cells or any other human cells.

23. A method for the evaluation of an HIV vaccine, wherein a Hu-SCID-mouse as defined in any of claims 14 to 22 is inoculated with the HIV vaccine and thereafter challenged with HI-virus.

24. The method of claim 23, wherein the HIV vaccine is an HIV-1 envelop peptides/proteins cocktail, preferably wherein the proteins or peptides are of recombinant origin.

25. The method of any of claims 14 or 24, wherein the evaluation of the vaccine is determined by determining the efficacy of the vaccine.

26. The method of claim 25, wherein the efficacy is determined by determining the protection to HIV challenge.

27. The method of any of claims 23 to 26, wherein the Hu-SCID-mouse is inoculated with the HIV vaccine 1 to 4 weeks after the last engrafting of the human immunocompetent cells.

28. The method of any of claims 23 to 27, wherein an infectious dose of $5x10^2$ - 10^4 $TCID_{50}$ or higher, in particular up to up to 10^7 $TCID_{50}$ for challenging of one Hu-SCID animal is used.

29. The method of any of claims 23 to 28, wherein the efficacy of the HIV vaccine is determined by determining the viral load and / or the specificity an / or the intensity of an immune response in a body fluid sample of the mouse.

30. The method of claim 29, wherein the body fluid sample is blood serum.

31. The method of any of claims 23 to 30, wherein the inoculation with a vaccine is performed in the presence of an adjuvant.

32. The method of any of claims 23 to 31, wherein HIV-specific immune response is detectable within the period of several weeks after the last vaccination.

33. The method of any of claims 23 to 32, wherein the evaluation of the vaccine is carried out by means of RT-PCR, Real Time PCR or ELISA, in particular by means of RT-PCR, Real Time PCR and ELISA.

34. The method of any of claims 14 to 33, wherein at least one HIV laboratory strain playback is used to infect and/or expose the Hu-SCID mice with/to HIV.

Figures

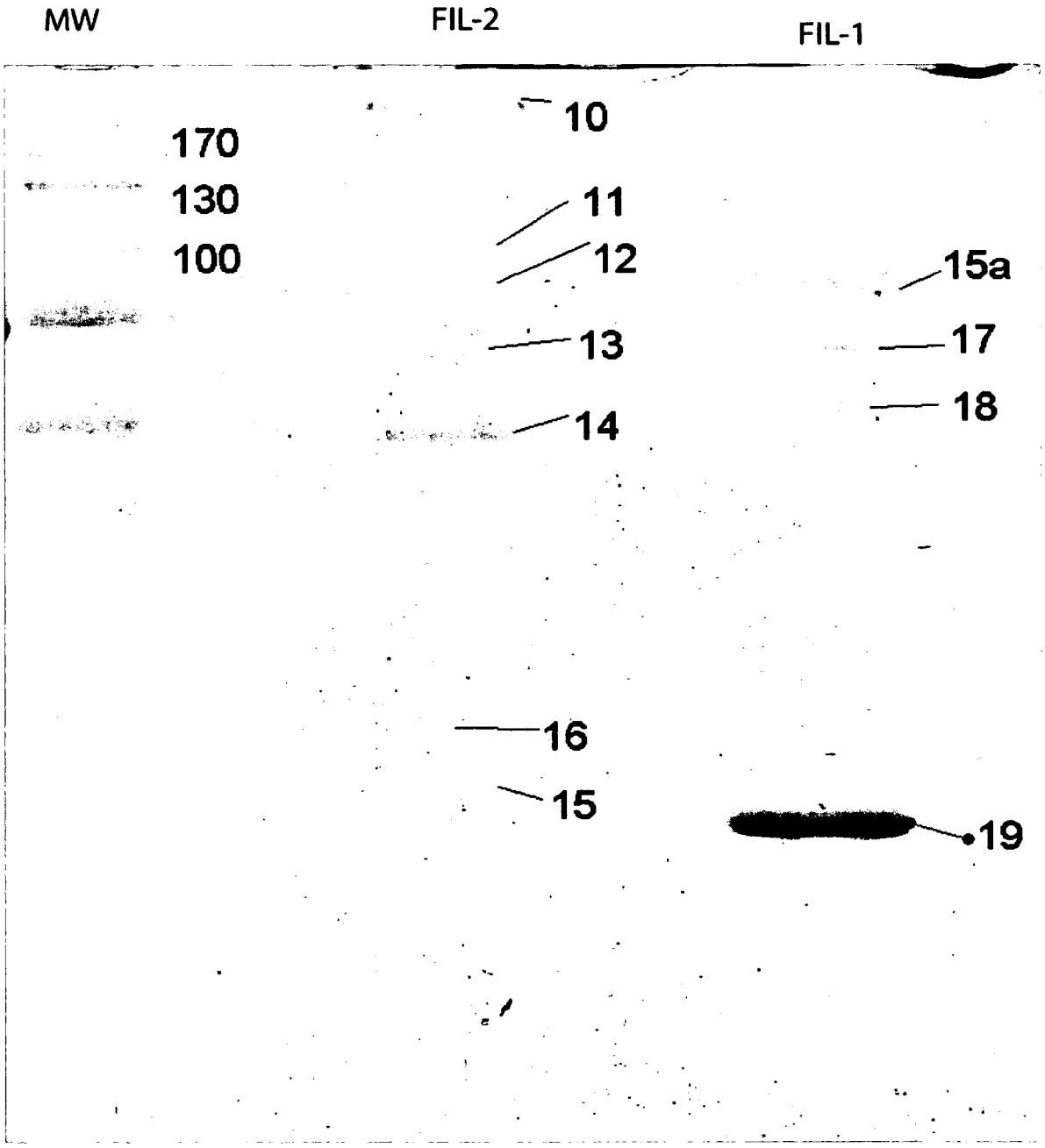


Figure 1 A

Continuation of Figure 1

B

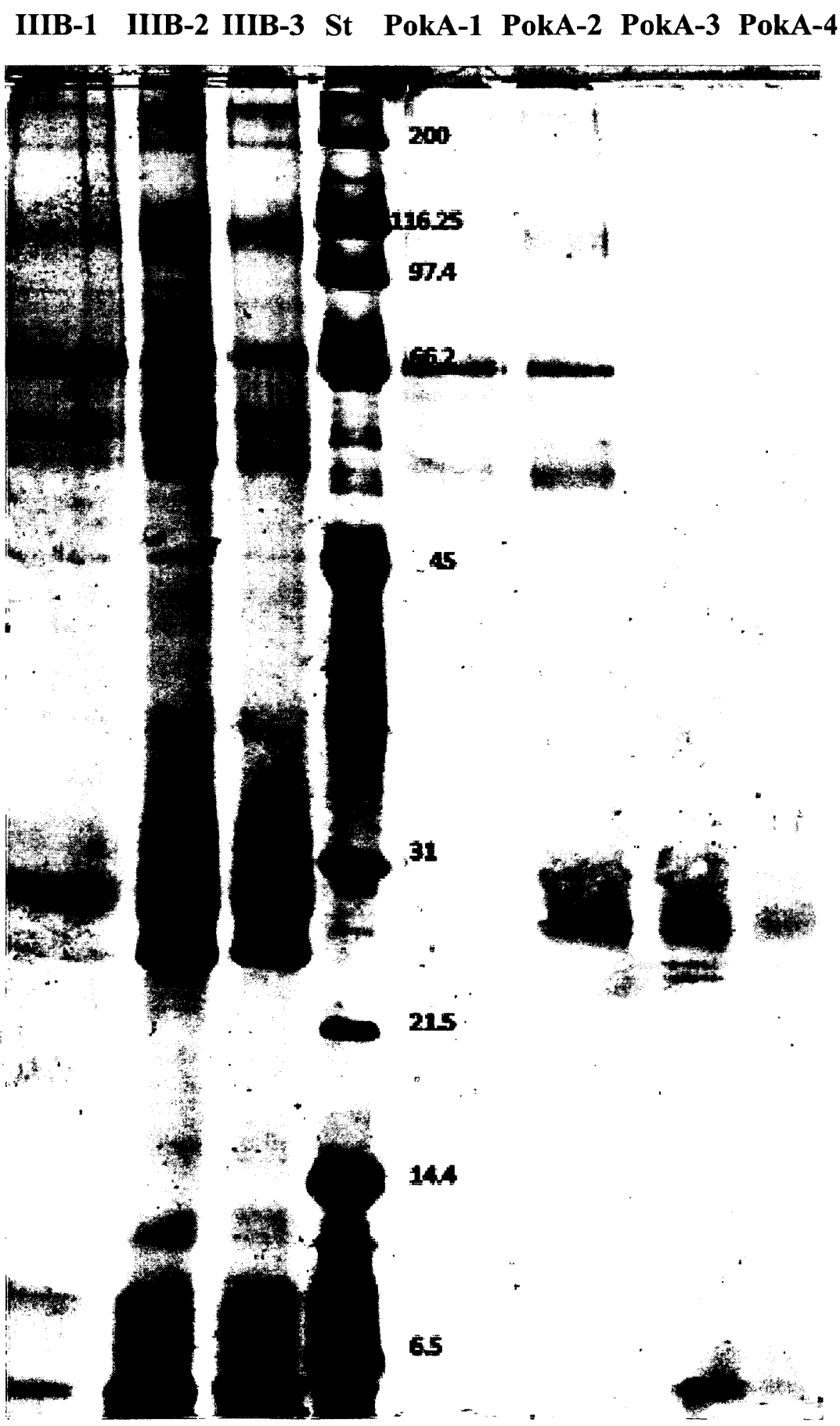


Figure 2: SCID Mice Immune Deficiency Compared to BalbC Immune Response for Isolated U455 Lab. Strain Proteins

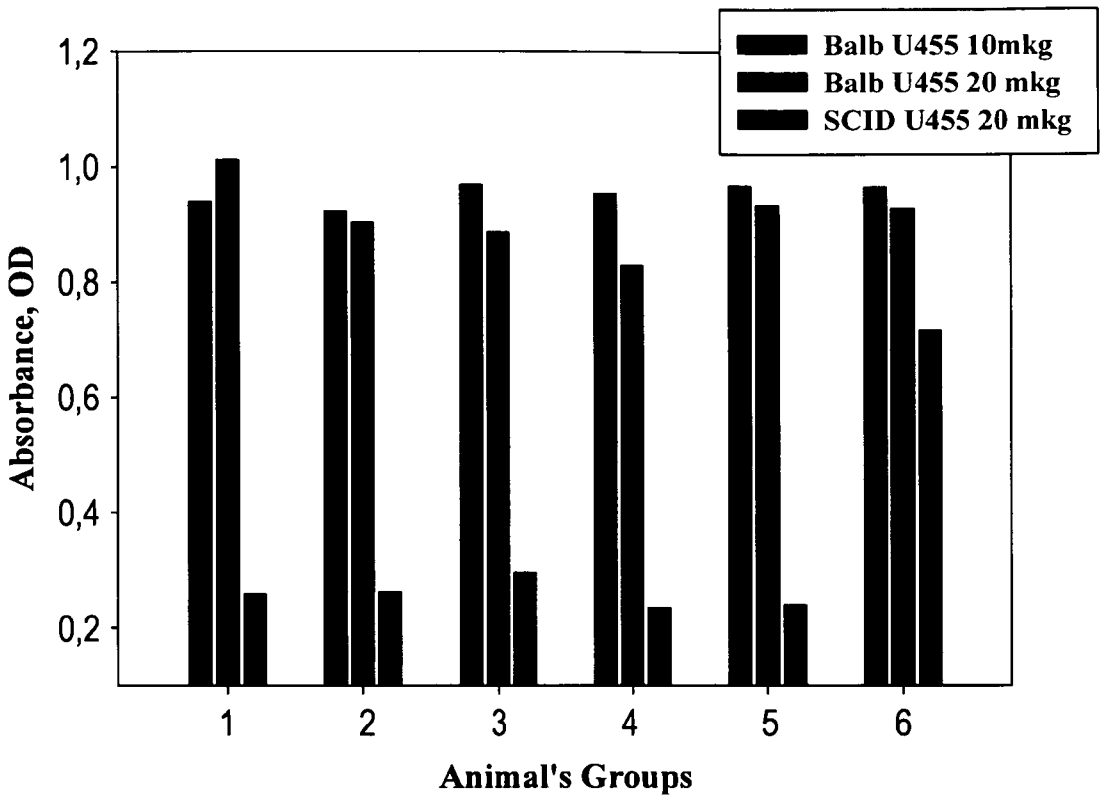


Figure 3: SCID-PBMC and BalbC Immune Response for Immunization with Coctails of Recombinant p120-1 and p120-2 Proteins

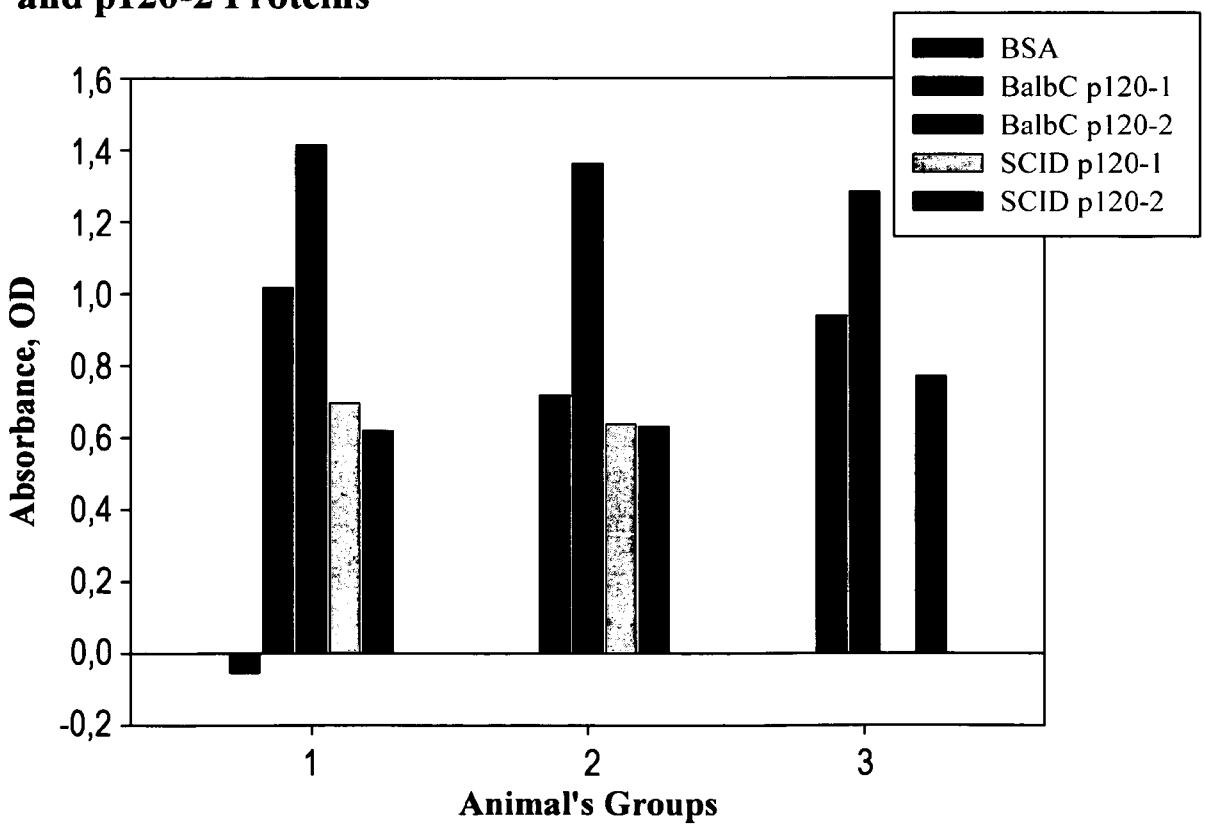


Figure 4: PokA-79 Viral Load Rates on U937 and MT-4 Background, 3 Challenges 7 Days b/w Period

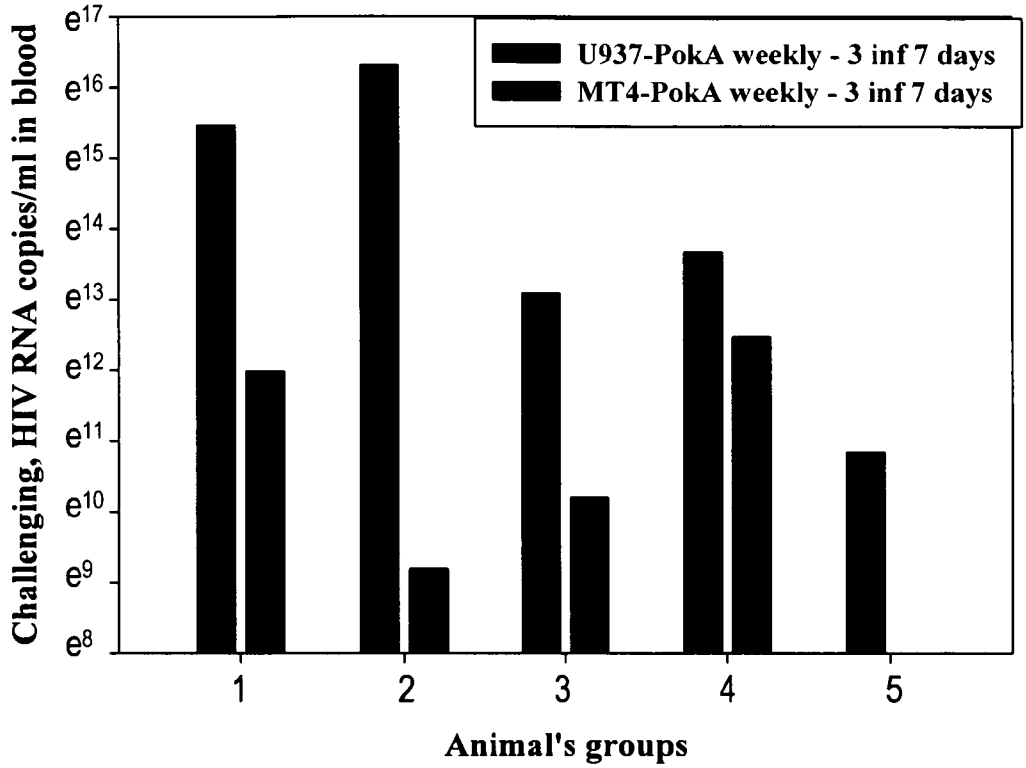


Figure 5: PokA-79 Viral Load Rates on U937 and MT-4 Background, 2 Challenges 3 Days b/w Period

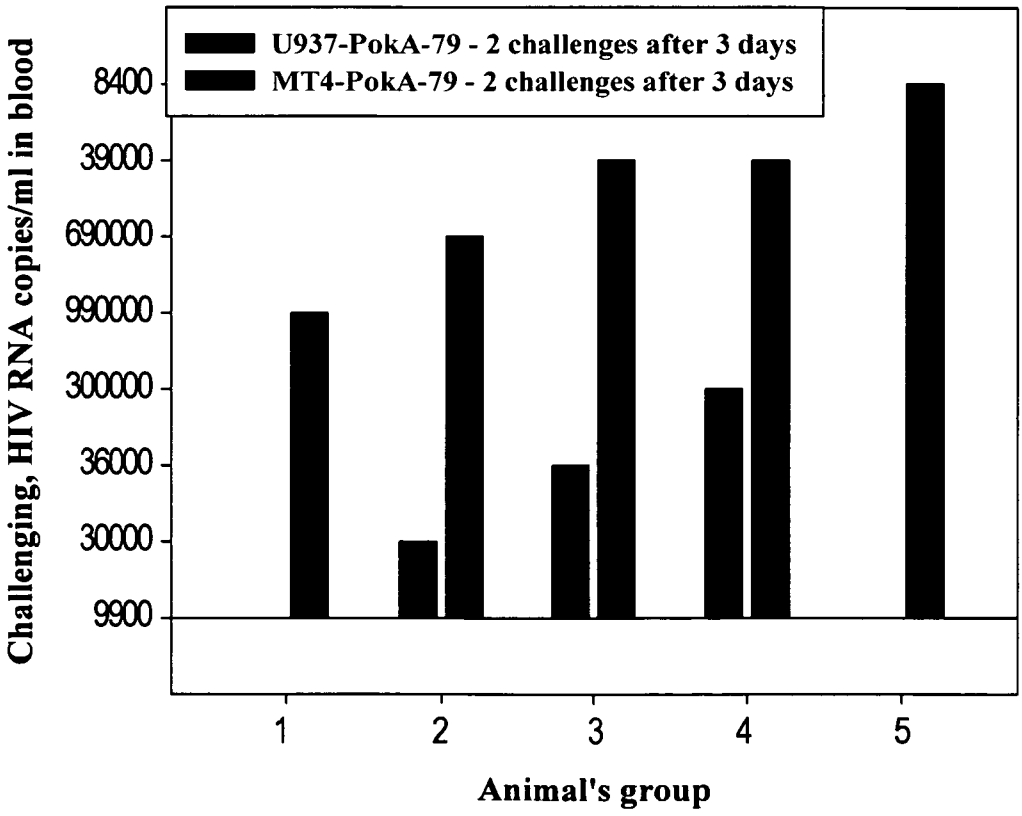


Figure 6: PokA-79 - MT4 and U937-background's Viral Load Rates for Different Periods between Challenging in SCID Mice

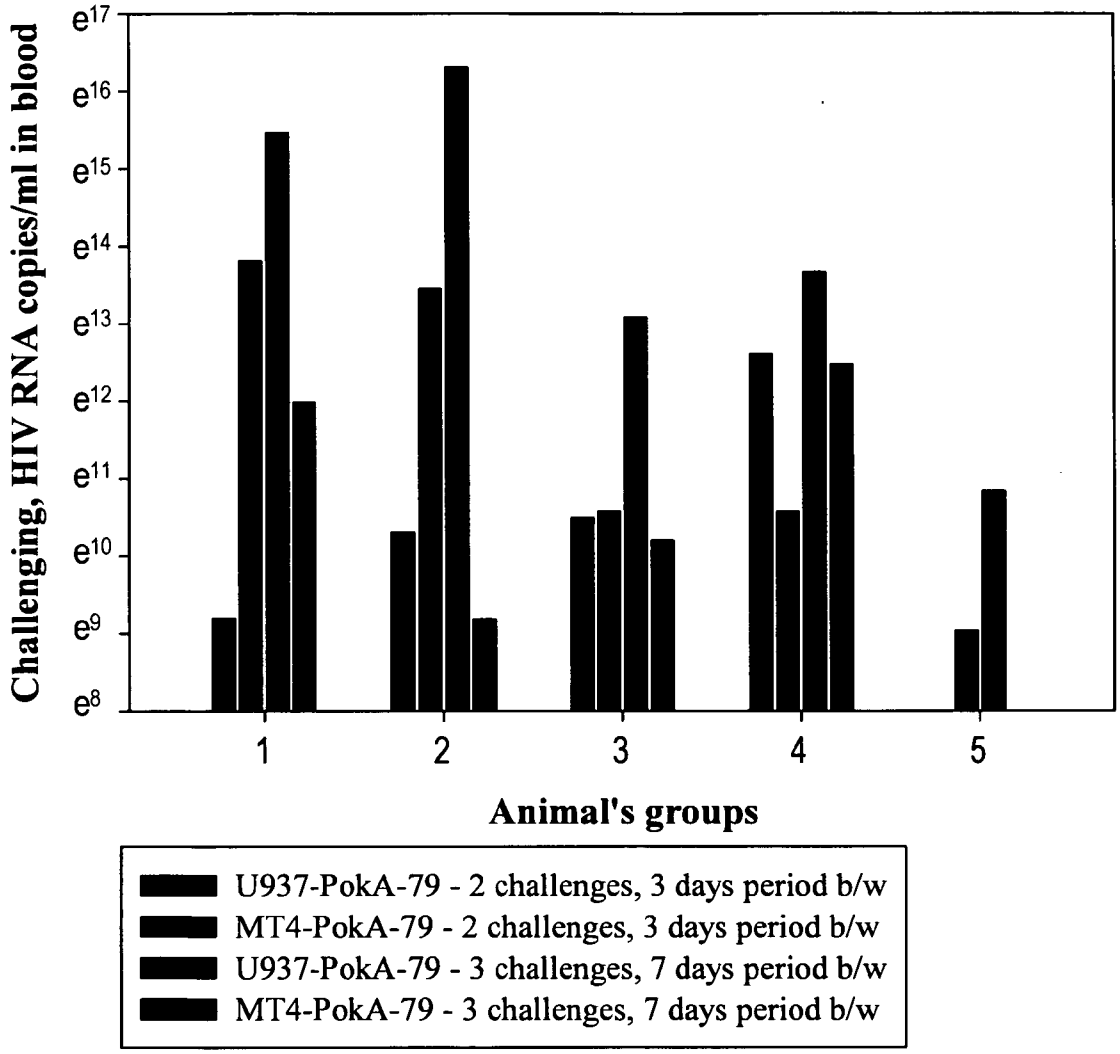


Figure 7: PokA-79 in vitro Replication Dynamics, Lymphocyte MT-4, Macrophage U937, PBMC and DC Background

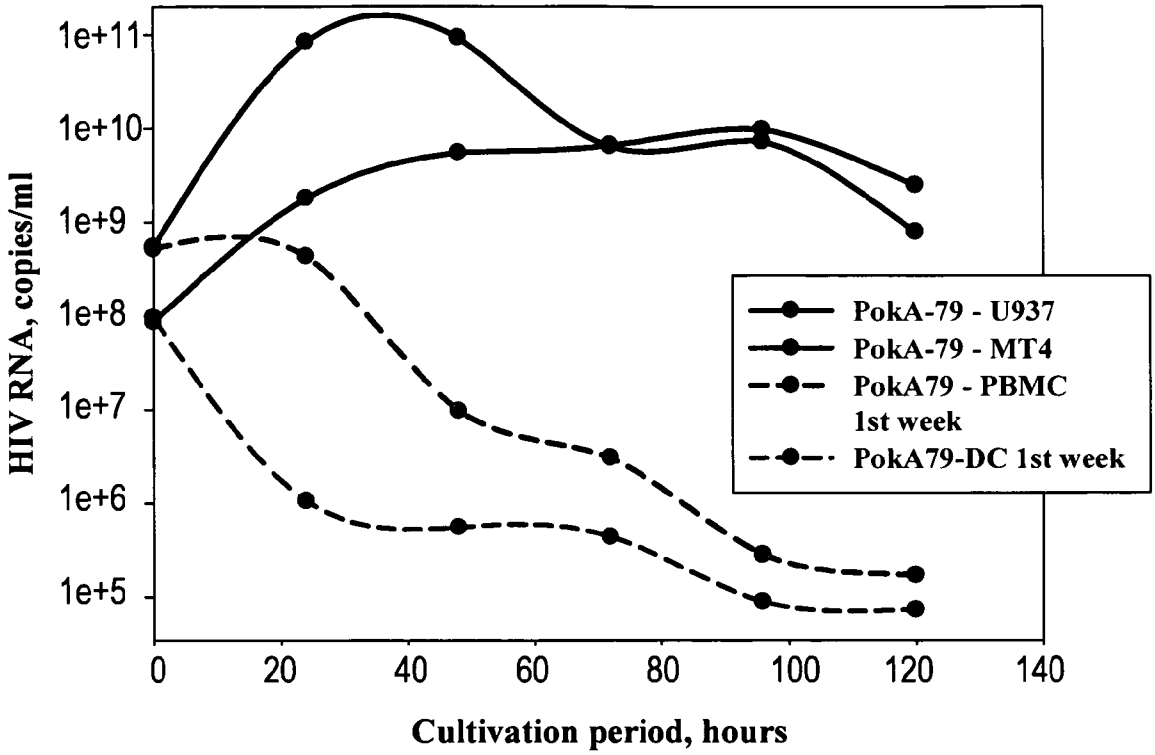


Figure 8: PokA-79 in vitro Replication Dynamics, p24 Expression, Lymphocyte MT-4 and Macrophage U937 Background

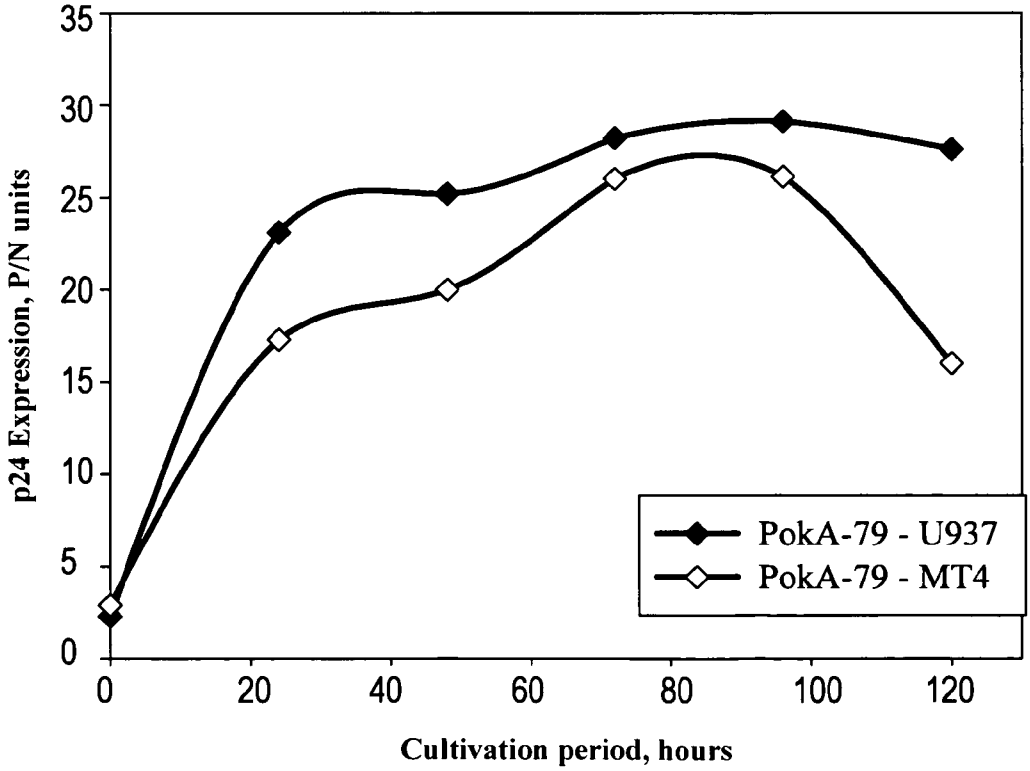
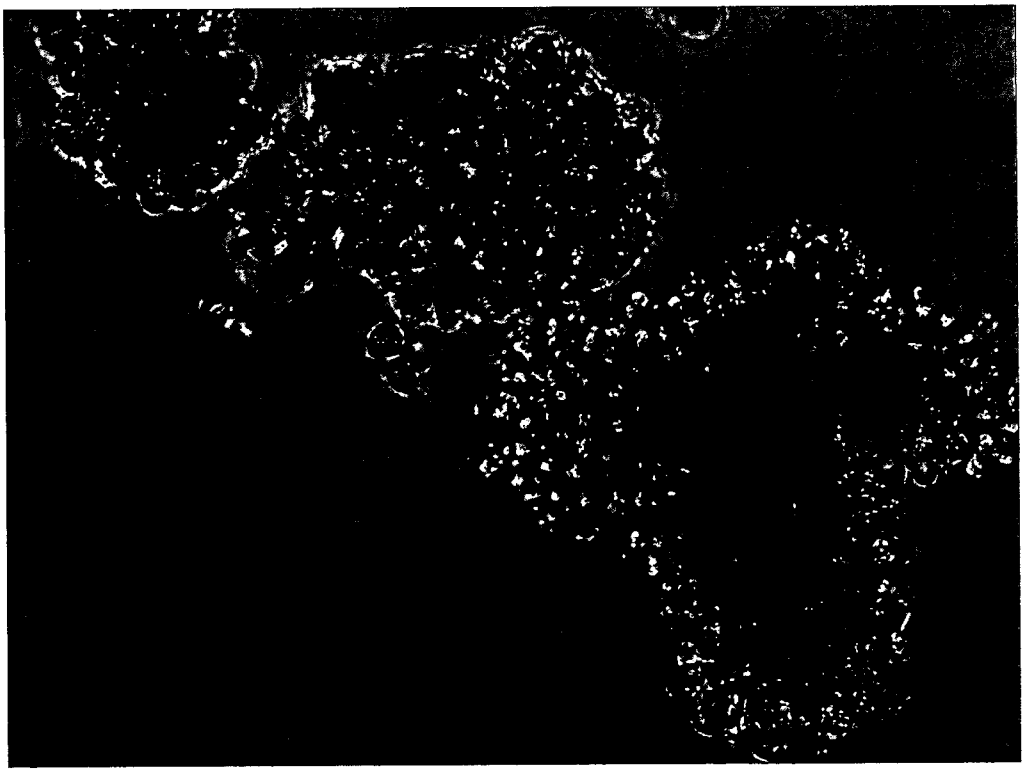


Figure 9

A



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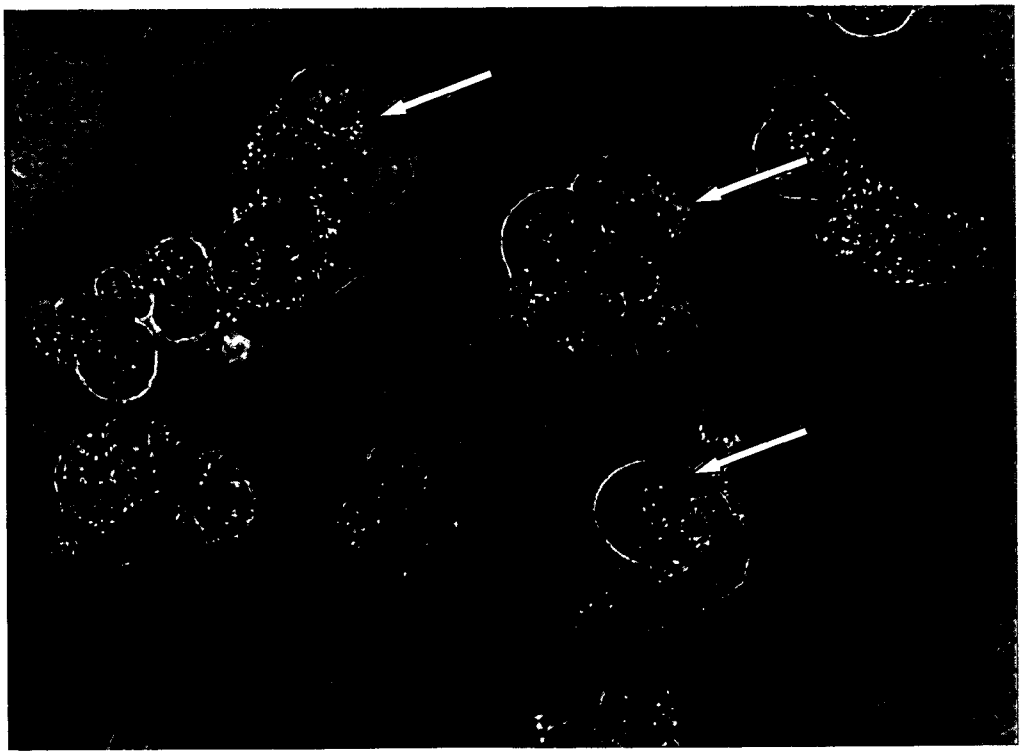
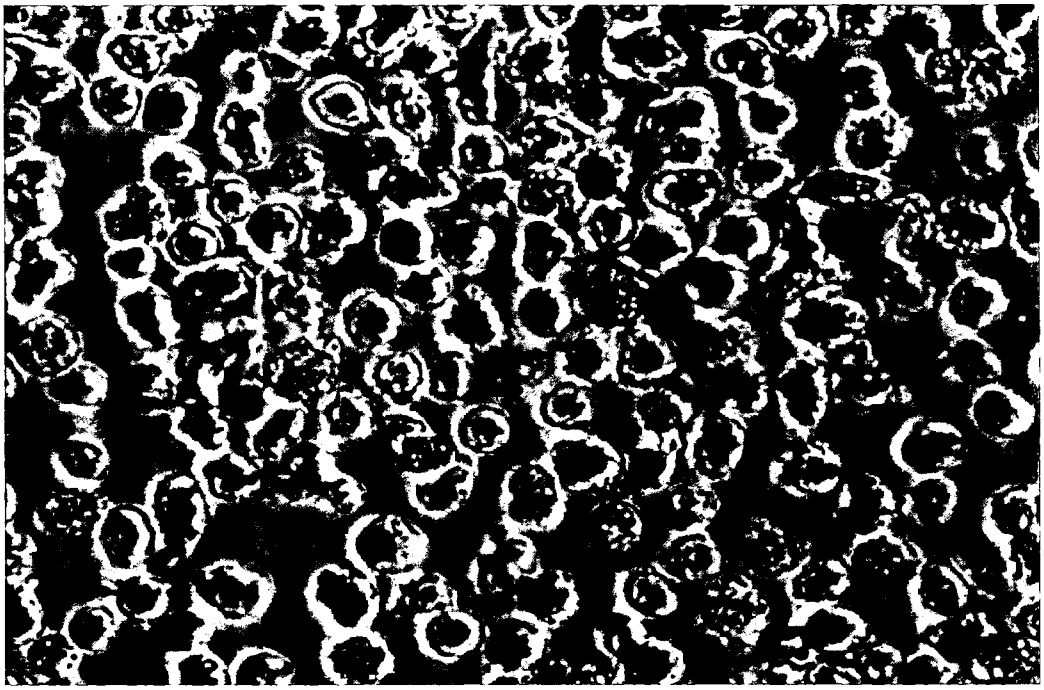


Figure 10

A



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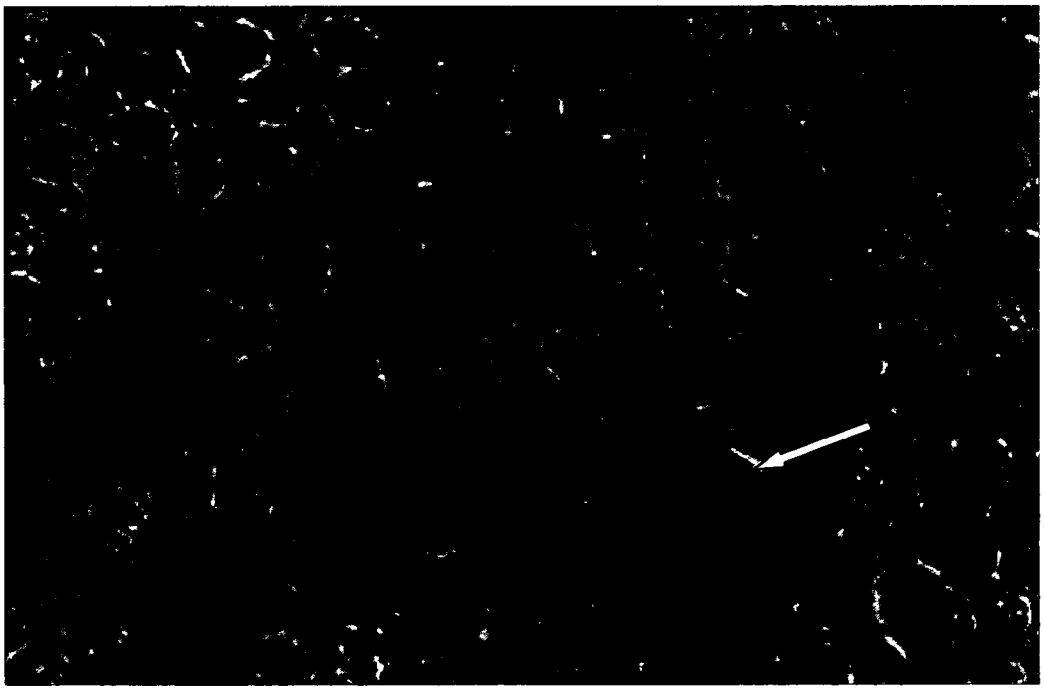


Continuation of Figure 10

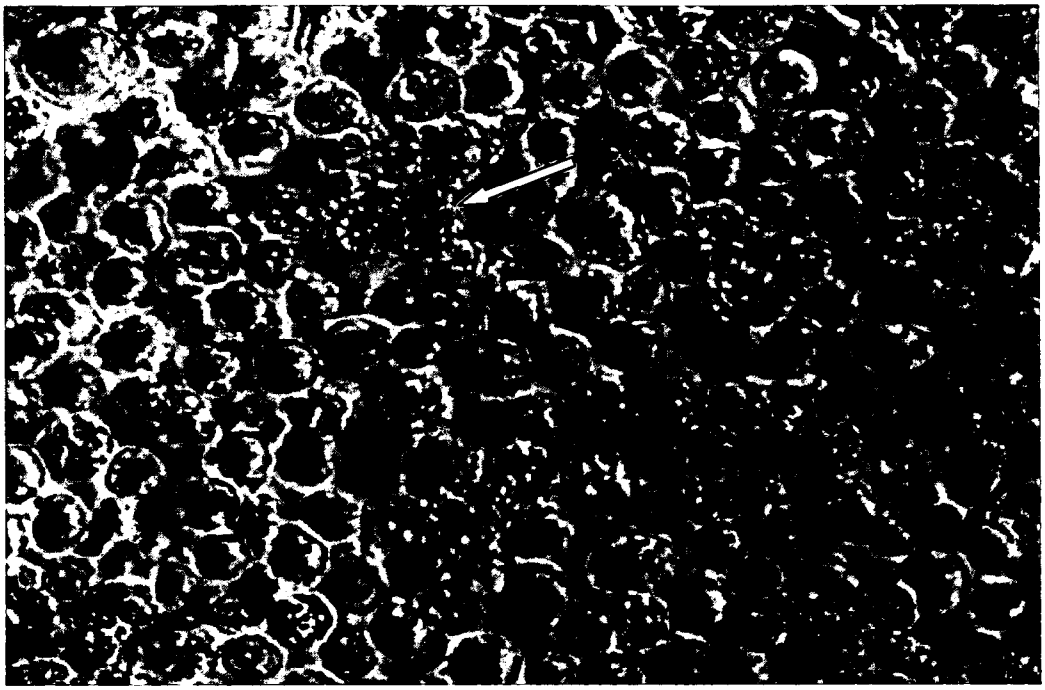
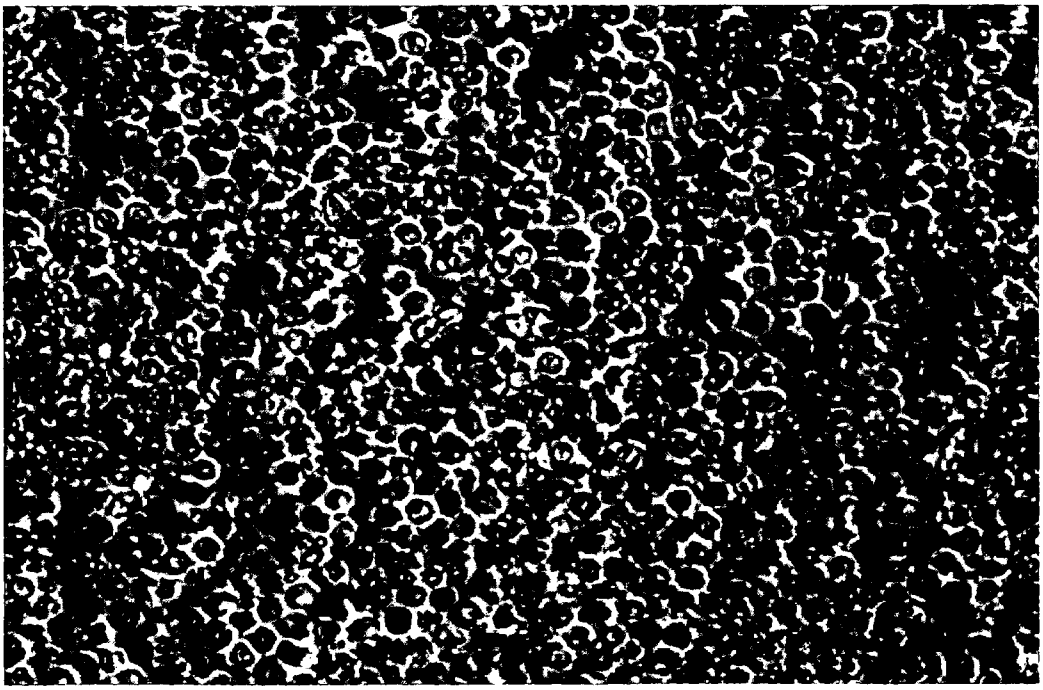
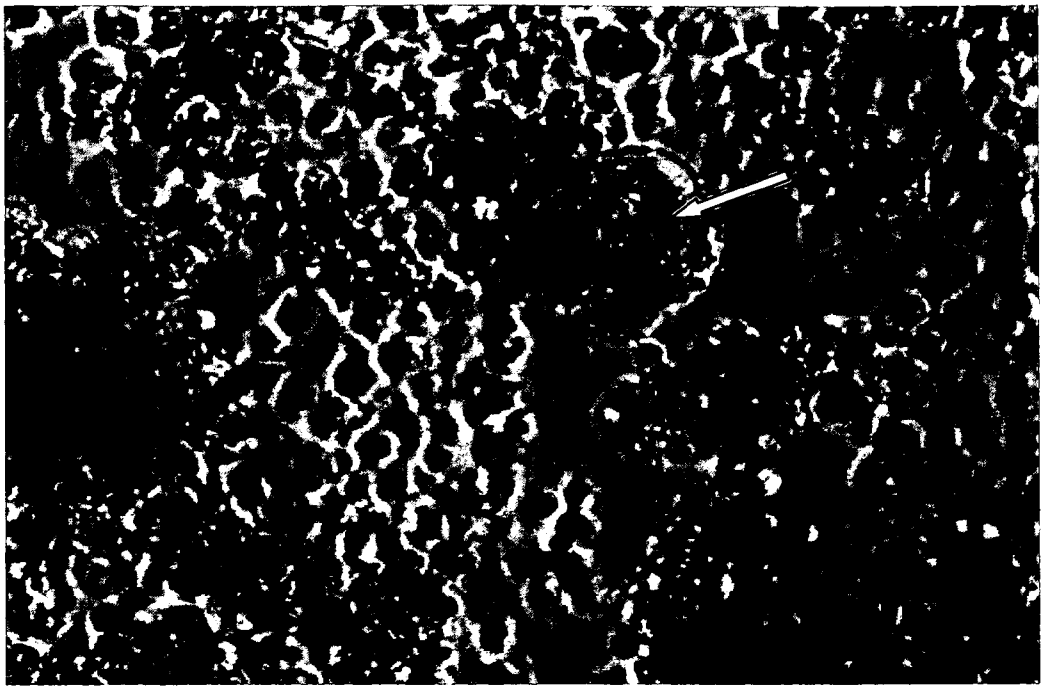
C 

Figure 11

A 

Continuation of Figure 11

B



C

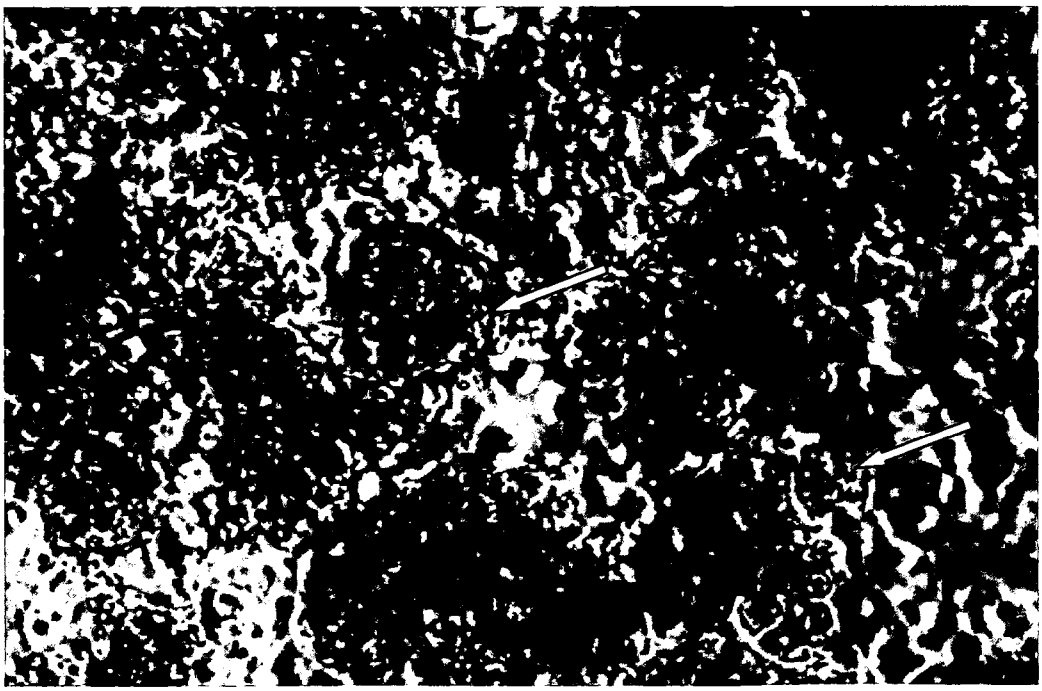
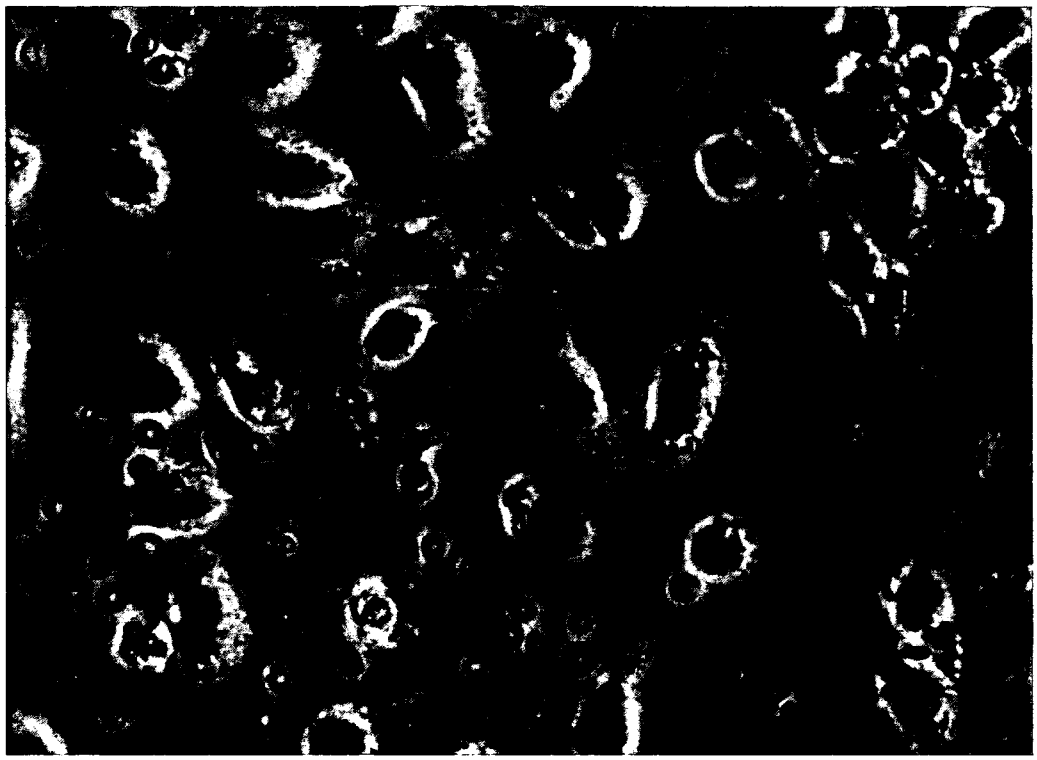


Figure 12

A



B

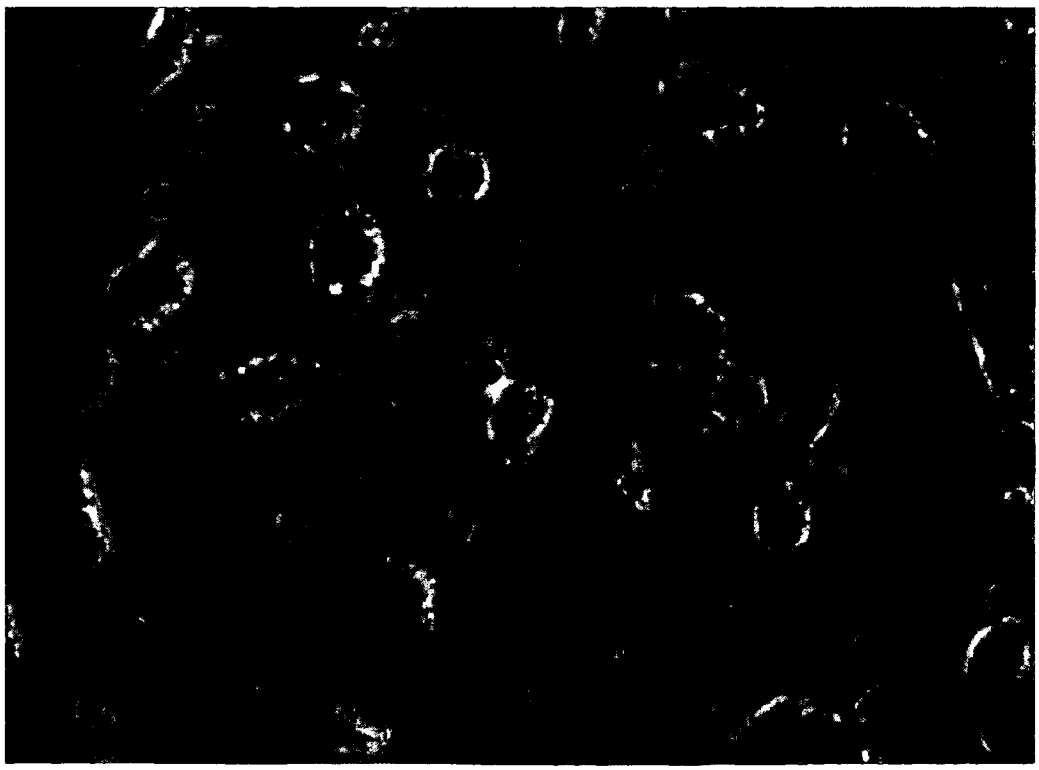


Figure 13: Laboratory Strains U455 and IIIB/H9 Viral Load Rates on MT-4 and U937 Cell Cultures background, 4 Challenges with 7 Days b/w Period

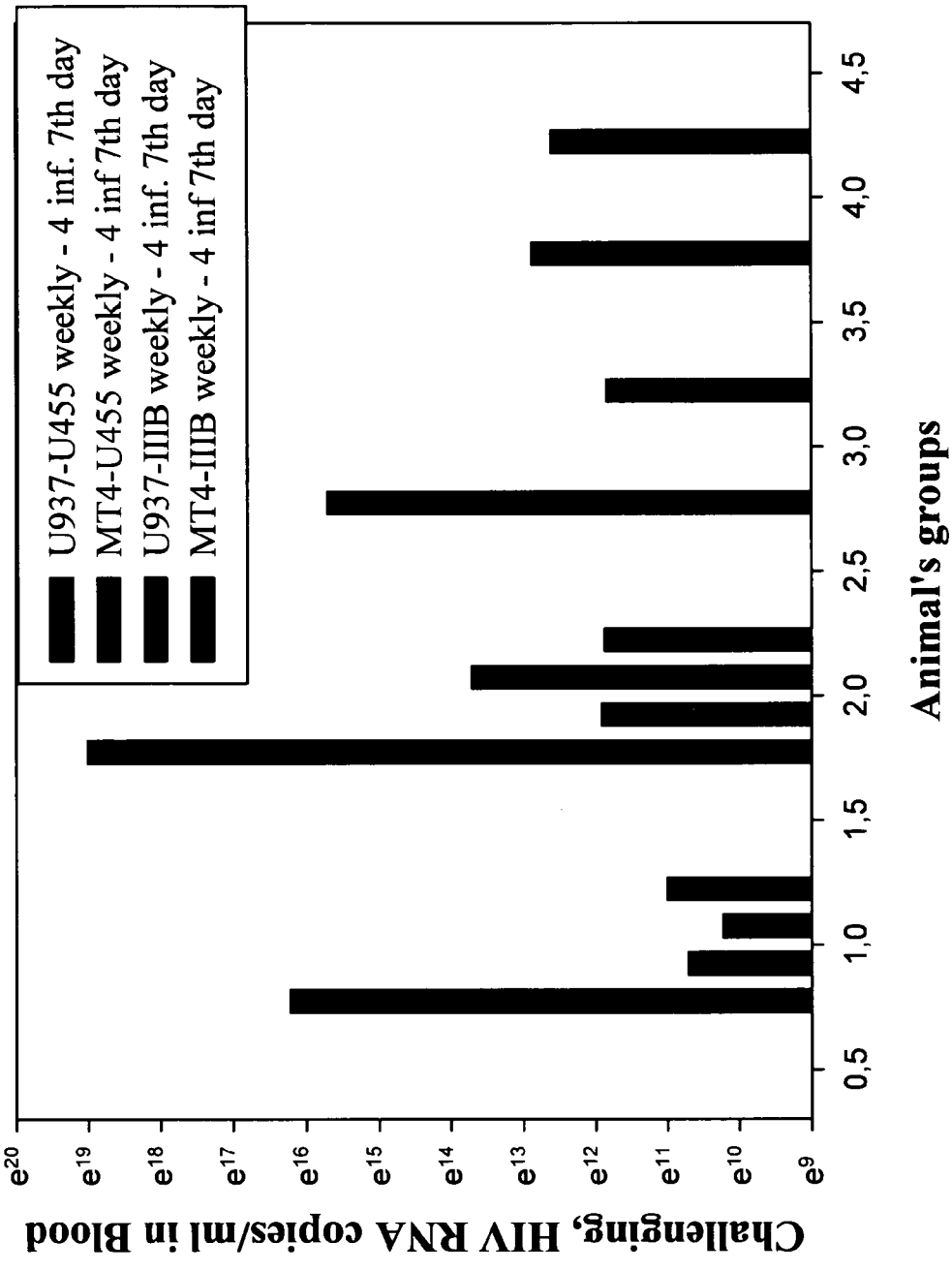


Figure 14: U455 and H9/IIIB in vitro Replication Dynamics on Lymphocytes MT-4 and Macrophage U937 Backgrounds

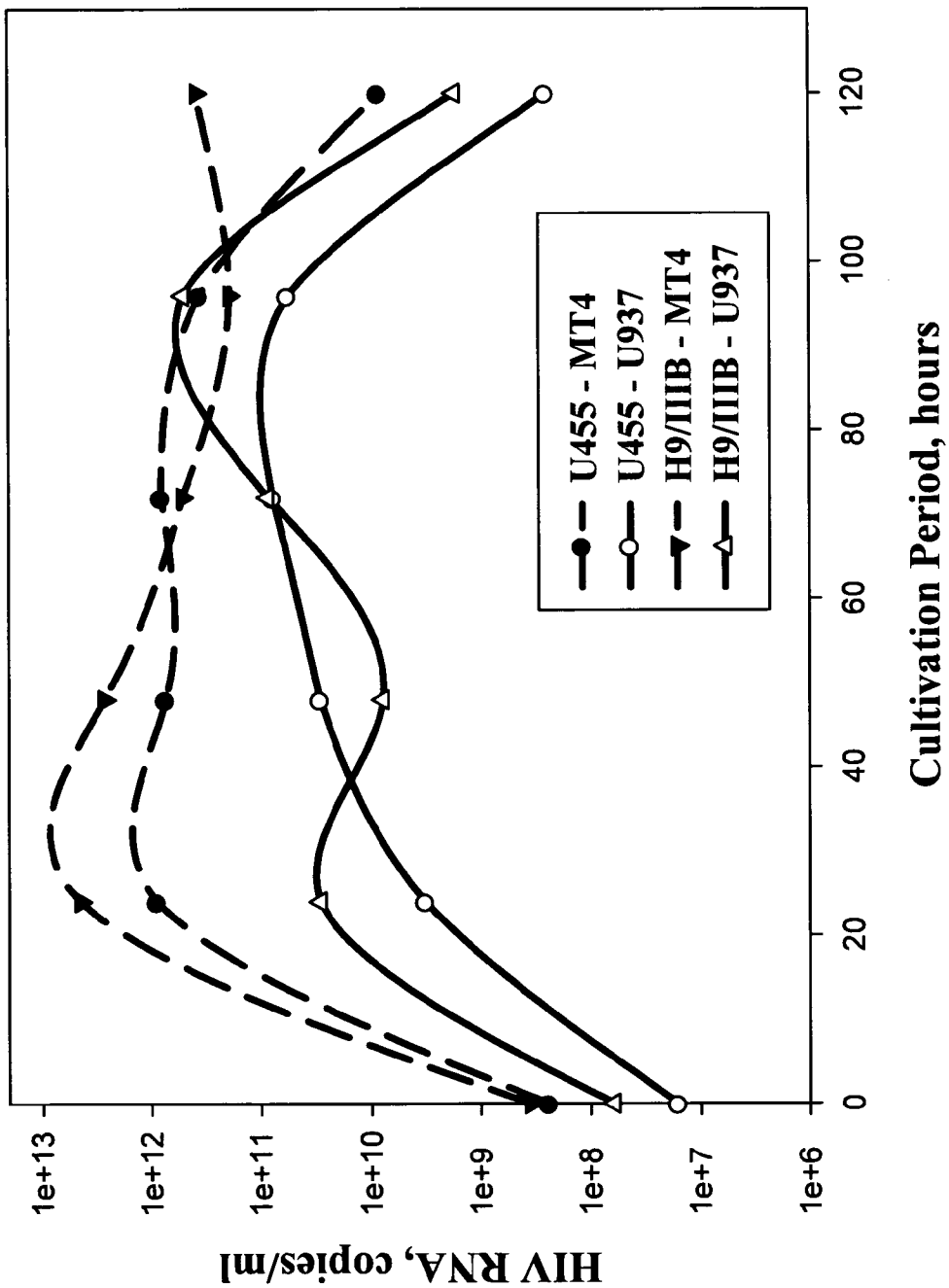


Figure 15: U455 and H9/IIIB in vitro Replication Dynamics, p24 Expression on Lymphocyte MT-4 and Macrophage U937 Cultures

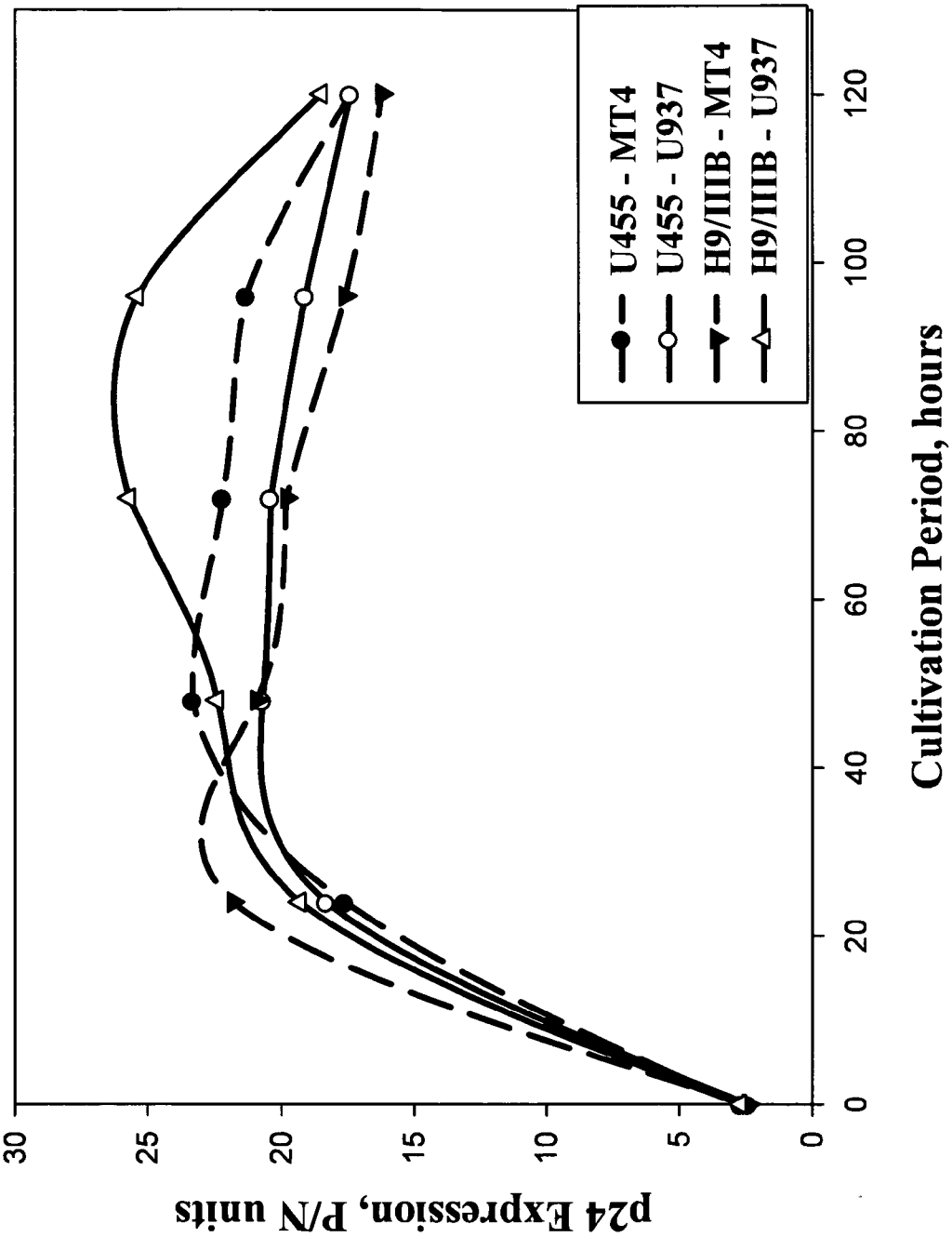


Figure 16: PokA-79 Viral Load Rates on Cell Culture U937 and MT4 Background Depending on the Number of Challenging

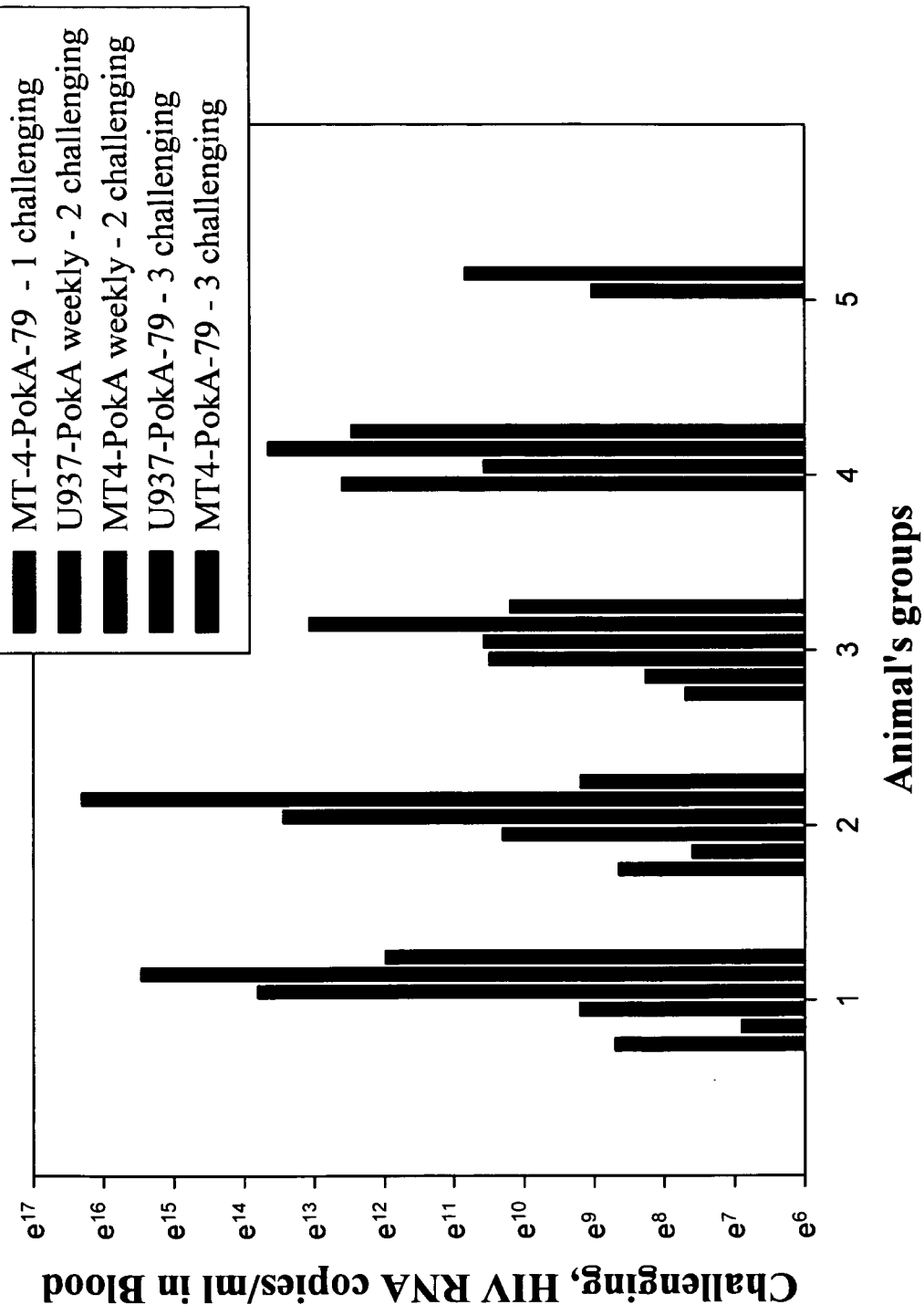


Figure 17: SCID-PBMC and SCID-cell Culture Background Viral Load Rates for Challenging with PokA-PBMC and PokA-79 Strains, Respectively

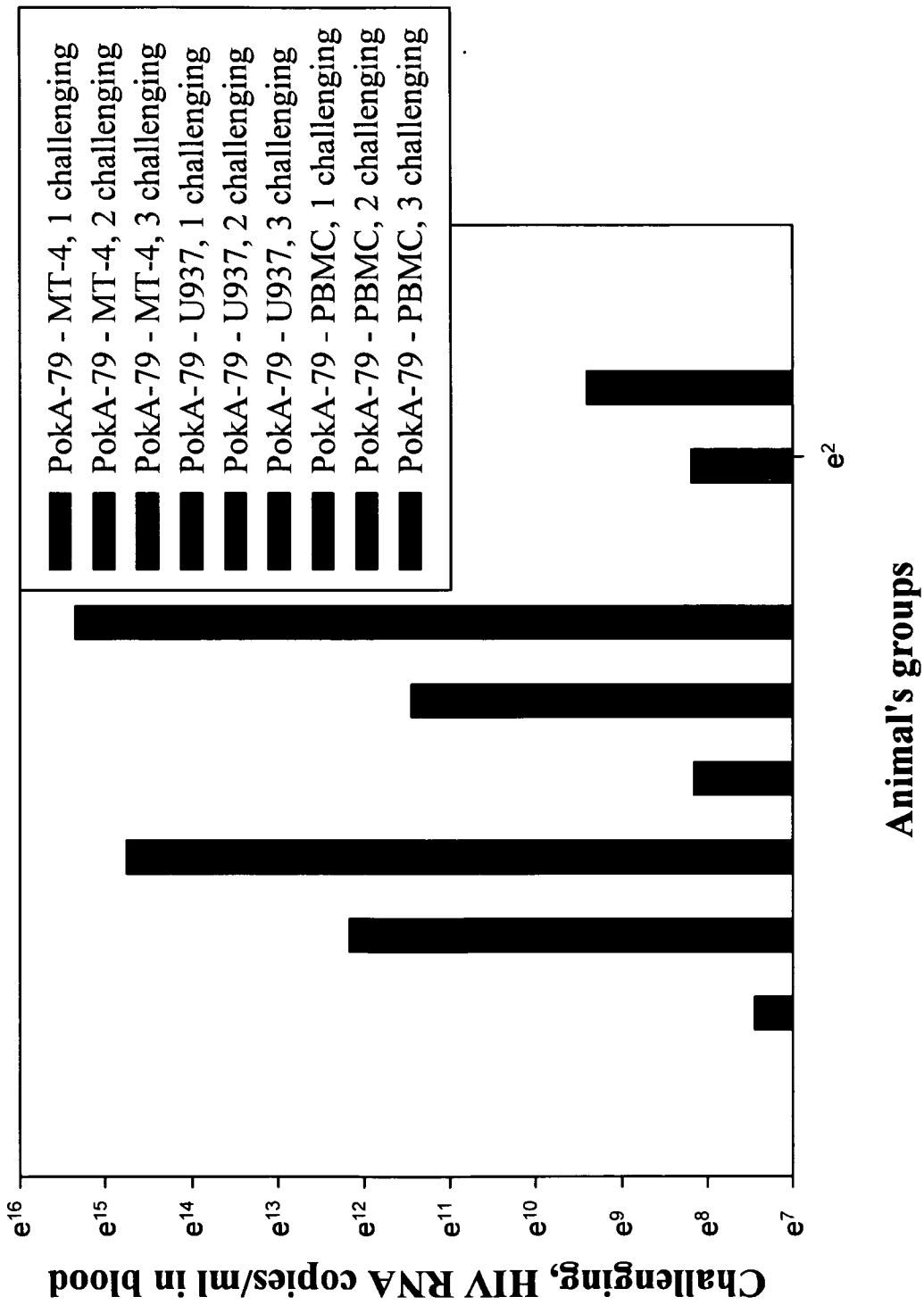
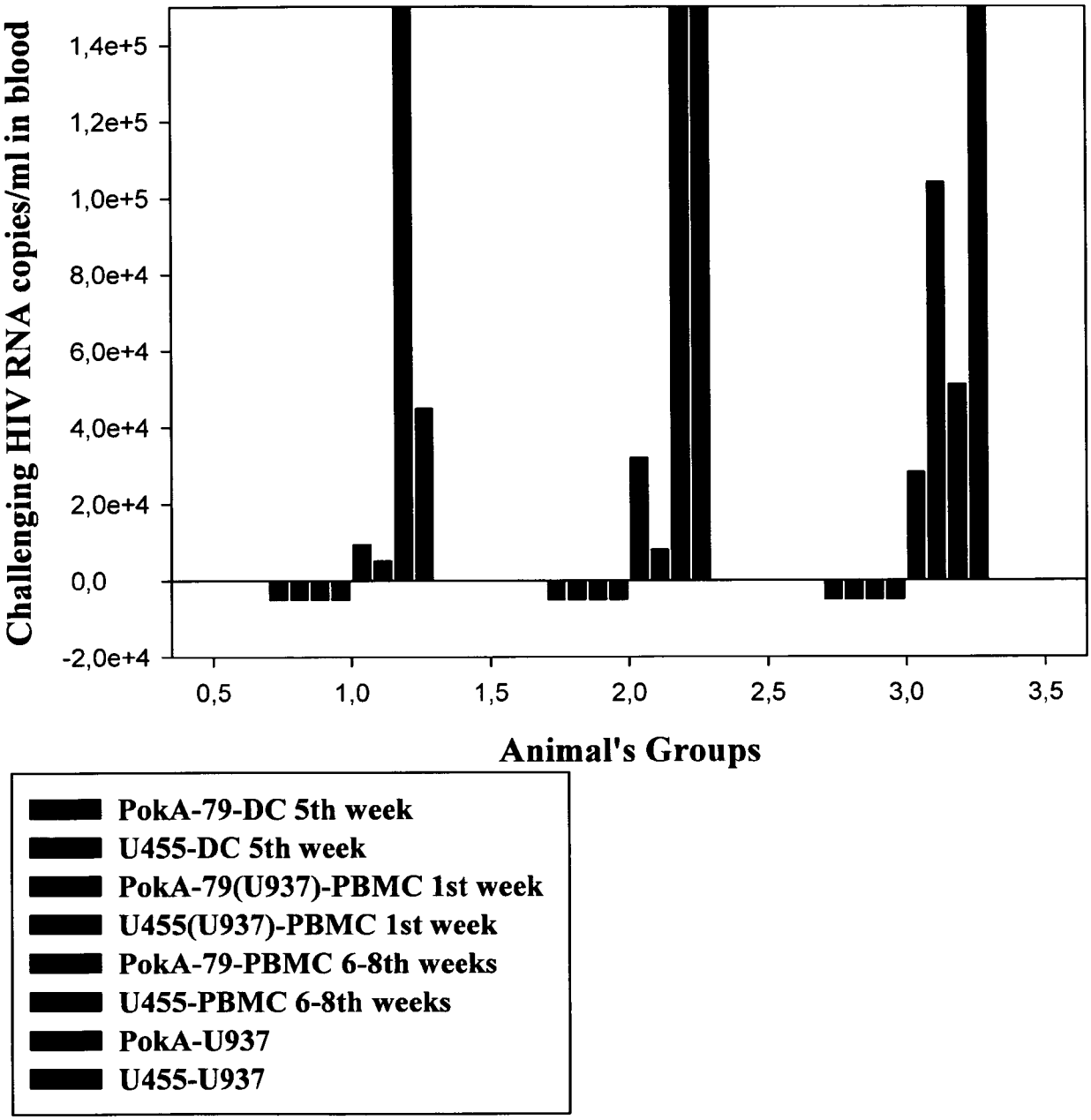


Figure 18: Negative Challenging Results for Dendrite Cells and Fresh PBMC PokA-79-U937 and U455-U937 Lab. Strains Compared to 6-8th PBMC Passage and U937 Background



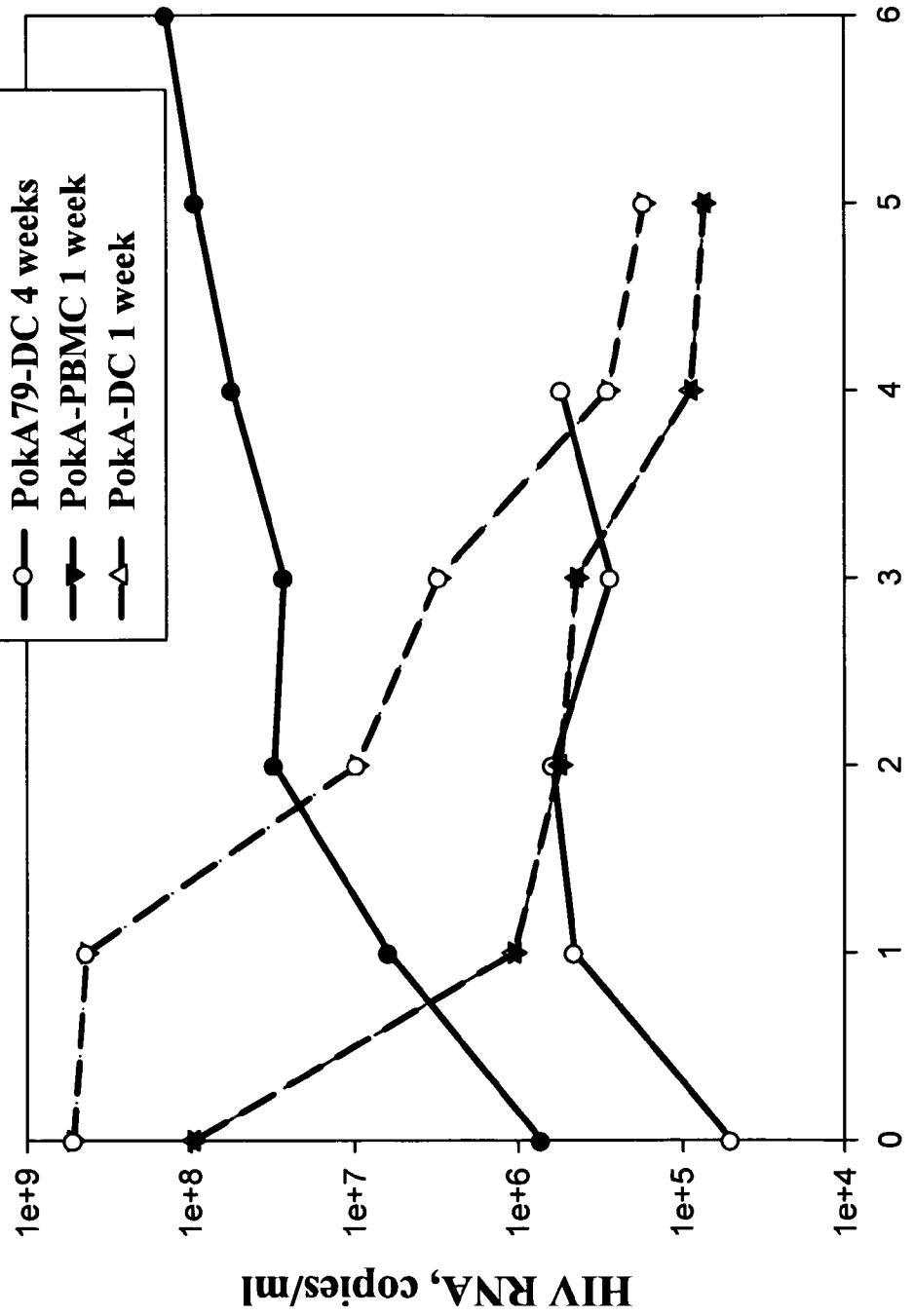


Figure 19: PokA-79 in vitro Passages Replication Dynamics, DC and PBMC background, 1st Week and 5-6th weeks Passages

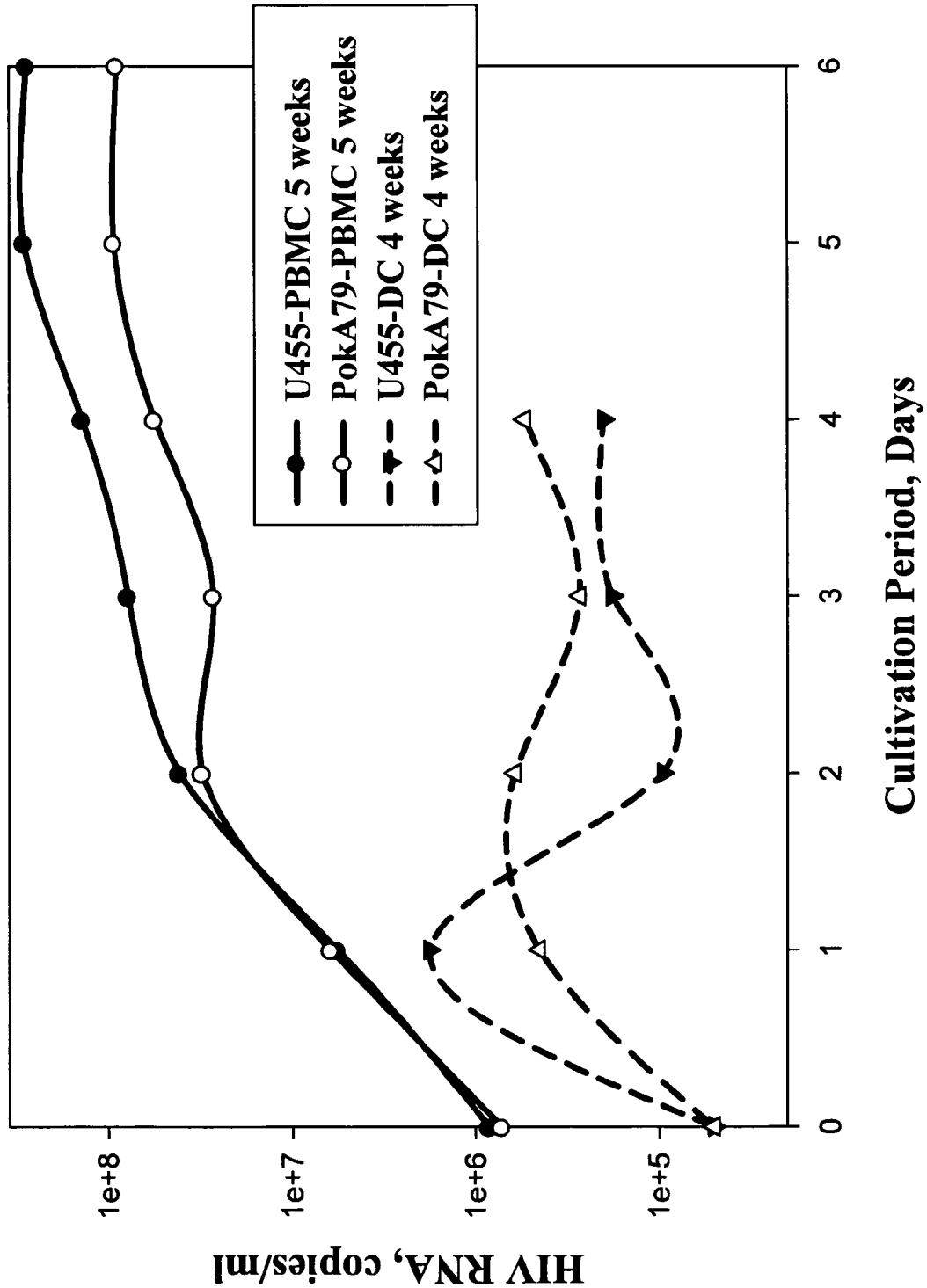
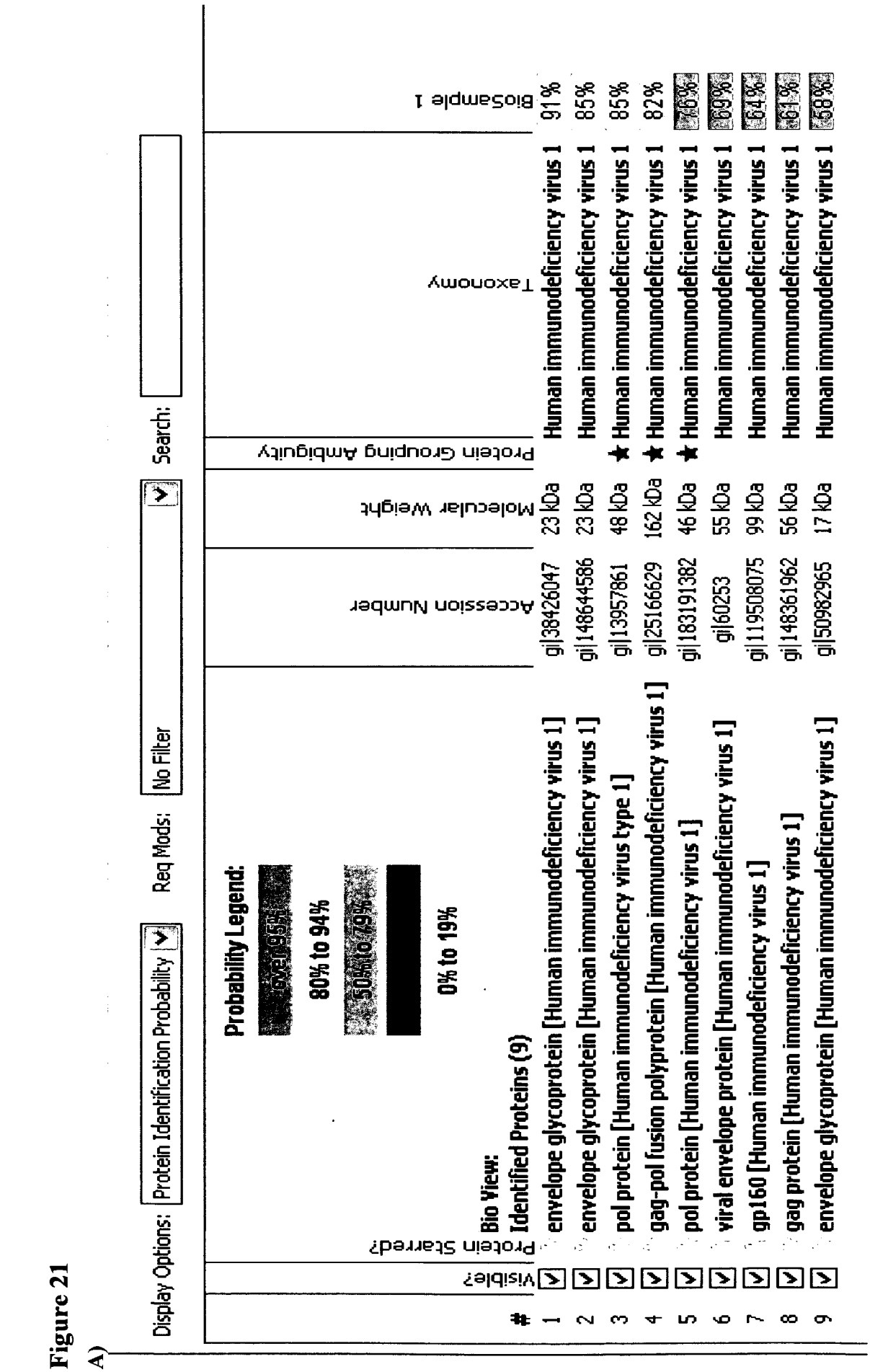


Figure 20: U455 and PokA-79 in vitro Replication Dynamics, Dendrite Cells (DC) or PBMC background, 5th and 6th passages

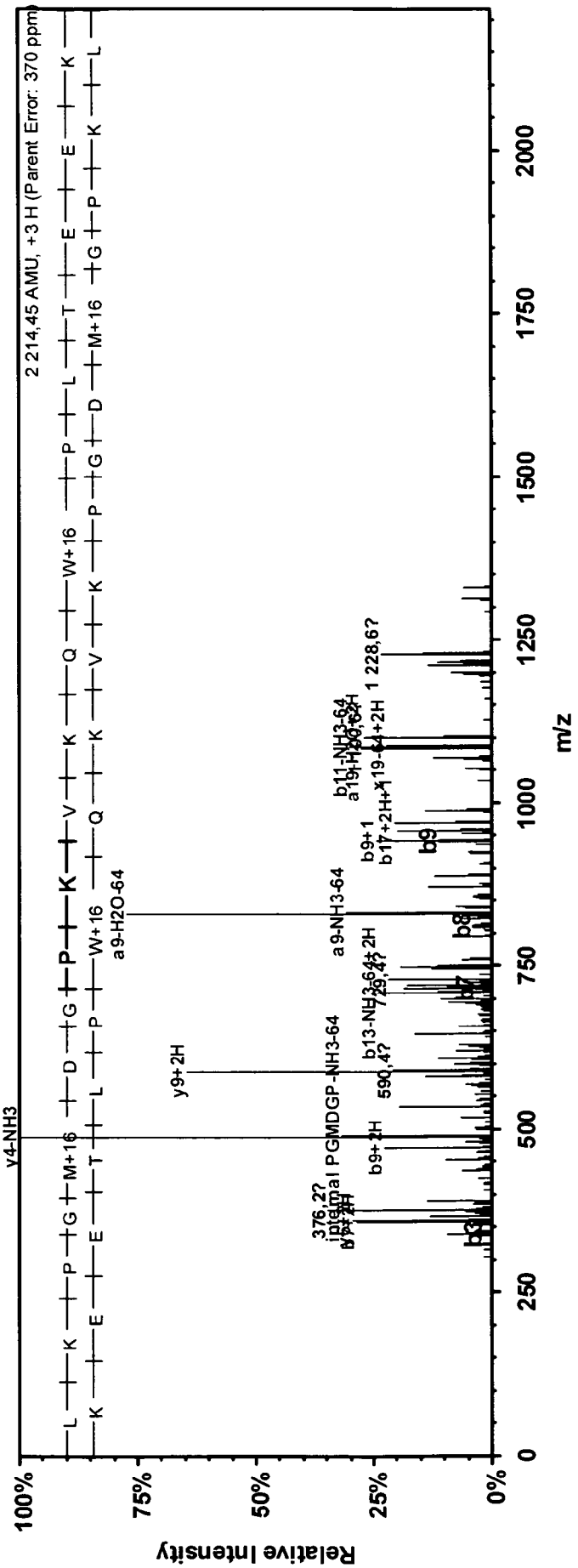
Figure 21

A)


Display Options:
Protein Identification Probability
Req Mods:
No Filter
Search:
Probability Legend:
Level 95%
80% to 94%
50% to 79%
0% to 19%
Bio View:
Identified Proteins (9)
Visible? Protein Starred? Accession Number Molecular Weight Protein Grouping Ambiguity Taxonomy BioSample 1
1 envelope glycoprotein [Human immunodeficiency virus 1] gi|38426047 23 kDa Human immunodeficiency virus 1 91%
2 envelope glycoprotein [Human immunodeficiency virus 1] gi|148644586 23 kDa Human immunodeficiency virus 1 85%
3 pol protein [Human immunodeficiency virus type 1] gi|13957861 48 kDa ★ Human immunodeficiency virus 1 85%
4 gag-pol fusion polyprotein [Human immunodeficiency virus 1] gi|25166629 162 kDa ★ Human immunodeficiency virus 1 82%
5 pol protein [Human immunodeficiency virus 1] gi|183191382 46 kDa ★ Human immunodeficiency virus 1 76%
6 viral envelope protein [Human immunodeficiency virus 1] gi|60253 55 kDa Human immunodeficiency virus 1 69%
7 gp160 [Human immunodeficiency virus 1] gi|119508075 99 kDa Human immunodeficiency virus 1 64%
8 gag protein [Human immunodeficiency virus 1] gi|148361962 56 kDa Human immunodeficiency virus 1 61%
9 envelope glycoprotein [Human immunodeficiency virus 1] gi|50982965 17 kDa Human immunodeficiency virus 1 58%

Continuation of Figure 21

B)



Continuation of Figure 21

C)

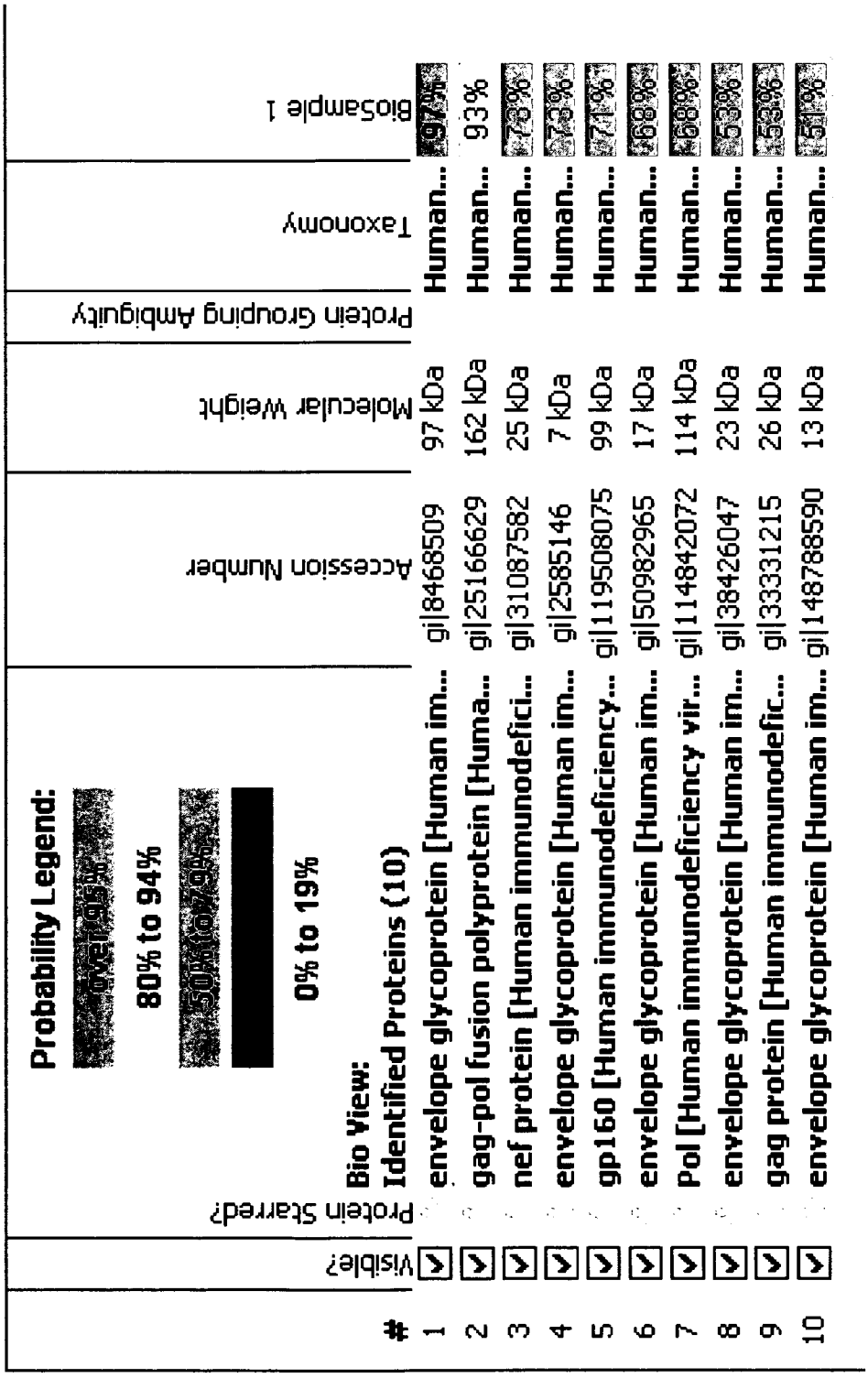
gi|119508075 (64%), 98 959,1 Da
gp160 [Human immunodeficiency virus 1]
2 unique peptides, 2 unique spectra, 3 total spectra, 47/874 amino acids (5% coverage)

MRVMGIQRNY	PLLWRWGLMI	FGMLIICNAN	HRQMWVTVYY	GVPVWRDAET
TLFCASDAKA	YDKEVHNVWA	THACVPTDPD	PQELPLGNVT	ENFNIWDNSM
VEQMHEDIIS	LWDQSLKPCV	QLTPLCVTLN	CSNVSNETNS	NNTGVNRTIY
EEMKGEIKNC	SFNMTTELRD	KKQKVYALFY	KLDIVQMGSS	SSSQDNSSSL
YRLINCNTSA	VTQTCPKVSF	EPIPIHYCAP	AGFAILKCND	KEFNGTGPCK
NVSTVQCTHG	IRPVVSTQLL	LNGSLAEEKI	AIRSKNISDN	AKTIIVQLTE
PVEINCTRPG	NNTRRSVRIG	PGQTFYATGD	IIGDIRQAHC	NVSREKWNST
LQKVATRLGE	YFKNKTTIIF	TNHAGGDLEI	TTHSFICGGE	FFYCNTTNLF
NSTWEKKKGT	WNDTTANPPN	GTITLQCRIK	QIVNMWQRVG	QAIYAPPIPG
IISCNSSITG	LLLTRDGGEG	NETRKNETFR	PGGGDMRDNW	RSELYKYKVV
KIEPLGVAPT	YAKRRVVERE	KR**AVG**[■]**GAVL**	**LGFLGAAGST**	**MGAASITLTV**
QARQLLSGIV	QQQSNLLKAI	EAQQQLLRLT	VWGIKQLQAR	VLALERYLGD
QQLLGLWGCS	GK**L**[■]**TTTVP**	**WNSSSWSNK**NY	SDIWENMTWL	QWDREISNYT
HTIYTLIEEA	QNQQEKNEND	LLALDKWASL	WNWFDITKWL	WYIKMFIMIV
GGLIGLRIVF	AVLTIIKRVR	QGYSPLSFQT	LTHHQREPDR	PERIEEGGGE
QDRDRSVRLV	SGFLALAWDD	LRSLCLFSYH	RLRDLLLIAA	RTVELLGHSS
LKGLRLGWEA	LKYLGNLLSY	WGQELKNSAI	SLFDTIAITV	ANWTDRVIEL
GQRIGRAICN	IPRRIRQGLE	RSLL		

SUBSTITUTE SHEET (RULE 26)

Continuation of Figure 21

D)



Continuation of Figure 21

E)

gi|8468509 (97%), 96 819,6 Da
envelope glycoprotein [Human immunodeficiency virus type 1]
3 unique peptides, 3 unique spectra, 3 total spectra, 73/854 amino acids (9% coverage)

M R V K E I R K **N Y** **L W R G G T L L L** **G M L I C S A A E** **K** S W V T V Y Y G V P V W K E A T T T L
F C A S D A K A Y D T E A H N V W A T H A C V P T D P N P Q E V E L K N V T E N F N M W K N N M V E
Q M H E D I I S L W D Q S L K P C V K L T P L C V T L N C T D V E R N A T N I T I S S E K T M D K G
E I K N C S F N I T T N L R D K V Q K E Y A L F Y K L D V V Q I D N T S Y R L I S C N T S V I T Q A
C P K I S F E P I P I H Y C A P A G F A I L K C K D K N F N G K G P C T N V S T V Q C T H G I R P V
V S T Q L L L N G S L A E D D V V I R S E N F T N N A K T I I V Q L K E A V Q I N C T R P N N N T R
K G I H I G P G R A F Y T T G D I I G D I R Q A Y C N L S R T Q W N K T L E Q V V I K L G E Q F G N
K T I K F Q P S S G G D P E I V T H T L N C G G G F F Y C N S T P L F N S I W N I T G N I T E E S N
N T K G N D T I I L T C R I K Q I I N M W Q G V G K A M Y A P P I R G Q I R C V S N I T G L L L T R
D G G N S N N T E S N N T E V F R P G G G N M R D N W R S E L Y K Y K V V K I E P L G V A P T K A K
R R V V Q R E K R **A** **V G I G A V F L G F** **L G A A G S T M G A** **A S I T L T V Q A R** Q L L S G I V Q Q Q
S N L L R A I E A Q Q H L L Q L T V W G I K Q L Q A R V L A V E R Y L R D Q Q L L G I W G C S G K L
I C T T T V P W N A S W S N K S L D M I W N N M T W M E W E K E I S N Y T N V I Y N L I E E S Q N Q
Q E K N E H E L L E L D K W A S L W N W F D I T K W L W Y I K I F I M I V G G L V G L R I V F A V L
S I V N R V R Q G Y S P L S F Q T L L P A T R G P D R P E G T A E E G G E R D R D R S G R L V D G F
L A L I W D D L R **S** **L L F S Y H R L R** **D L L L I V T R** I V E L L G R R G W E V L K Y W W N L L Q Y
W R K E L K N S A V S L L N A T A I A V A E G T D R V I E V V Q R I Y R A I L H I P R R I R Q G L E
R A L L

Figure 22

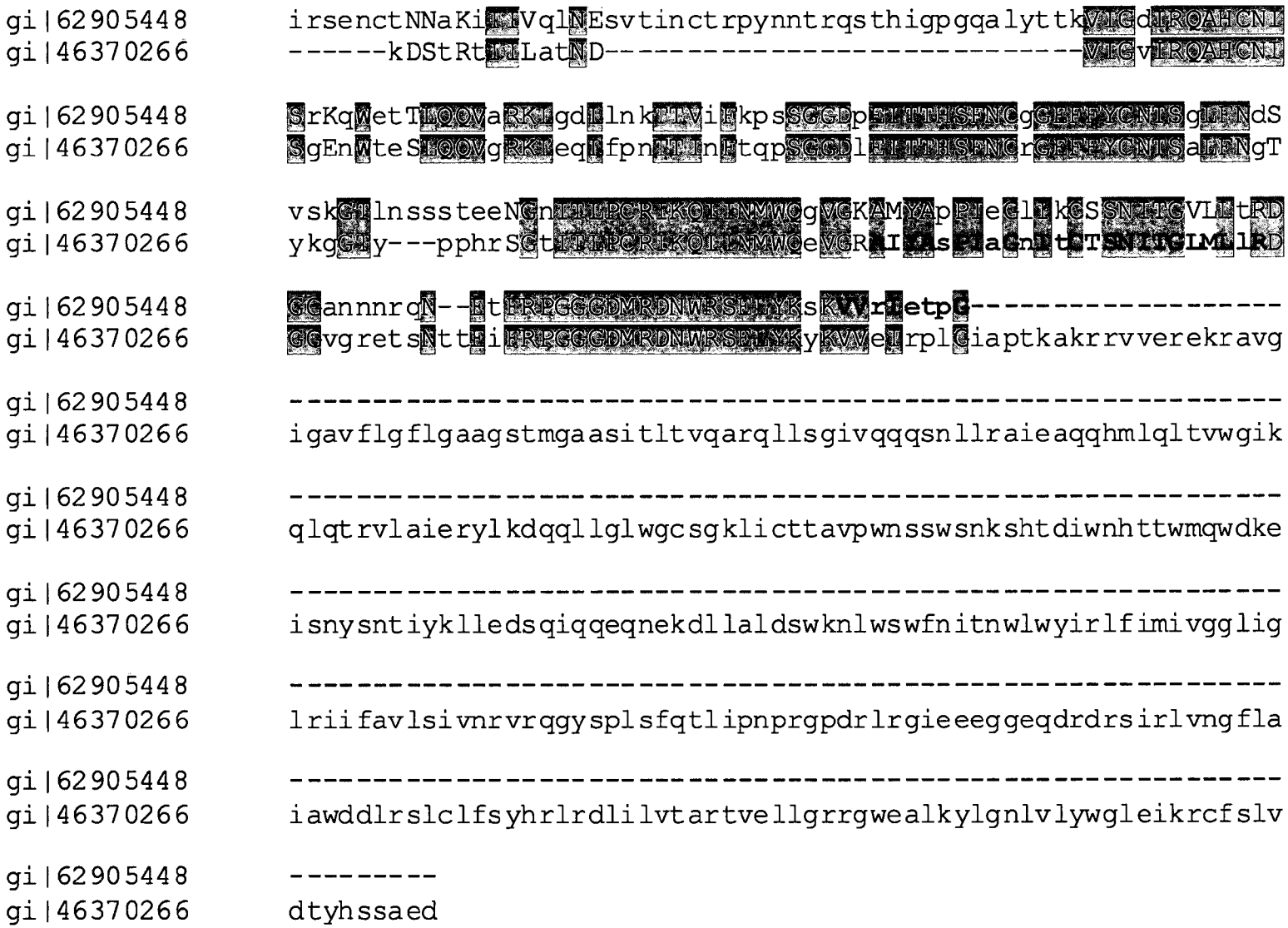
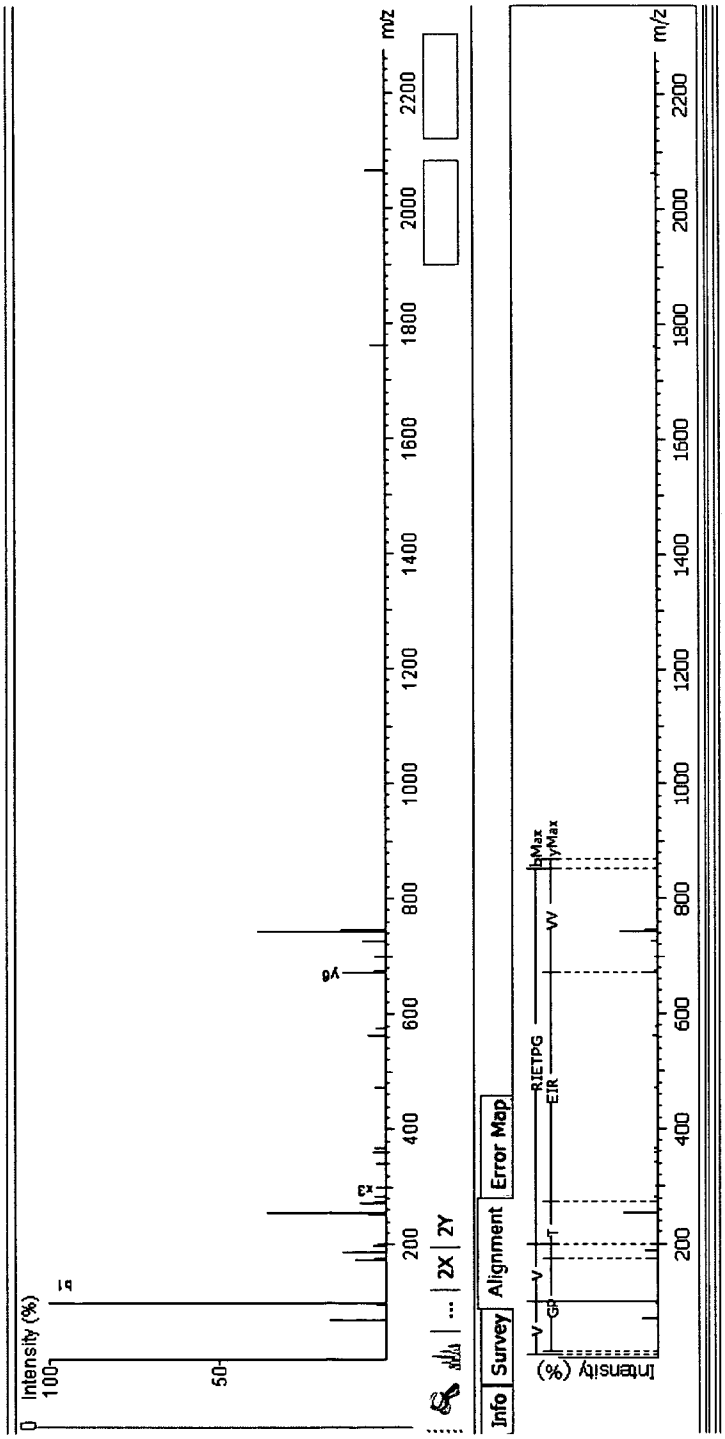


Figure 23
A)

Spider search

#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	72.08	100.11	82.07	72.08	117.10	V						8
2	72.08	199.14	181.13	171.15	216.17	V	771.44	753.43	754.41	755.41	386.22	7
3	129.11	355.25	337.24	327.25	372.27	R	672.38	654.36	655.34	656.34	336.68	6
4	86.10	468.33	450.32	440.33	485.36	I	516.27	498.26	499.24	500.24	258.63	5
5	102.06	597.37	579.36	569.38	614.40	E	403.18	385.17	386.16	387.16	202.09	4
6	74.06	698.42	680.41	670.43	715.45	T	274.17	256.13	257.11	258.11	137.57	3
7	70.07	795.47	777.46	767.48	812.50	P	173.16	155.08	156.07	157.07	87.05	2
8	30.03					G	76.04	58.03	59.01	60.01	38.52	1

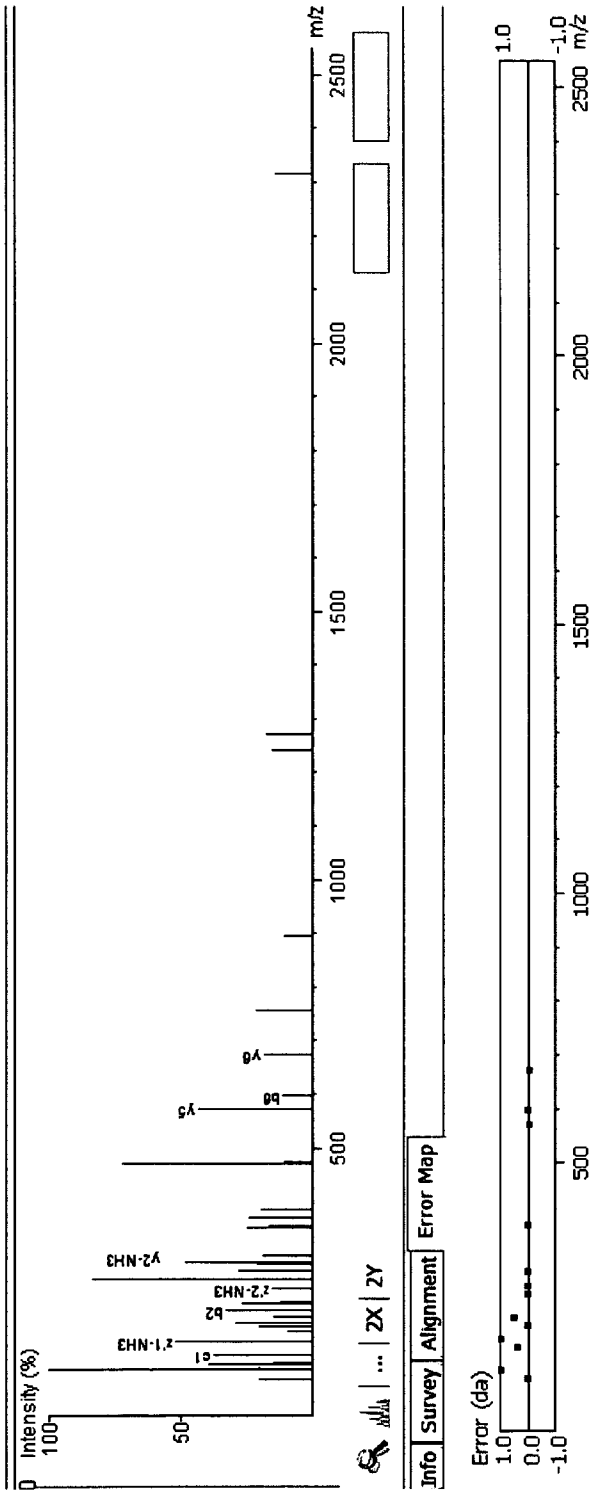


Continuation of Figure 23

B)

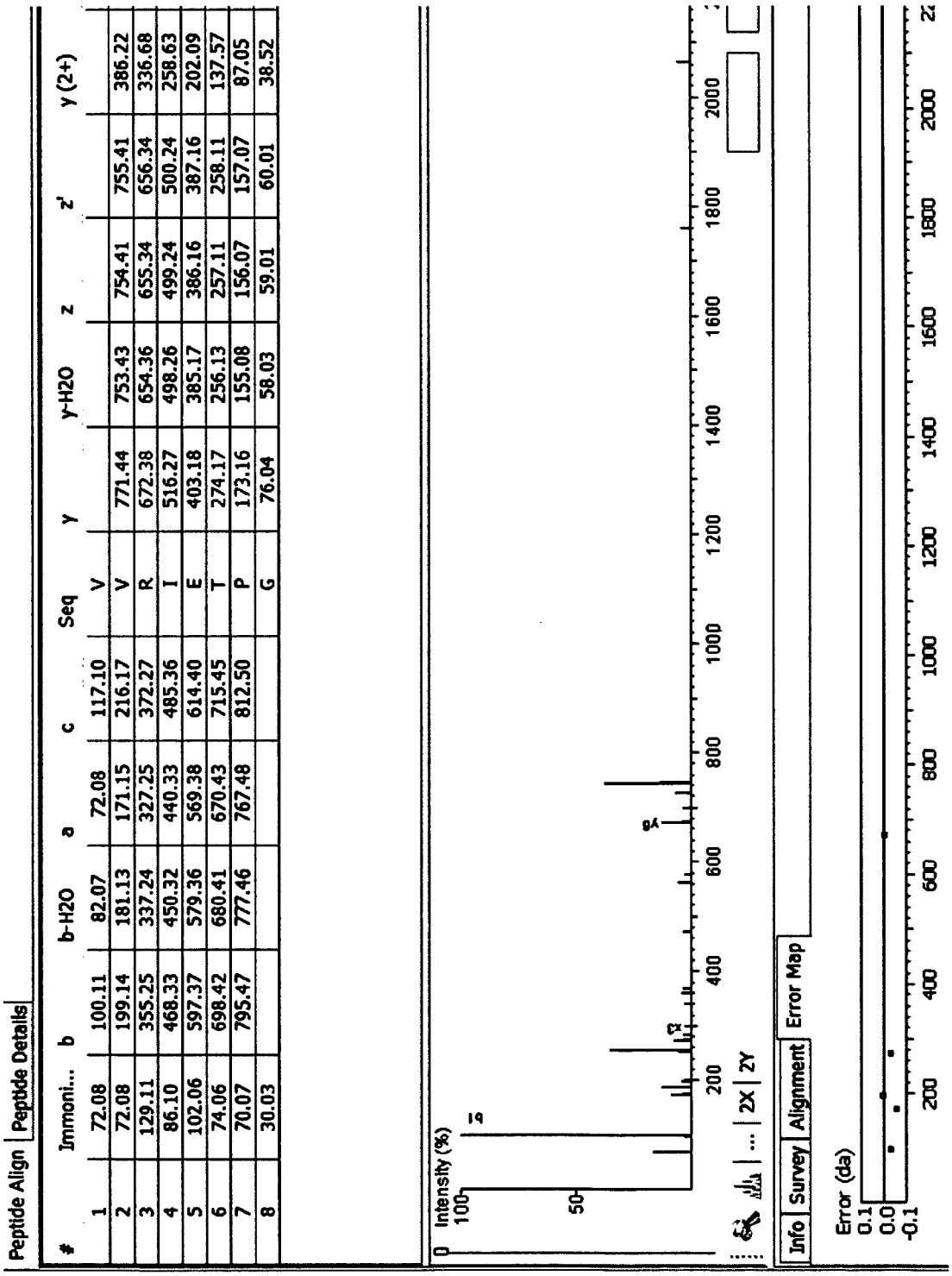
Spider Search

#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	72.08	100.12	82.07	72.08	116.11	V						8
2	70.07	197.12	179.12	169.13	213.63	P	769.38	751.37	752.36	753.36	385.19	7
3	74.06	298.18	280.17	270.14	315.20	T	672.41	654.32	655.30	656.30	336.67	6
4	88.04	413.20	395.19	385.26	430.23	D	571.35	553.27	554.26	555.26	286.14	5
5	70.07	510.26	492.25	482.26	527.28	P	456.26	438.25	439.23	440.23	228.63	4
6	60.04	597.31	579.28	569.29	614.32	S	359.20	341.19	342.18	343.18	180.10	3
7	70.07	694.34	676.33	666.35	711.37	P	272.17	254.16	255.14	256.14	136.59	2
8	129.11					R	174.14	157.11	158.09	158.69	88.06	1



Continuation of Figure 23

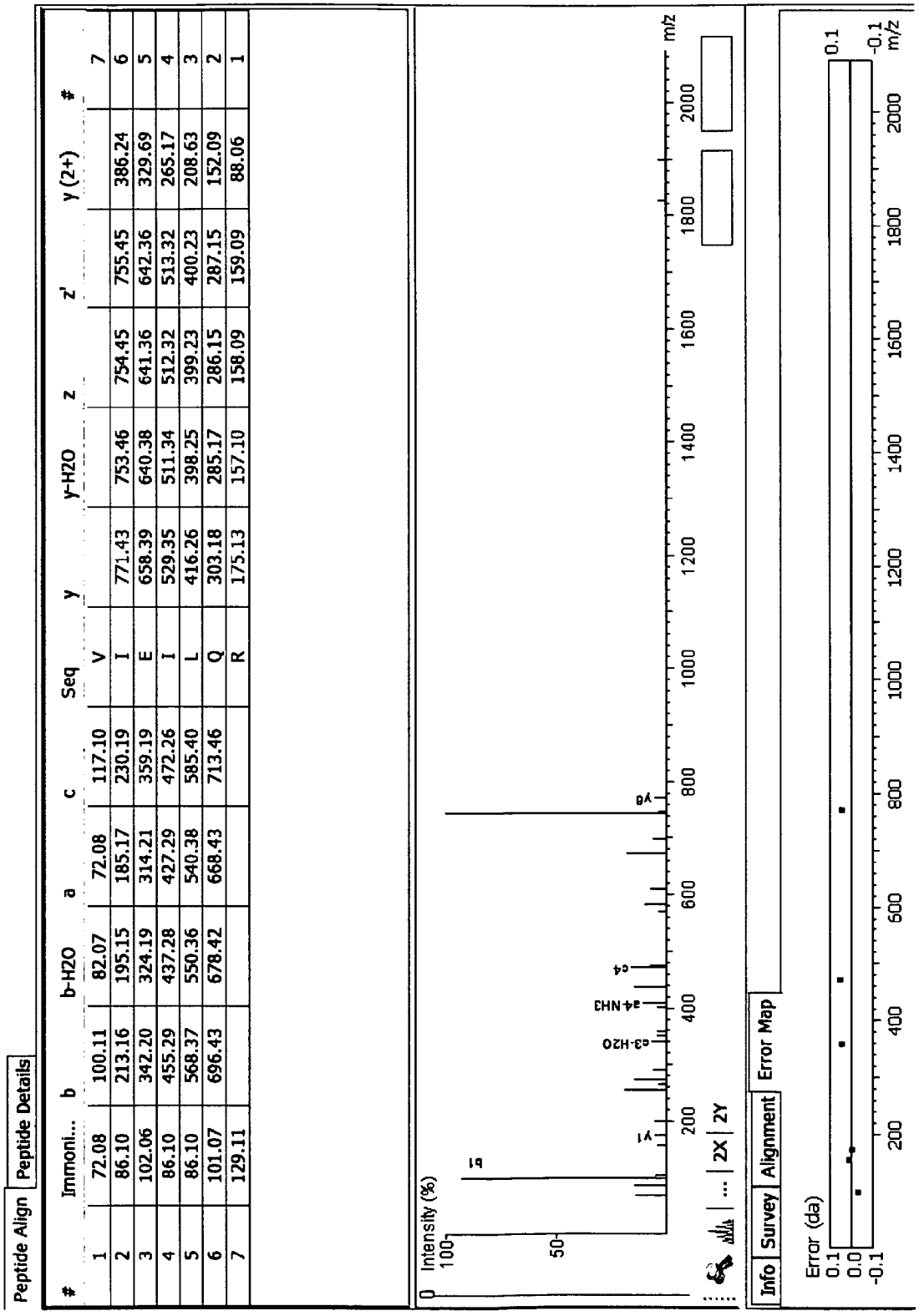
C)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)
1	72.08	100.11	82.07	72.08	117.10	V	771.44	753.43	754.41	755.41	386.22
2	72.08	199.14	181.13	171.15	216.17	V	672.38	654.36	655.34	656.34	336.68
3	129.11	355.25	337.24	327.25	372.27	R	516.27	498.26	499.24	500.24	258.63
4	86.10	468.33	450.32	440.33	485.36	I	403.18	385.17	386.16	387.16	202.09
5	102.06	597.37	579.36	569.38	614.40	E	274.17	256.13	257.11	258.11	137.57
6	74.06	698.42	680.41	670.43	715.45	T	173.16	155.08	156.07	157.07	87.05
7	70.07	795.47	777.46	767.48	812.50	P	76.04	58.03	59.01	60.01	38.52
8	30.03					G					

Continuation of Figure 23

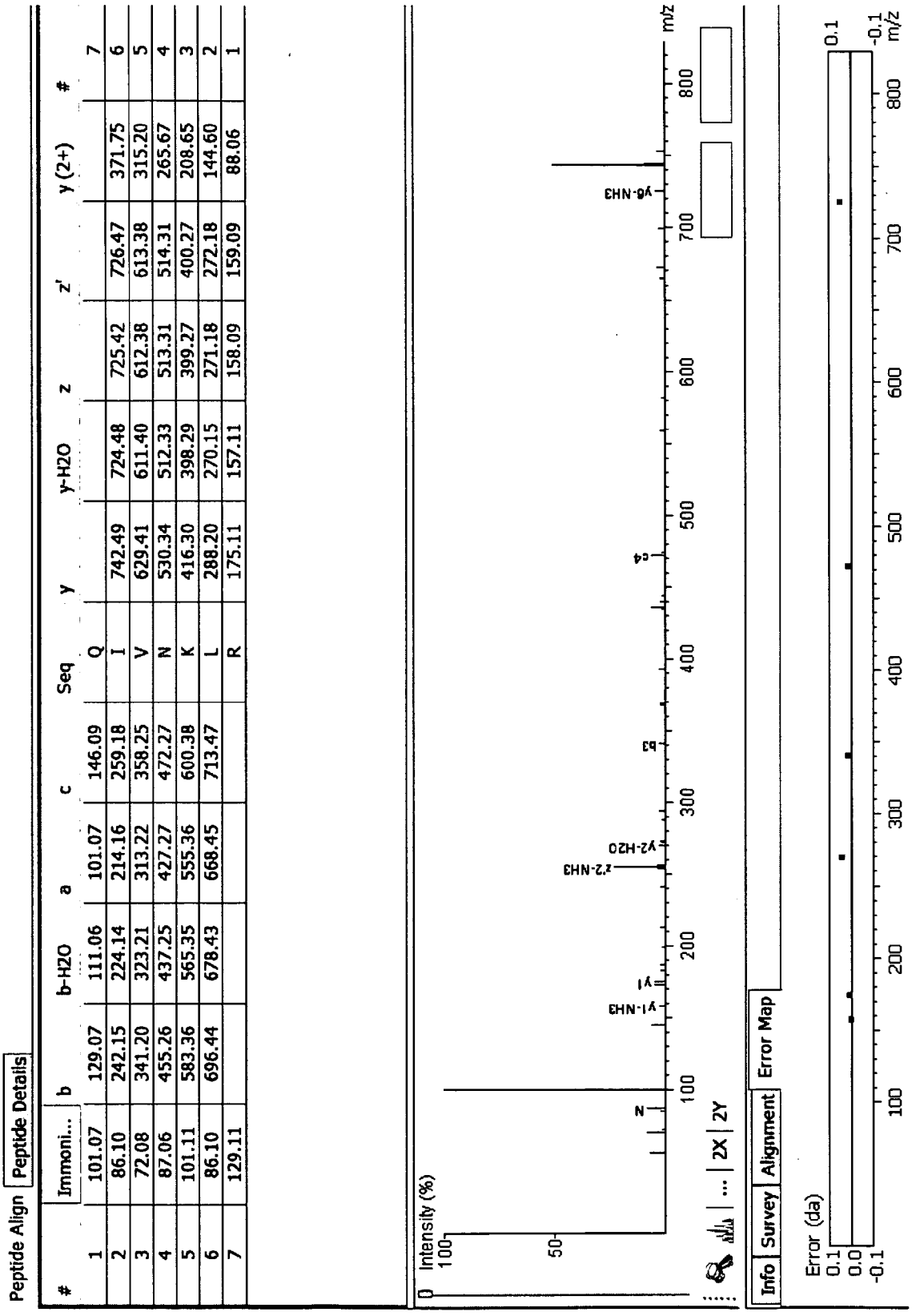
D)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	72.08	100.11	82.07	72.08	117.10	V						7
2	86.10	213.16	195.15	185.17	230.19	I	771.43	753.46	754.45	755.45	386.24	6
3	102.06	342.20	324.19	314.21	359.19	E	658.39	640.38	641.36	642.36	329.69	5
4	86.10	455.29	437.28	427.29	472.26	I	529.35	511.34	512.32	513.32	265.17	4
5	86.10	568.37	550.36	540.38	585.40	L	416.26	398.25	399.23	400.23	208.63	3
6	101.07	696.43	678.42	668.43	713.46	Q	303.18	285.17	286.15	287.15	152.09	2
7	129.11					R	175.13	157.10	158.09	159.09	88.06	1

Continuation of Figure 23

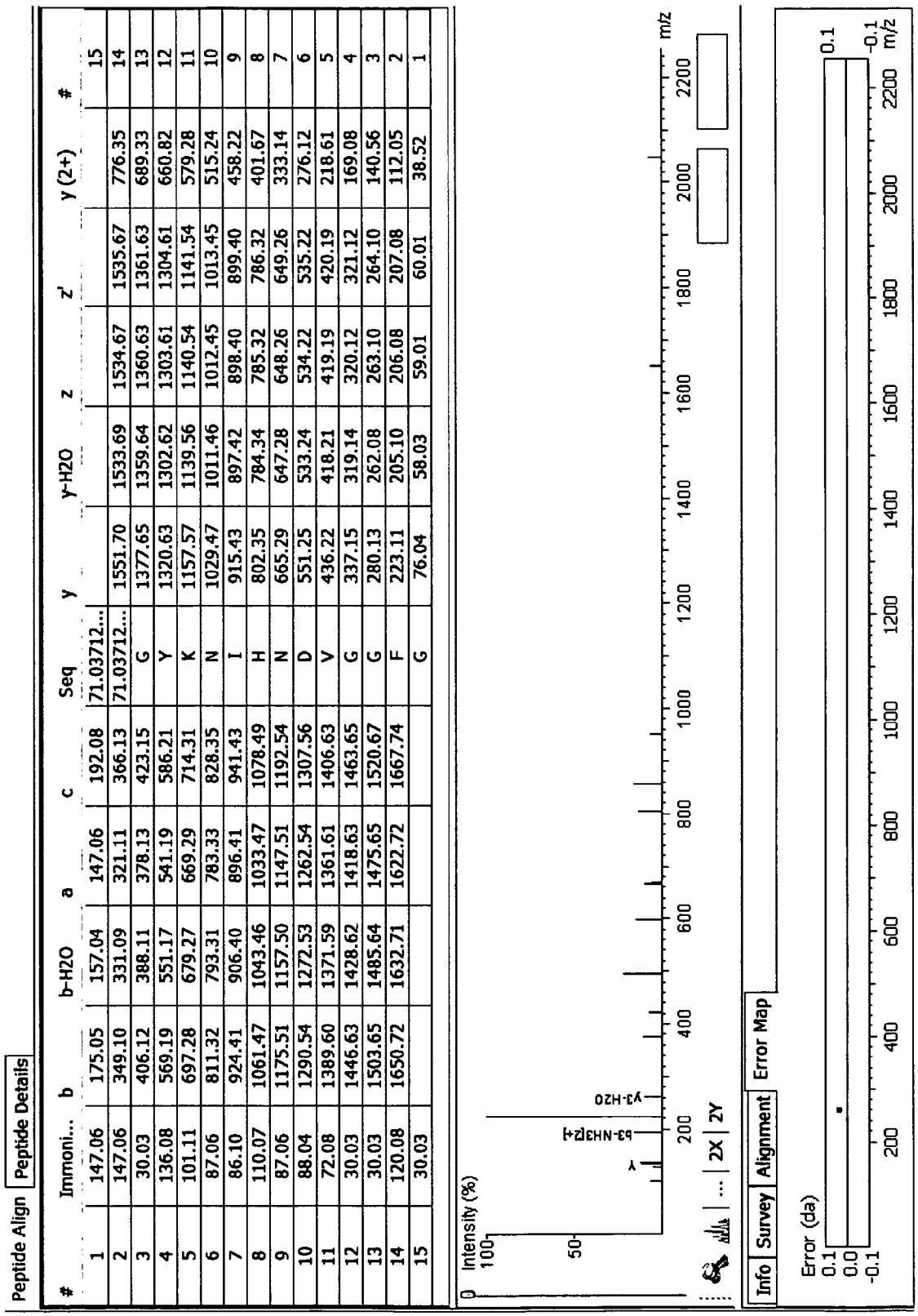
E)



#		Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	101.07		129.07	111.06	101.07	146.09	Q						7
2	86.10		242.15	224.14	214.16	259.18	I	742.49	724.48	725.42	726.47	371.75	6
3	72.08		341.20	323.21	313.22	358.25	V	629.41	611.40	612.38	613.38	315.20	5
4	87.06		455.26	437.25	427.27	472.27	N	530.34	512.33	513.31	514.31	265.67	4
5	101.11		583.36	565.35	555.36	600.38	K	416.30	398.29	399.27	400.27	208.65	3
6	86.10		696.44	678.43	668.45	713.47	L	288.20	270.15	271.18	272.18	144.60	2
7	129.11						R	175.11	157.11	158.09	159.09	88.06	1

Continuation of Figure 23

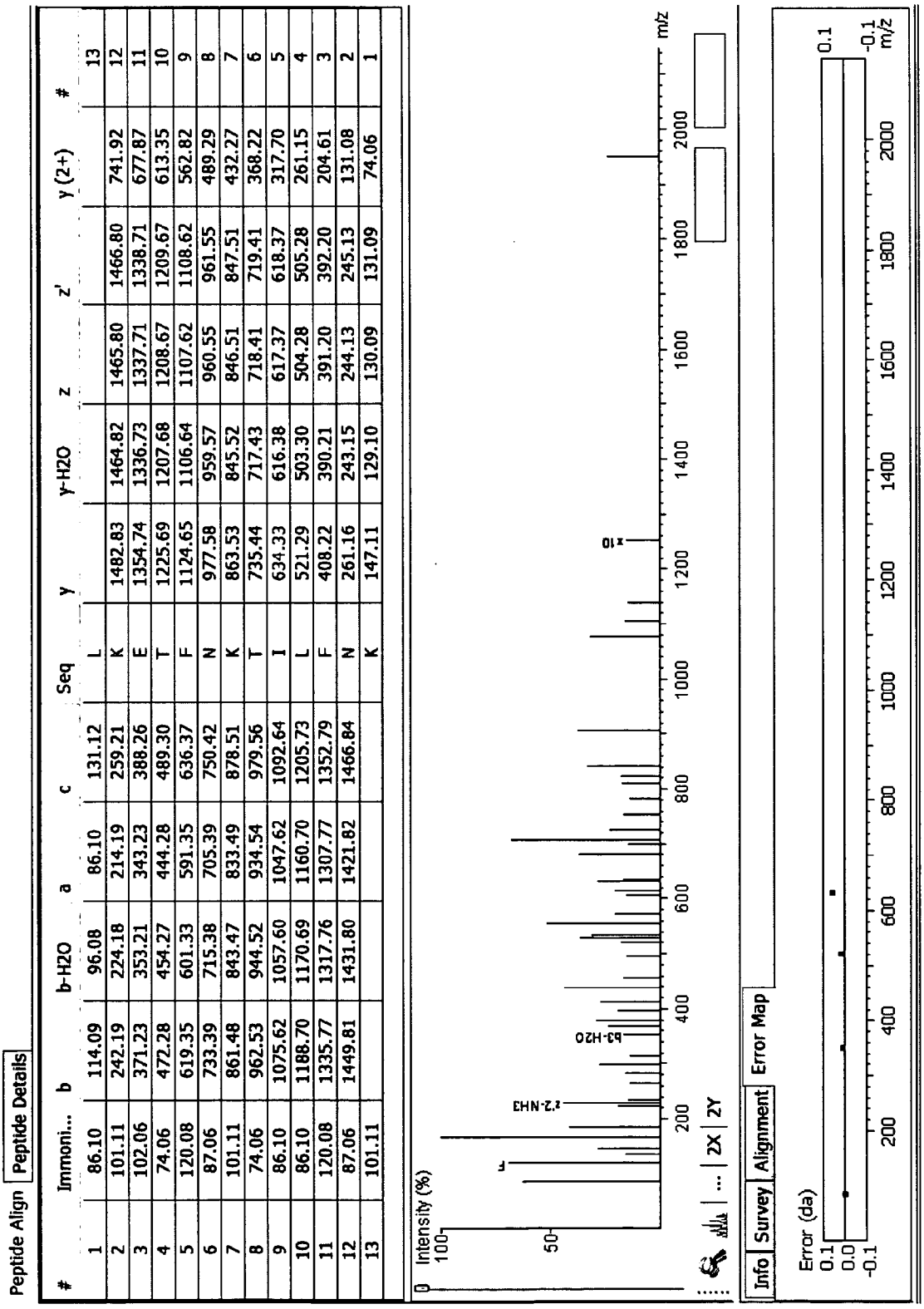
F)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	147.06	175.05	157.04	147.06	192.08	71.03712...						15
2	147.06	349.10	331.09	321.11	366.13	71.03712...	1551.70	1533.69	1534.67	1535.67	776.35	14
3	30.03	406.12	388.11	378.13	423.15	G	1377.65	1359.64	1360.63	1361.63	689.33	13
4	136.08	569.19	551.17	541.19	586.21	Y	1320.63	1302.62	1303.61	1304.61	660.82	12
5	101.11	697.28	679.27	669.29	714.31	K	1157.57	1139.56	1140.54	1141.54	579.28	11
6	87.06	811.32	793.31	783.33	828.35	N	1029.47	1011.46	1012.45	1013.45	515.24	10
7	86.10	924.41	906.40	896.41	941.43	I	915.43	897.42	898.40	899.40	458.22	9
8	110.07	1061.47	1043.46	1033.47	1078.49	H	802.35	784.34	785.32	786.32	401.67	8
9	87.06	1175.51	1157.50	1147.51	1192.54	N	665.29	647.28	648.26	649.26	333.14	7
10	88.04	1290.54	1272.53	1262.54	1307.56	D	551.25	533.24	534.22	535.22	276.12	6
11	72.08	1389.60	1371.59	1361.61	1406.63	V	436.22	418.21	419.19	420.19	218.61	5
12	30.03	1446.63	1428.62	1418.63	1463.65	G	337.15	319.14	320.12	321.12	169.08	4
13	30.03	1503.65	1485.64	1475.65	1520.67	G	280.13	262.08	263.10	264.10	140.56	3
14	120.08	1650.72	1632.71	1622.72	1667.74	F	223.11	205.10	206.08	207.08	112.05	2
15	30.03					G	76.04	58.03	59.01	60.01	38.52	1

Continuation of Figure 23

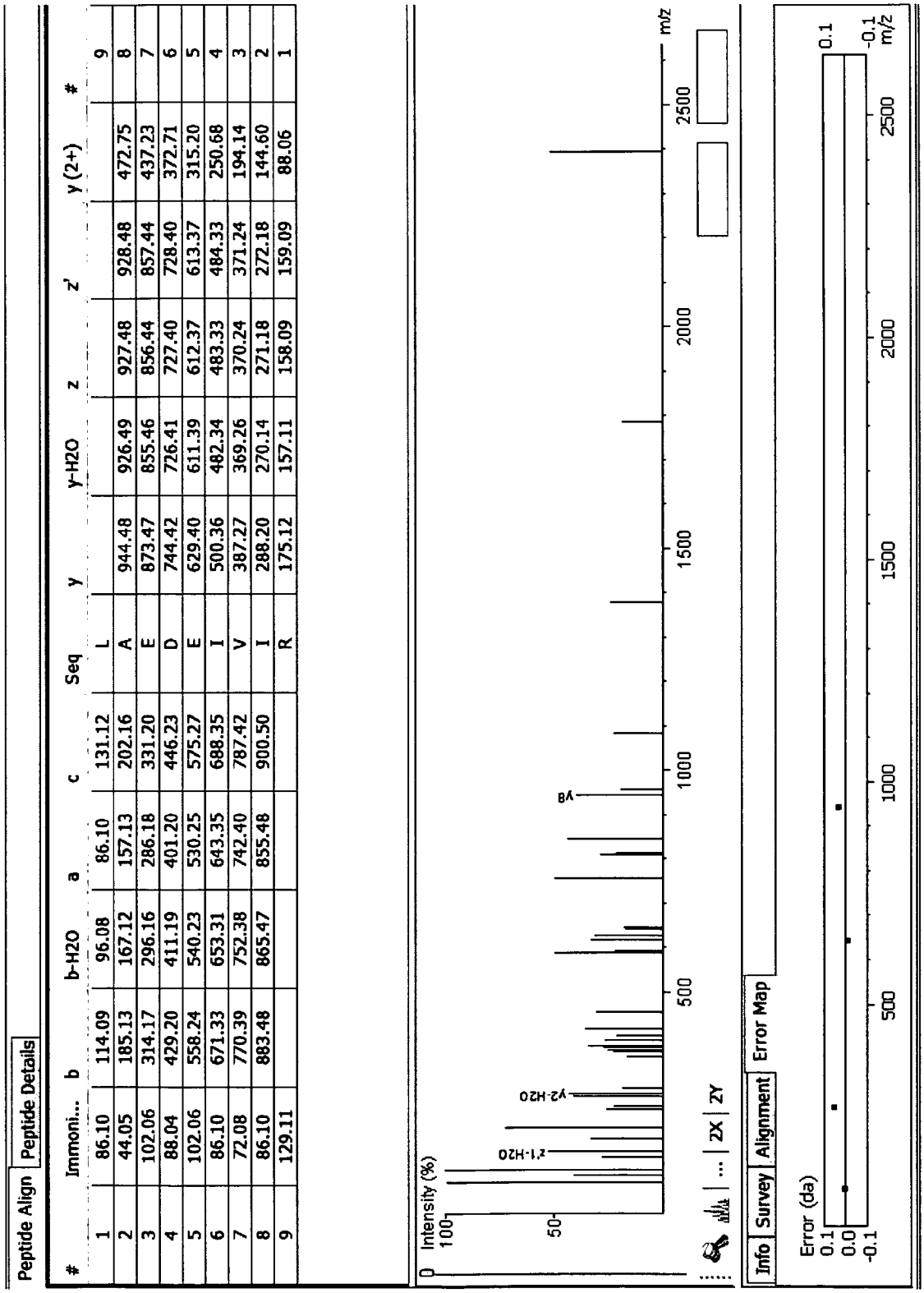
G)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	86.10	114.09	96.08	86.10	131.12	L						13
2	101.11	242.19	224.18	214.19	259.21	K	1482.83	1464.82	1465.80	1466.80	741.92	12
3	102.06	371.23	353.21	343.23	388.26	E	1354.74	1336.73	1337.71	1338.71	677.87	11
4	74.06	472.28	454.27	444.28	489.30	T	1225.69	1207.68	1208.67	1209.67	613.35	10
5	120.08	619.35	601.33	591.35	636.37	F	1124.65	1106.64	1107.62	1108.62	562.82	9
6	87.06	733.39	715.38	705.39	750.42	N	977.58	959.57	960.55	961.55	489.29	8
7	101.11	861.48	843.47	833.49	878.51	K	863.53	845.52	846.51	847.51	432.27	7
8	74.06	962.53	944.52	934.54	979.56	T	735.44	717.43	718.41	719.41	368.22	6
9	86.10	1075.62	1057.60	1047.62	1092.64	I	634.33	616.38	617.37	618.37	317.70	5
10	86.10	1188.70	1170.69	1160.70	1205.73	L	521.29	503.30	504.28	505.28	261.15	4
11	120.08	1335.77	1317.76	1307.77	1352.79	F	408.22	390.21	391.20	392.20	204.61	3
12	87.06	1449.81	1431.80	1421.82	1466.84	N	261.16	243.15	244.13	245.13	131.08	2
13	101.11					K	147.11	129.10	130.09	131.09	74.06	1

Continuation of Figure 23

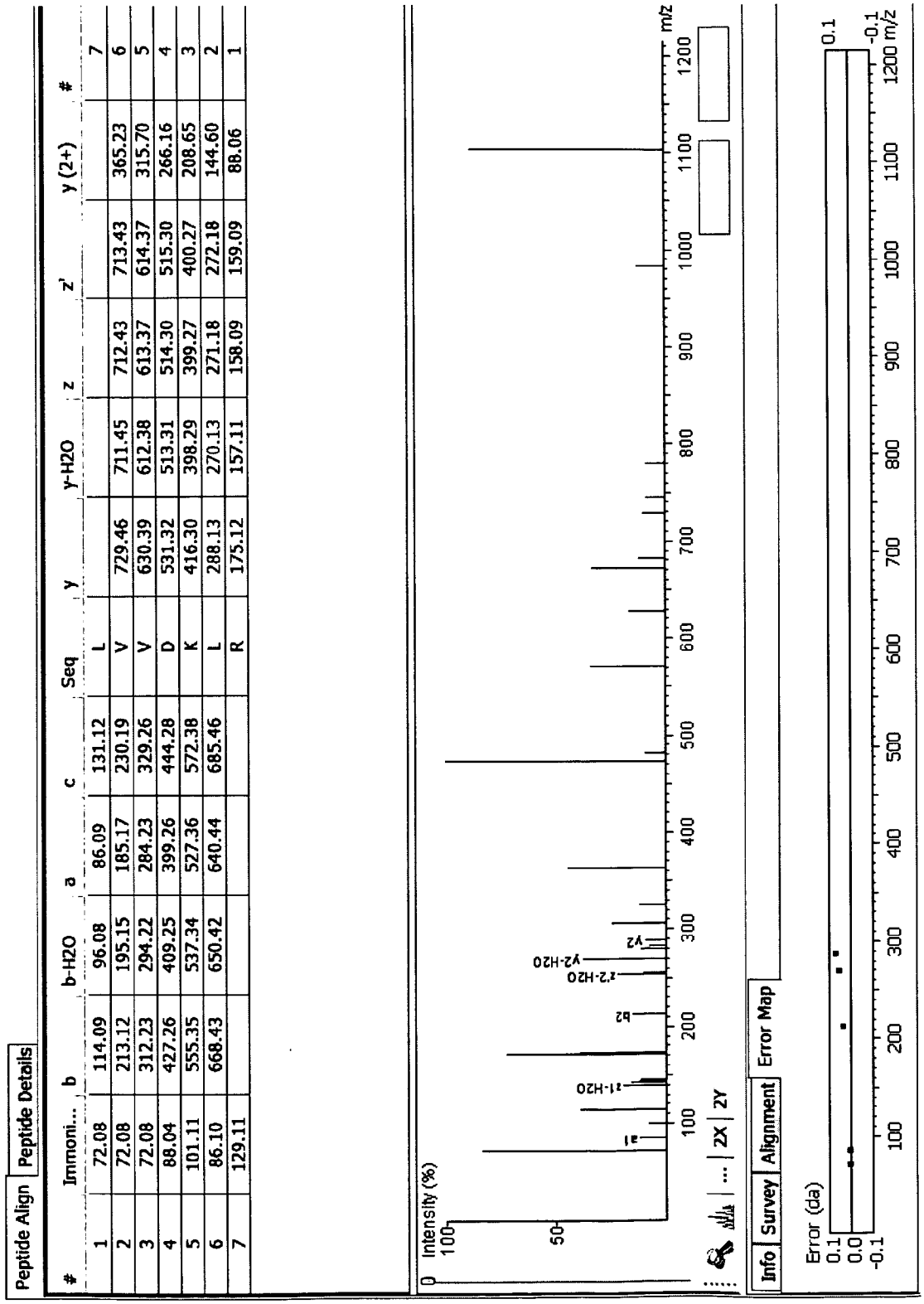
H)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	86.10	114.09	96.08	86.10	131.12	L						9
2	44.05	185.13	167.12	157.13	202.16	A	944.48	926.49	927.48	928.48	472.75	8
3	102.06	314.17	296.16	286.18	331.20	E	873.47	855.46	856.44	857.44	437.23	7
4	88.04	429.20	411.19	401.20	446.23	D	744.42	726.41	727.40	728.40	372.71	6
5	102.06	558.24	540.23	530.25	575.27	E	629.40	611.39	612.37	613.37	315.20	5
6	86.10	671.33	653.31	643.35	688.35	I	500.36	482.34	483.33	484.33	250.68	4
7	72.08	770.39	752.38	742.40	787.42	V	387.27	369.26	370.24	371.24	194.14	3
8	86.10	883.48	865.47	855.48	900.50	I	288.20	270.14	271.18	272.18	144.60	2
9	129.11					R	175.12	157.11	158.09	159.09	88.06	1

Continuation of Figure 23

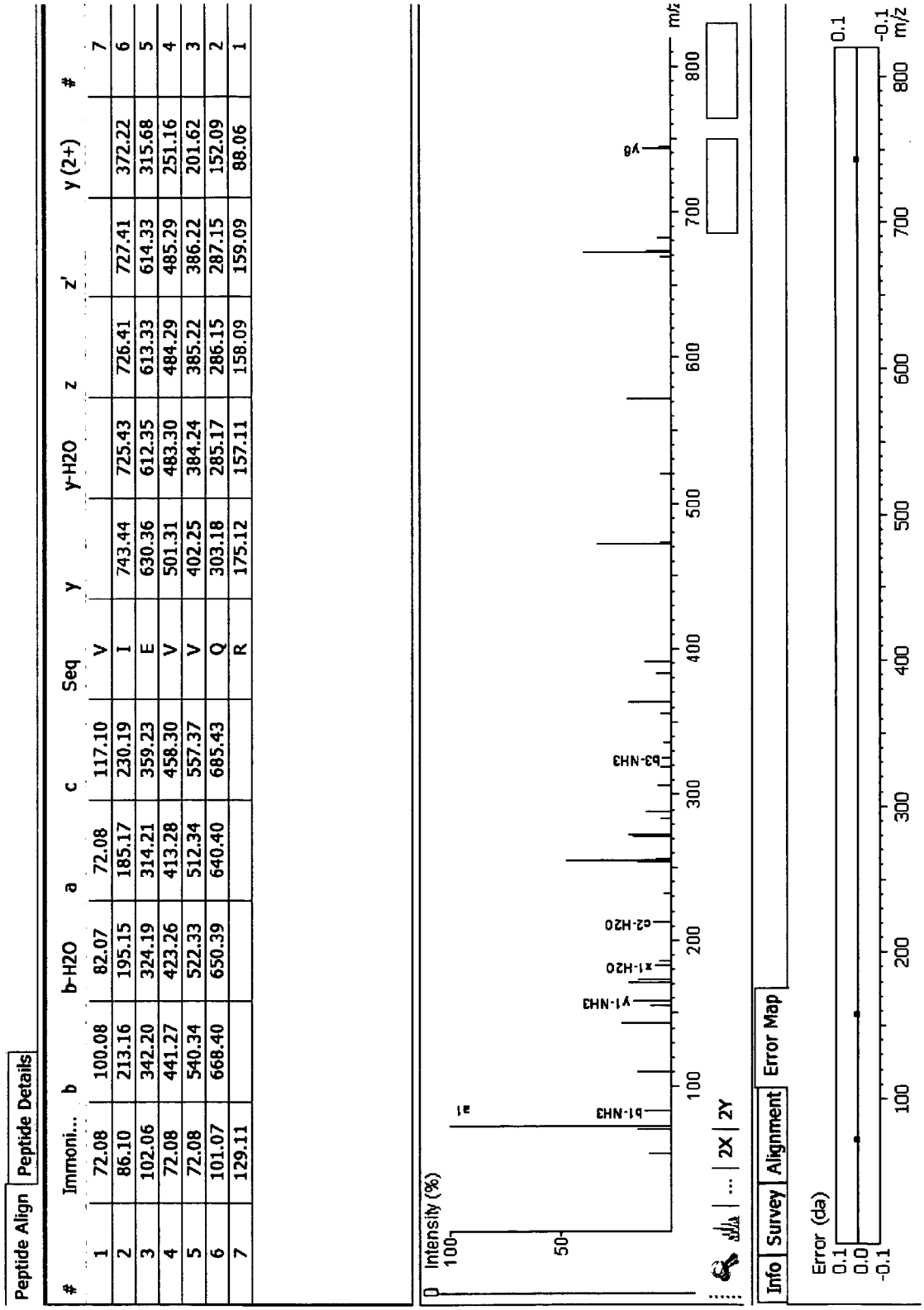
I)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	72.08	114.09	96.08	86.09	131.12	L						7
2	72.08	213.12	195.15	185.17	230.19	V	729.46	711.45	712.43	713.43	365.23	6
3	72.08	312.23	294.22	284.23	329.26	V	630.39	612.38	613.37	614.37	315.70	5
4	88.04	427.26	409.25	399.26	444.28	D	531.32	513.31	514.30	515.30	266.16	4
5	101.11	555.35	537.34	527.36	572.38	K	416.30	398.29	399.27	400.27	208.65	3
6	86.10	668.43	650.42	640.44	685.46	L	288.13	270.13	271.18	272.18	144.60	2
7	129.11					R	175.12	157.11	158.09	159.09	88.06	1

Continuation of Figure 23

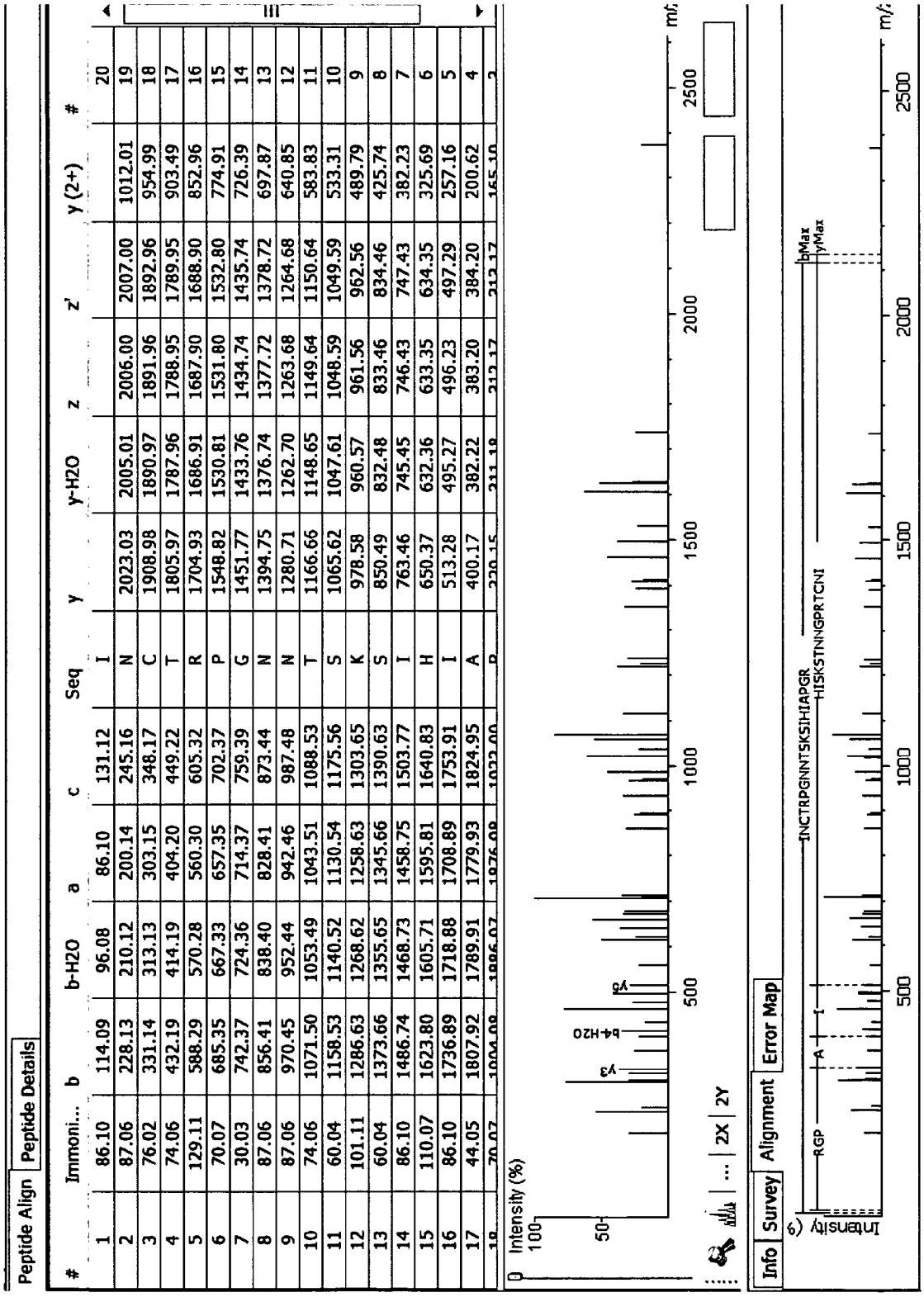
J)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	72.08	100.08	82.07	72.08	117.10	V						7
2	86.10	213.16	195.15	185.17	230.19	I	743.44	725.43	726.41	727.41	372.22	6
3	102.06	342.20	324.19	314.21	359.23	E	630.36	612.35	613.33	614.33	315.68	5
4	72.08	441.27	423.26	413.28	458.30	V	501.31	483.30	484.29	485.29	251.16	4
5	72.08	540.34	522.33	512.34	557.37	V	402.25	384.24	385.22	386.22	201.62	3
6	101.07	668.40	650.39	640.40	685.43	Q	303.18	285.17	286.15	287.15	152.09	2
7	129.11					R	175.12	157.11	158.09	159.09	88.06	1

Continuation of Figure 23

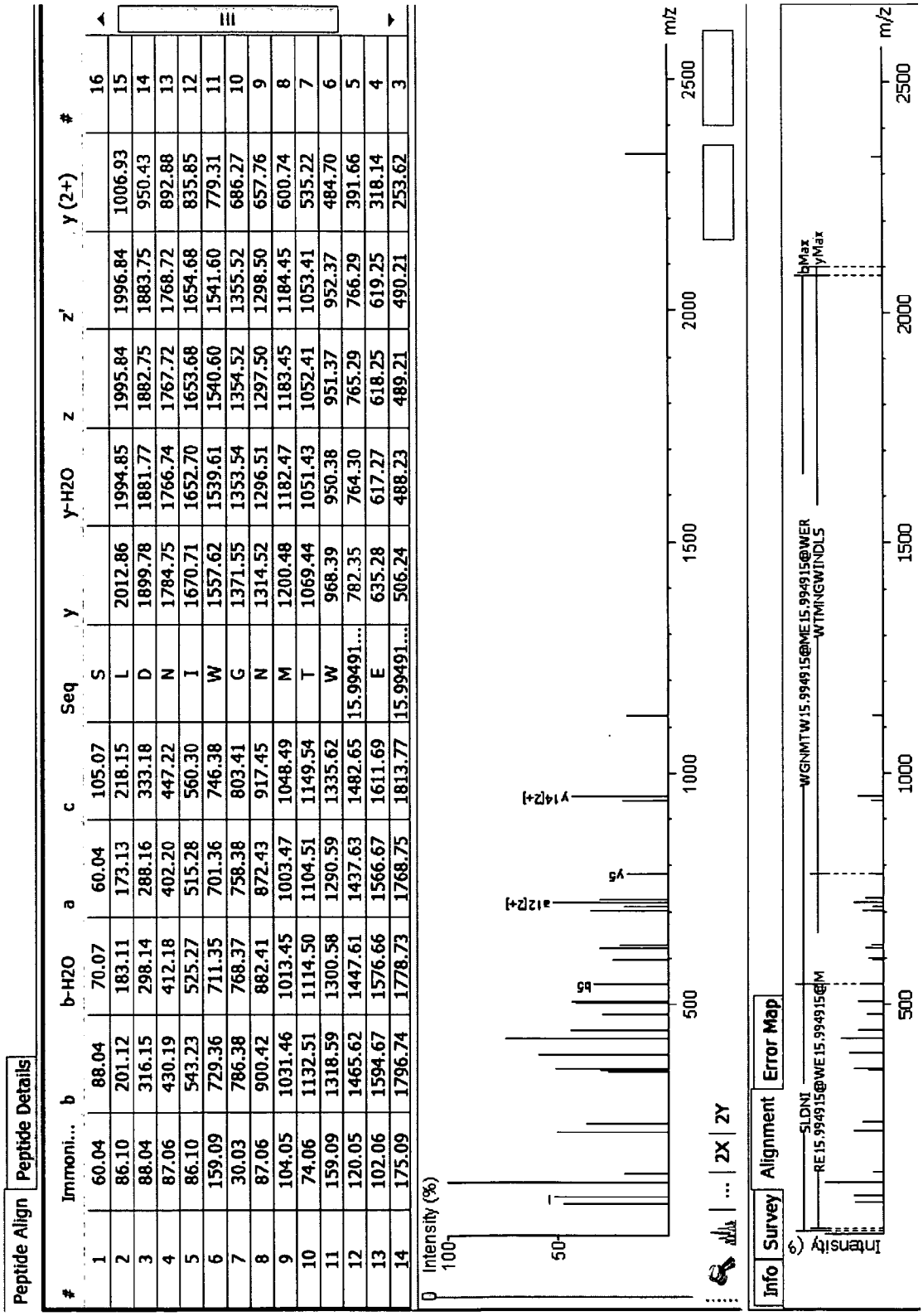
K)



#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	86.10	114.09	96.08	86.10	131.12	I						20
2	87.06	228.13	210.12	200.14	245.16	N	2023.03	2005.01	2006.00	2007.00	1012.01	19
3	76.02	331.14	313.13	303.15	348.17	C	1908.98	1890.97	1891.96	1892.96	954.99	18
4	74.06	432.19	414.19	404.20	449.22	T	1805.97	1787.96	1788.95	1789.95	903.49	17
5	129.11	588.29	570.28	560.30	605.32	R	1704.93	1686.91	1687.90	1688.90	852.96	16
6	70.07	685.35	667.33	657.35	702.37	P	1548.82	1530.81	1531.80	1532.80	774.91	15
7	30.03	742.37	724.36	714.37	759.39	G	1451.77	1433.76	1434.74	1435.74	726.39	14
8	87.06	856.41	838.40	828.41	873.44	N	1394.75	1376.74	1377.72	1378.72	697.87	13
9	87.06	970.45	952.44	942.46	987.48	N	1280.71	1262.70	1263.68	1264.68	640.85	12
10	74.06	1071.50	1053.49	1043.51	1088.53	T	1166.66	1148.65	1149.64	1150.64	583.83	11
11	60.04	1158.53	1140.52	1130.54	1175.56	S	1065.62	1047.61	1048.59	1049.59	533.31	10
12	101.11	1286.63	1268.62	1258.63	1303.65	K	978.58	960.57	961.56	962.56	489.79	9
13	60.04	1373.66	1355.65	1345.66	1390.63	S	850.49	832.48	833.46	834.46	425.74	8
14	86.10	1486.74	1468.73	1458.75	1503.77	I	763.46	745.45	746.43	747.43	382.23	7
15	110.07	1623.80	1605.71	1595.81	1640.83	H	650.37	632.36	633.35	634.35	325.69	6
16	86.10	1736.89	1718.88	1708.89	1753.91	I	513.28	495.27	496.23	497.29	257.16	5
17	44.05	1807.92	1789.91	1779.93	1824.95	A	400.17	382.22	383.20	384.20	200.62	4
18	70.07	1904.98	1886.97	1876.98	1922.00	P	229.15	211.10	212.12	213.12	155.10	3

Continuation of Figure 23

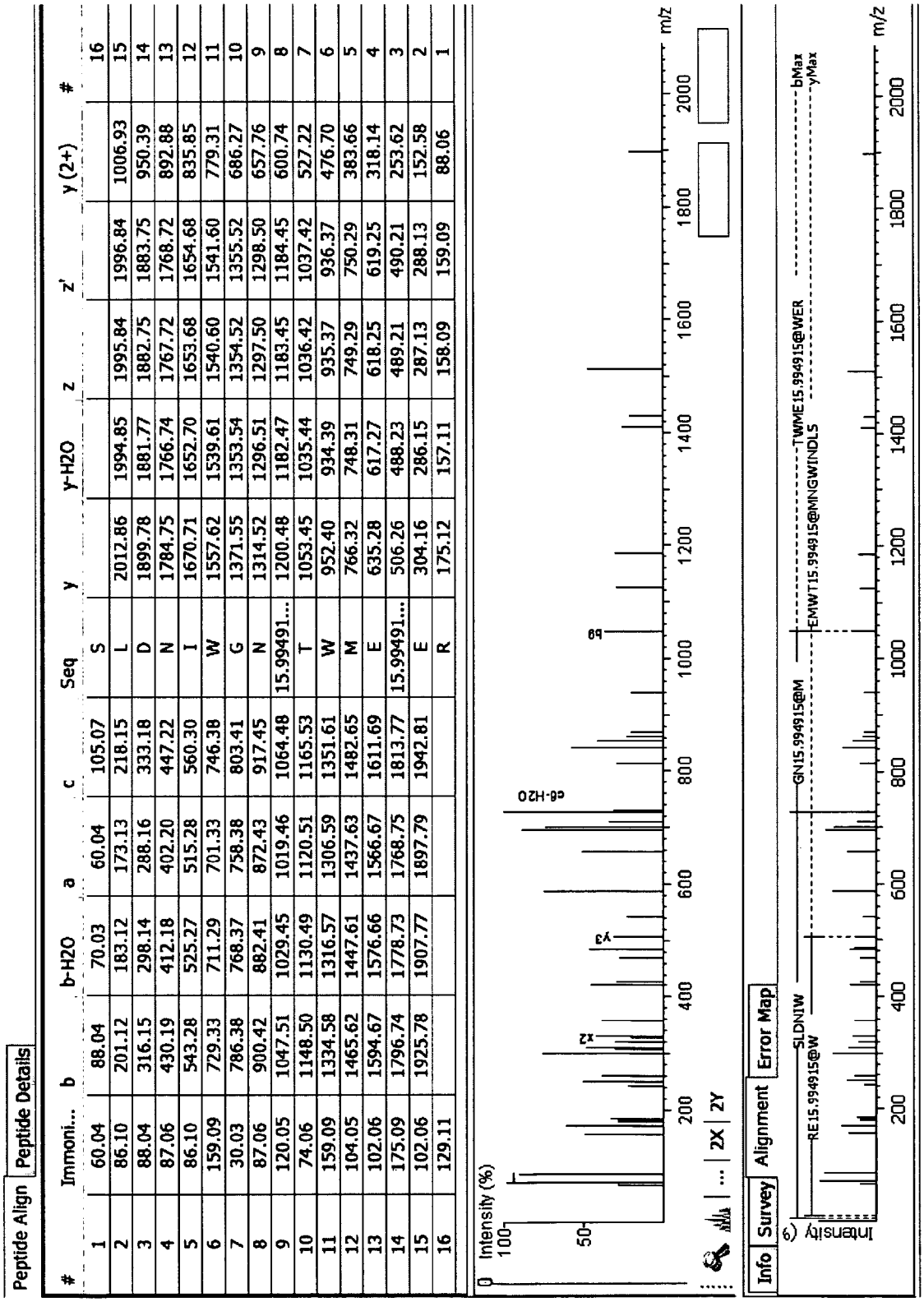
L)



#	Immoni... b		b-H2O	a	c	Seq	S	y	y-H2O	z	z'	y (2+)	#
1	60.04	88.04	70.07	60.04	105.07								16
2	86.10	201.12	183.11	173.13	218.15		L	2012.86	1994.85	1995.84	1996.84	1006.93	15
3	88.04	316.15	298.14	288.16	333.18		D	1899.78	1881.77	1882.75	1883.75	950.43	14
4	87.06	430.19	412.18	402.20	447.22		N	1784.75	1766.74	1767.72	1768.72	892.88	13
5	86.10	543.23	525.27	515.28	560.30		I	1670.71	1652.70	1653.68	1654.68	835.85	12
6	159.09	729.36	711.35	701.36	746.38		W	1557.62	1539.61	1540.60	1541.60	779.31	11
7	30.03	786.38	768.37	758.38	803.41		G	1371.55	1353.54	1354.52	1355.52	686.27	10
8	87.06	900.42	882.41	872.43	917.45		N	1314.52	1296.51	1297.50	1298.50	657.76	9
9	104.05	1031.46	1013.45	1003.47	1048.49		M	1200.48	1182.47	1183.45	1184.45	600.74	8
10	74.06	1132.51	1114.50	1104.51	1149.54		T	1069.44	1051.43	1052.41	1053.41	535.22	7
11	159.09	1318.59	1300.58	1290.59	1335.62		W	968.39	950.38	951.37	952.37	484.70	6
12	120.05	1465.62	1447.61	1437.63	1482.65		15.994915...	782.35	764.30	765.29	766.29	391.66	5
13	102.06	1594.67	1576.66	1566.67	1611.69		E	635.28	617.27	618.25	619.25	318.14	4
14	175.09	1796.74	1778.73	1768.75	1813.77		15.994915...	506.24	488.23	489.21	490.21	253.62	3

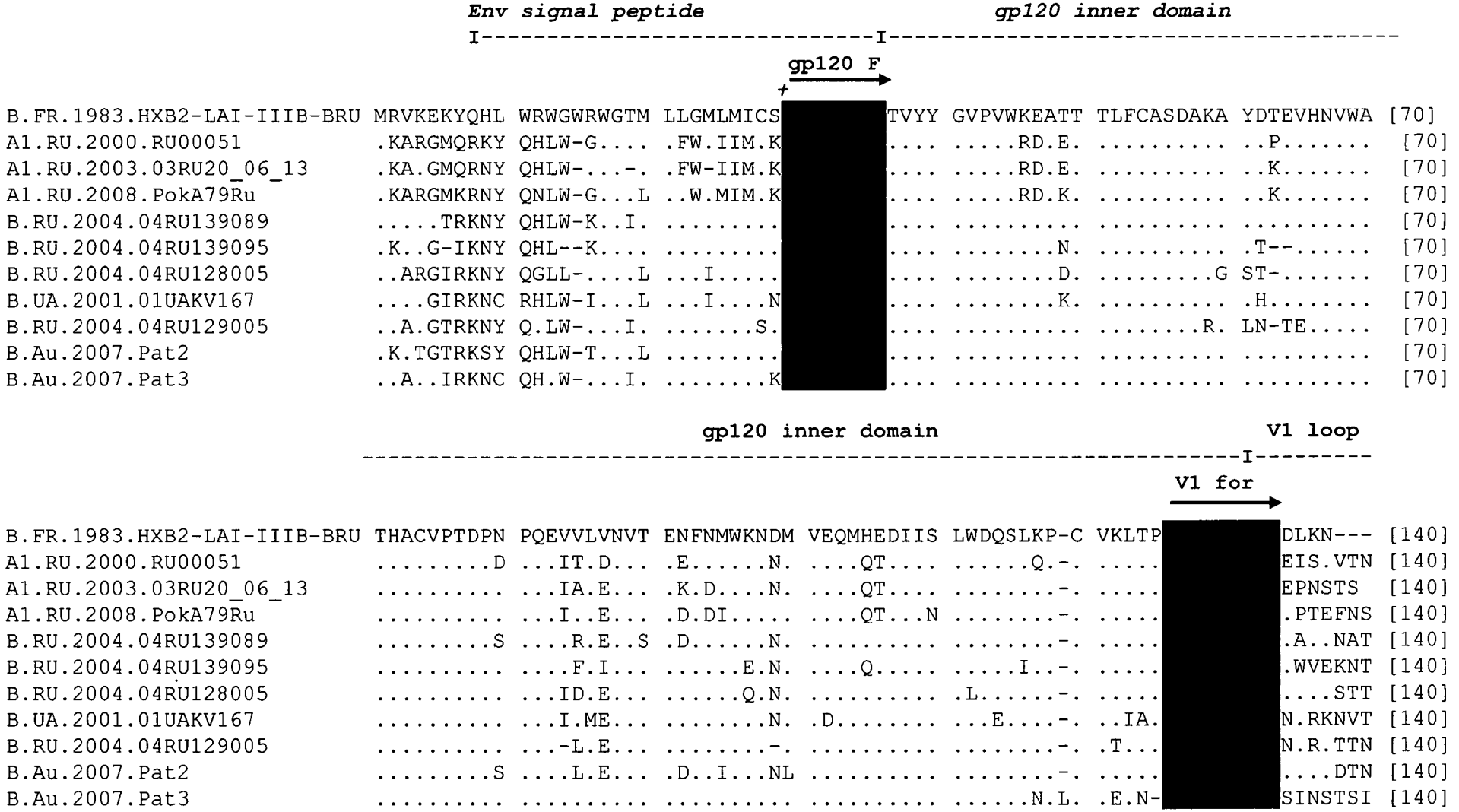
Continuation of Figure 23

M)

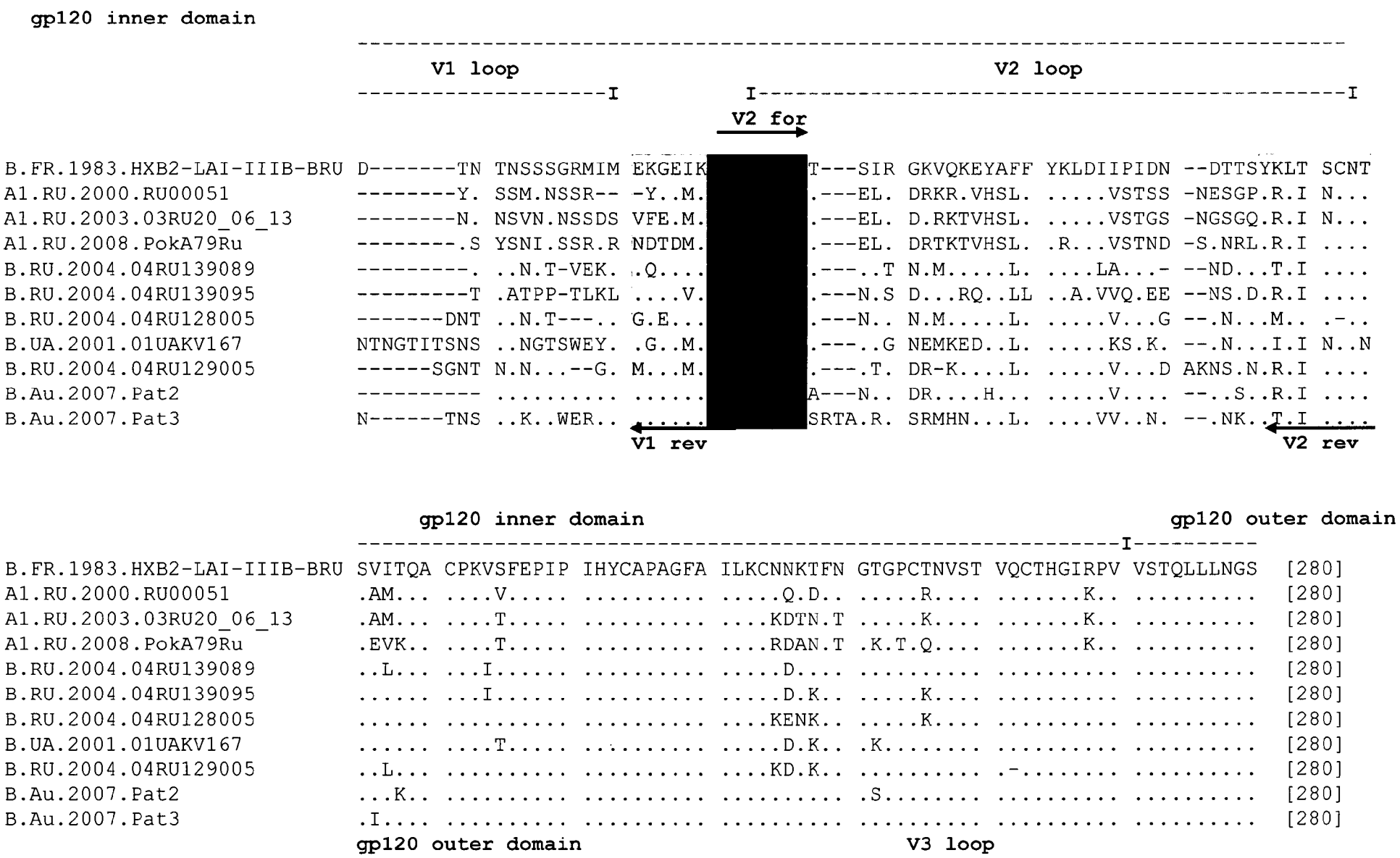


#	Immoni...	b	b-H2O	a	c	Seq	y	y-H2O	z	z'	y (2+)	#
1	60.04	88.04	70.03	60.04	105.07	S						16
2	86.10	201.12	183.12	173.13	218.15	L	2012.86	1994.85	1995.84	1996.84	1006.93	15
3	88.04	316.15	298.14	288.16	333.18	D	1899.78	1881.77	1882.75	1883.75	950.39	14
4	87.06	430.19	412.18	402.20	447.22	N	1784.75	1766.74	1767.72	1768.72	892.88	13
5	86.10	543.28	525.27	515.28	560.30	I	1670.71	1652.70	1653.68	1654.68	835.85	12
6	159.09	729.33	711.29	701.33	746.38	W	1557.62	1539.61	1540.60	1541.60	779.31	11
7	30.03	786.38	768.37	758.38	803.41	G	1371.55	1353.54	1354.52	1355.52	686.27	10
8	87.06	900.42	882.41	872.43	917.45	N	1314.52	1296.51	1297.50	1298.50	657.76	9
9	120.05	1047.51	1029.45	1019.46	1064.48	15.99491...	1200.48	1182.47	1183.45	1184.45	600.74	8
10	74.06	1148.50	1130.49	1120.51	1165.53	T	1053.45	1035.44	1036.42	1037.42	527.22	7
11	159.09	1334.58	1316.57	1306.59	1351.61	W	952.40	934.39	935.37	936.37	476.70	6
12	104.05	1465.62	1447.61	1437.63	1482.65	M	766.32	748.31	749.29	750.29	383.66	5
13	102.06	1594.67	1576.66	1566.67	1611.69	E	635.28	617.27	618.25	619.25	318.14	4
14	175.09	1796.74	1778.73	1768.75	1813.77	15.99491...	506.26	488.23	489.21	490.21	253.62	3
15	102.06	1925.78	1907.77	1897.79	1942.81	E	304.16	286.15	287.13	288.13	152.58	2
16	129.11					R	175.12	157.11	158.09	159.09	88.06	1

Figure 24 **Primers disposition for PCR products of immunogenic composition's tailoring mapped for several sequences in alignment.**



Continuation of Figure 24



Continuation of Figure 24

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                               -----------------------------I-------------------------------------I
                                                          V3 for
                                                         ------>
B.FR.1983.HXB2-LAI-IIIB-BRU  LAEEEVVIRS VNFTDNAKTI IVQLNTSV[redacted]NNTR KRIRIQR-GP GRAFVTIG-K IGNMRQAHCN [350]
A1.RU.2000.RU00051           ...GN.T... E.I...T... ....SKP.[redacted].... TS...G---. .QT.YAT.DI ..DT.K.... [350]
A1.RU.2003.03RU20_06_13      ...K..-M.. E.I...G.-. ....TEP.[redacted].... TS...G---. .QT.YAT-DV ..DI.K.Y.- [350]
A1.RU.2008.PokA79Ru          ...K.IM... K.LSN...N. .....EP.[redacted]IK.. .G.HVGHT.. .Q.IYVT.AV T.DI.K.... [350]
B.RU.2004.04RU139089         ...G...... E.......N. L....EA.[redacted].... .S.HM---.L ....YAT.DI ..DI...... [350]
B.RU.2004.04RU139095         .....I.... E.L...V... ..H..E..[redacted].... .G.P.---.. ...WYAT.DI T.DI.K.... [350]
B.RU.2004.04RU128005         .......V.. R..S....N. ....KDP.[redacted].... .S.S.---.. .-..YAT.DI ..DI.-.... [350]
B.UA.2001.01UAKV167          ...GDI.... E.....V... .....E.I[redacted].... .G.H.---.. .GT.FAT.AI ...I...... [350]
B.RU.2004.04RU129005         .......... A...N..... .....E..[redacted].... .S.P.---.. ....Y.T.DI ..DI.....- [350]
B.Au.2007.Pat2               ....G..... E.......N. ....SV.I[redacted].... XS.X.G---. ....YAT.QI ..DI...... [350]
B.Au.2007.Pat3               .......... Q..SN..R.. ....KEA.[redacted].... .S.PMG---. .K..F.T.DI ..DI...Y.. [350]

                                                          gp120 outer domain          V4 loop
                             ---------------------------------------------------I------------------
                                                                                V4 for
                                                                               ------>
B.FR.1983.HXB2-LAI-IIIB-BRU  ISRAKWNNTL KQIASKLREQ FGNNKTIIFK Q-SSGGDPEI VTHSFNCGG[redacted]QLF NSTWFNSTW- [420]
A1.RU.2000.RU00051           V..TE.G.AT EE.RKQ.QTY .P.-...S.. N-.....L.. T........[redacted]G.. .NAS-.N--- [420]
A1.RU.2003.03RU20_06_13      V...A.S-.. QK.STQ..KY .N.-...-.. N-.....L.V T........[redacted]D.. ....-DG--- [420]
A1.RU.2008.PokA79Ru          V......T.. QKLSER..KY .V.-...... N-H...A.. T........[redacted]E.. ....YKN--- [420]
B.RU.2004.04RU139089         ..AT...... A...K..Q.H .R.-...V.N H-........ .M.......[redacted]K.. .R..LTN--- [420]
B.RU.2004.04RU139095         ......D-E. .KVVK..... .K.-...... .-........ .M.T.....[redacted]... ....NTN--- [420]
B.RU.2004.04RU128005         L.G.D.TK.. E..VK..-.. YNK--..V.. .-.......- IM.......[redacted]K.. ....E.N--- [420]
B.UA.2001.01UAKV167          L...Q..K.. E..VI..GK. .NS-T....D .QPP...... LM.T.....[redacted]K.. ....N.G..T [420]
B.RU.2004.04RU129005         L.ST...D.. R..VE..... ...-...K.N .-........ .M.......[redacted]P.. ....NS.HG- [420]
B.Au.2007.Pat2               ...TE..... QEV.K..E.. .K.-...V.N .-........ .M.......[redacted]K.. ....NSN--- [420]
B.Au.2007.Pat3               ...KN..D.. R..VK..... V..-.K.V.N .-........ .M.T.....[redacted]D.. ....LAN--- [420]
                               <------
                               V3 rev
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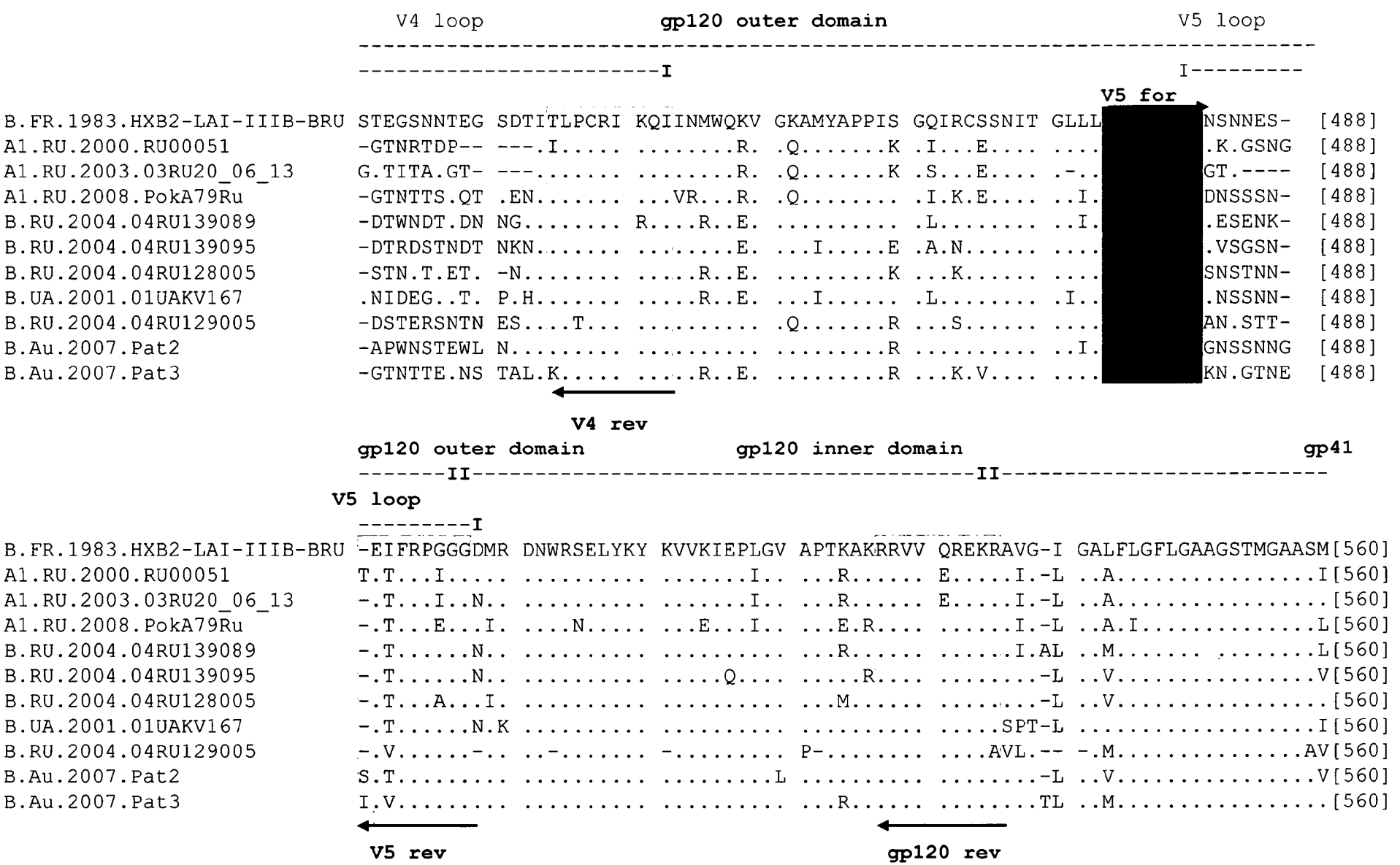
Continuation of Figure 24



Continuation of Figure 24

gp41

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B.FR.1983.HXB2-LAI-IIIB-BRU TLTVQARQLL SGIVQQQNNL LRAIEAQQHL LQLTVWGIKQ LQARILAVER YLKDQQLLGI WGCSGKLICT [630]
A1.RU.2000.RU00051          .......... .......S.. .......... .K........ ....V..... .......... .......... [630]
A1.RU.2003.03RU20_06_13     .......... .......S.. .......... .K........ ....V..... .......... .......... [630]
A1.RU.2008.PokA79Ru         .......K.. .......S.. .......... .K........ ....V..... .......... .......... [630]
B.RU.2004.04RU139089        .......L.. .......... .......... .......... ....V..... .......... .......... [630]
B.RU.2004.04RU139095        .......... .......... .......... .......... ....V..... .......... .......... [630]
B.RU.2004.04RU128005        .......... .........- .......... .......... ....V..... .......... .......... [630]
B.UA.2001.01UAKV167         .......... .......... .......... .......... ....V..... ..G....... ...A...... [630]
B.RU.2004.04RU129005        .......... .........N .......... .......... ....V..... .......... .......... [630]
B.Au.2007.Pat2              .......L.. .......S.. .......... .......... ....V..... ..R....... .......... [630]
B.Au.2007.Pat3              .......... .......... .K........ .......... ....V..... .......... .......... [630]
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gp41

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B.FR.1983.HXB2-LAI-IIIB-BRU TAVPWNASWS NKSLEQIWNH TTWMEWDREI NNYTSLIHSL IEESQNQQEK NEQELLELDK WASLWNWFNI [700]
A1.RU.2000.RU00051          .N....S... ...QSD..DN L...Q..... H...DI.YD. ..K....... ...D..A... .........D. [700]
A1.RU.2003.03RU20_06_13     .N....S... ...QSE..DN M...Q..K.V I...-I.YD. ..K....... ...D..A... .....-..D. [700]
A1.RU.2008.PokA79Ru         .N....S... ....RA..DN M......K.V H...NI.YQ. .......... ......AF.. .........D. [700]
B.RU.2004.04RU139089        .......... ....NE..DN M.....E... ....G..YT. .......... ......A..T ..N..S..D. [700]
B.RU.2004.04RU139095        .T....T... ....DE..DN M.....EK.. ....G..YR. .......... ......A... .........S. [700]
B.RU.2004.04RU128005        .......... ....NE..DN M...Q.R-.. D...-T.YN. L......... .......... .........D. [700]
B.UA.2001.01UAKV167         .T.....T.. ..T.K...DN M.....E... ....DI.YN. ..Q....... .......... .........S. [700]
B.RU.2004.04RU129005        .N....T... ..--SN..DN M.....E... D...D..YN. ..K....... .......... .........D. [700]
B.Au.2007.Pat2              ......I... ....DD..GN M...Q.E... D...D..YT. L......... .......... .G......S. [700]
B.Au.2007.Pat3              .N....TT.. ...YNE..DN M...Q.E... D.H.NT.YT. LV........ ..KD...... .D......S. [700]
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Continuation of Figure 24

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                                                          gp41
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B.FR.1983.HXB2-LAI-IIIB-BRU TNWLWYIKLF IMIVGGLVGL RIVFAVLSIV NRVRQGYSPL SFQTHLPTPR GPDRPEGIEE EGGERDRDRS [770]
A1.RU.2000.RU00051          S......... ......I... ........VI .......... .-..LT.N.E -....GR..D G...-GK... [770]
A1.RU.2003.03RU20_06_13     S.......I. .I...S.I.. .-I....Y.I ..A....... .L..LT.H.E .....GR.K. ....QG.... [770]
A1.RU.2008.PokA79Ru         SK.....RI. ......I... ....T....I .......... .L..LT.H.E ....LG..K. ....QG.... [770]
B.RU.2004.04RU139089        S.......I. ...I...... ..I.T..... .......... ....R..VR. E....GE... ........T. [770]
B.RU.2004.04RU139095        S.......I. .......... ..I....... .......... ....R...Q. E....-EG.. .....G..T. [770]
B.RU.2004.04RU128005        ........I. .I........ .......... .......... .......AR. .......T.. .........K. [770]
B.UA.2001.01UAKV167         ........I. ......I... ..I.T.F... .......... ....R...Q. .........G ....Q..... [770]
B.RU.2004.04RU129005        ........I. .....-.... ....T..... .......... ....L.RA.. .......T.. ....-..... [770]
B.Au.2007.Pat2              ........I. .......... ....T.I... .......... ....RH.G.. .......... ....QG.... [770]
B.Au.2007.Pat3              .K......I. .......... ....T.I... .......... ....R..V.G .......... ...K.G.... [770]

                                                 gp41
                          ----------------------------------------------------------------------------

B.FR.1983.HXB2-LAI-IIIB-BRU IRLVNGSLAL IWDDLRSLCL FSYHRLRDLL LIVTRIVELL G-------RR GWEALKYWWN LLQYWSQELK [840]
A1.RU.2000.RU00051          ....G-F... -....-.... .L......FI ..A..T.... .HSSLKGL.L ...G...L.. ..V..-R... [840]
A1.RU.2003.03RU20_06_13     ....S.F... A......... ........FI -.AA.T.... .RSSLKGL.L ...G...LG. ..G..G.... [840]
A1.RU.2008.PokA79Ru         ...AS.F... A.E....... ........FI S.AA.T.... .RSSLKGL.L V..G...LG. ..G..G.... [840]
B.RU.2004.04RU139089        TP..A.F... .......... .......... ...V...... .-------.. .......... .......... [840]
B.RU.2004.04RU139095        ......F... ........-. .I........ .......... .-------.. .......... .......... [840]
B.RU.2004.04RU128005        G...D.F..I ..V..-.... .......... .......... .-------.. ...I...... .......... [840]
B.UA.2001.01UAKV167         GP..T.F..I ..V....... .......... ...A...... .-------.. .......... .......... [840]
B.RU.2004.04RU129005        -H..D.F..I ..V....... .......... .LI..T.... .-------.. .......... .......... [840]
B.Au.2007.Pat2              SP..D.F..I L.V....... .......... .......... .-------.. ...I...... .......... [840]
B.Au.2007.Pat3              G.SA..F.I. .....W...R ...L...... ...A...... .-------.. .......... .......... [840]
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Continuation of Figure 24

```
                                                    gp41
                                   ------------------------------------------------I

B.FR.1983.HXB2-LAI-IIIB-BRU NSAVSLLNAT AIAVAEGTDR VIEVVQGACR AIRHIPRRIR QGLERILL [888]
A1.RU.2000.RU00051          S..IN.VDTI .....GW... ...IG.RV.. .H--...-.. ..F.KA.Q [888]
A1.RU.2003.03RU20_06_13     S..IN.IDTI .....RW..- ...IG.RL.. ...N...... ..-.KA.Q [888]
A1.RU.2008.PokA79Ru         ...IN.IDTI ..V..GW... ...IG.RF.. ...N...... ..A..A.Q [888]
B.RU.2004.04RU139089        ......F... .......... ....I.RT.. .VL....... .....A.Q [888]
B.RU.2004.04RU139095        K.....F.VI .......... ...LI.T.F. .FI....... .....A.. [888]
B.RU.2004.04RU128005        ...I...... .......... I..LL.R.G. ..I...T... .....A.. [888]
B.UA.2001.01UAKV167         S..I..F..I .........K I..IA.TIG. ..L....... .....F.. [888]
B.RU.2004.04RU129005        -..I...... .......... -..IGLRIF. ..L...T... .....T.. [888]
B.Au.2007.Pat2              K......... .V........ I.....RIG. .VL...T... .....A.. [888]
B.Au.2007.Pat3              .......... .......... IV.II.RL.. ..L....... ..F..A.I [888]
                                                                          gp41 rev
```

Continuation of Figure 24

Example 4: Glycosylated recombinant proteins molecular weight calculations.

http://www.encorbio.com/protocols/Prot-MW.htm

160 #1 (B.FR.83.HXB2_LAI_IIIB_BRU_K034) There are 856 Amino acids, and MW is 97241.93 Daltons, or 97.24 KiloDaltons; Charge is 30 assuming N and C termini are not blocked

MRVKEKY QHLWRWGWRW GTMLLGMLMI CSAT-EKLWV TVYYGVPVWK EATTTLFCAS DAKAYDTEVH NVWATHACVP
TDPNPQEVVL VNVTENFNMW KNDMVEQMHE DIISLWDQSL KPCVKLTPLC VSLKCTDLKN DTNTNSSSGRMI MEKGEIKNCS
FNISTSIRGK VQKEYAFFYK LDIIPIDNDTTSY KLTSCNTSVI TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI
RPVVSTQLLL NGSLAE-EEV VIRSVNFTDN AKTIIVQLNT SVEINCTRPN NNTRKRIRIQ RGPGRAFVTI GKIGNMRQA HCNISRAKWN
NTLKQIASKL REQFGNNKTIIFKQS SGGDPEIVT HSFNCGGEFF YCNSTQLFNS TWFNSTWSTEGS NNTEGSDTITLPCR IKQIINMWQK
VGKAMYAPPI SGQIRCSSNI TGLLLTRDGG NSNNESEIFRP GGGDMRDNWR SELYKYKVVK IEPLGVAPTK AKRRVVQRE KR[redacted]
LFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQNNLLR AIEAQQHLLQ LTVWGIKQLQ ARILAVERYL KDQQLLGIWG CSGKLICTTA
VPWNASWSNK SLEQIWNHTT WMEWDREINN YTSLIHSLIE ESQNQQEKNE QELLELDKWA SLWNWFNITN WLWYIKLFIM
IVGGLVGLRI VFAVLSIVNR VRQGYSPLSF QTHLPTPRGP DRPEGIEEEG GERDRDRSIR LVNGSLALIW DDLRSLCLFS YHRLRDLLLI
VTRIVELLGR RGW EALKYWWNLL QYWSQELKNS AVSLLNATAI AVAEGTDRVI EVVQGACRAI RHIPRRIRQG LERILL

120 #1 There are 511 Amino acids, and MW is 57695.64 Daltons, or 57.70 KiloDaltons

MRVKEKY QHLWRWGWRW GTMLLGMLMI CSAT-EKLWV TVYYGVPVWK EATTTLFCAS DAKAYDTEVH NVWATHACVP
TDPNPQEVVL VNVTENFNMW KNDMVEQMHE DIISLWDQSL KPCVKLTPLC VSLKCTDLKN DTNTNSSSGRMI MEKGEIKNCS
FNISTSIRGK VQKEYAFFYK LDIIPIDNDTTSY KLTSCNTSVI TQACPKVSFE PIPIHYCAPA GFAILKCNNK TFNGTGPCTN VSTVQCTHGI
RPVVSTQLLL NGSLAE-EEV VIRSVNFTDN AKTIIVQLNT SVEINCTRPN NNTRKRIRIQ RGPGRAFVTI GKIGNMRQA HCNISRAKWN
NTLKQIASKL REQFGNNKTIIFKQS SGGDPEIVT HSFNCGGEFF YCNSTQLFNS TWFNSTWSTEGS NNTEGSDTITLPCR IKQIINMWQK
VGKAMYAPPI SGQIRCSSNI TGLLLTRDGG NSNNESEIFRP GGGDMRDNWR SELYKYKVVK IEPLGVAPTK AKRRVVQRE KR

41#1 There are 345 Amino acids, and MW is 39564.30 Daltons, or 39.56 KiloDaltons

AV-G-IGA LFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQNNLLR AIEAQQHLLQ LTVWGIKQLQ ARILAVERYL KDQQLLGIWG
CSGKLICTTA VPWNASWSNK SLEQIWNHTT WMEWDREINN YTSLIHSLIE ESQNQQEKNE QELLELDKWA SLWNWFNITN
WLWYIKLFIM IVGGLVGLRI VFAVLSIVNR VRQGYSPLSF QTHLPTPRGP DRPEGIEEEG GERDRDRSIR LVNGSLALIW DDLRSLCLFS
YHRLRDLLLI VTRIVELLGR RGW EALKYWWNLL QYWSQELKNS AVSLLNATAI AVAEGTDRVI EVVQGACRAI RHIPRRIRQG LERILL

Continuation of Figure 24

160 #43(K.CM.96.MP535_AJ249239): There are 842 Amino acids, and MW is 95252.58 Daltons, or 95.25 KiloDaltons Charge is 22 assuming N and C termini are not blocked

MRVRGMQRNW QTLGNWG ILFLGILII CSNA-DKLWV TVYYGVPVWK EATPTLFCAS DAKAYEKEVH NVWATHACVP TDPNPQEVEM ENVTENFNMW KNNMVEQMHT DIISLWDESL KPCVELTPLC VTLNCTDYKG TNSTN NATSTV VSPAEIKNCS FNITTEIKDK KKKESALFYR LDVLPLNGEG NNSSTEY RLINCNTSTI TQTCPKVTFE PIPIHYCAPA GFAILKCKDK RFNGTGPCKN VSTVQCTHGI KPVVSTQLLL NGSLAE-EEI IIRSENITDN TKNIIVQLNE TVQINCTRPN NNTRKSIHM GPGKAFYTT GDIIGDIRQA HCNISGEKWN MTLSRVKEKL KEHFK NGTITFKPP NPGGDPEILT HMFNCAGEFF YCNTTKLFNE TGENGTITLPCR IKQIINMWQK VGKAIYAPPI AGSINCSSNI TGMILTRDGG NNTHN ETFRP GGGDMRDNWR SELYKYKVVQ IEPLGIAPTR ARRRVVQRE KR[redacted]VFFGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ LTVWGIKQLR ARILAVERYL KDQQLLGIWG CSGKLICTTN VPWNSSWSNK SWEEIWNNMT WMEWEKEIGN YSDTIYKLIE ESQTQQEKNE QDLLALDKWA SLWNWFDITK WLWYIKIFIM IIGGLIGLRI AFAVLSVVNR VRQGYSPLSF QTLIPTSRGA DRPEGIEEEG GEQDKNRSVR LVSGFLALAW DDLRNLCLFS YRQLRNLILI VTRILERGLRGGW EALKYLWNLV QYWSQELKNS AISLLNTTAI AVAGGTDRII EIGQRAFRAL LHIPRRIRQG LERALL

120 #43: There are 497 Amino acids, and MW is 55884.46 Daltons, or 55.88 KiloDaltons

MRVRGMQRNW QTLGNWG ILFLGILII CSNA-DKLWV TVYYGVPVWK EATPTLFCAS DAKAYEKEVH NVWATHACVP TDPNPQEVEM ENVTENFNMW KNNMVEQMHT DIISLWDESL KPCVELTPLC VTLNCTDYKG TNSTN NATSTV VSPAEIKNCS FNITTEIKDK KKKESALFYR LDVLPLNGEG NNSSTEY RLINCNTSTI TQTCPKVTFE PIPIHYCAPA GFAILKCKDK RFNGTGPCKN VSTVQCTHGI KPVVSTQLLL NGSLAE-EEI IIRSENITDN TKNIIVQLNE TVQINCTRPN NNTRKSIHM GPGKAFYTT GDIIGDIRQA HCNISGEKWN MTLSRVKEKL KEHFK NGTITFKPP NPGGDPEILT HMFNCAGEFF YCNTTKLFNE TGENGTITLPCR IKQIINMWQK VGKAIYAPPI AGSINCSSNI TGMILTRDGG NNTHN ETFRP GGGDMRDNWR SELYKYKVVQ IEPLGIAPTR ARRRVVQRE KR

41 #43: There are 345 Amino acids, and MW is 39386.14 Daltons, or 39.39 KiloDaltons

AVGLGA VFFGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ LTVWGIKQLR ARILAVERYL KDQQLLGIWG CSGKLICTTN VPWNSSWSNK SWEEIWNNMT WMEWEKEIGN YSDTIYKLIE ESQTQQEKNE QDLLALDKWA SLWNWFDITK WLWYIKIFIM IIGGLIGLRI AFAVLSVVNR VRQGYSPLSF QTLIPTSRGA DRPEGIEEEG GEQDKNRSVR LVSGFLALAW DDLRNLCLFS YRQLRNLILI VTRILERGLRGGW EALKYLWNLV QYWSQELKNS AISLLNTTAI AVAGGTDRII EIGQRAFRAL LHIPRRIRQG LERALL

Continuation of Figure 24

160 #6 (our A1.RU.03.03RU20_06_13_AY500393) There are 805 Amino acids, and MW is 90669.32 Daltons, or 90.67 KiloDaltons: Charge is 24 assuming N and C termini are not blocked

MKAKGMQRNY QHLWRWG--- -XMLFWXIIM CKAA-ENLWV TVYYGVPVWR DAETTLFCAS DAKAYDKEVH NVWATHACVP
TDPNPQEIAL ENVTEKFDMW KNNMVEQMQT DIISLWDQSL KPCVKLTPLC VTLNCAEPNS TSSNNNS--- ---------- ---VNSNSSD
SVFEEMKNCT FNMTTELRDK RKTVHSLFYK LDIVSTGS-- --NGS----- -------GQY RLINCNTSAM TQACPKVTFE PIPIHYCAPA
GFAILKCKDT NFTGTGPCKN VSTVQCTHGI KPVVSTQLLL NGSLAE-KEV MXRSENITDN GKXIIVQLTE PVNITCIRPG NNTRTSIRI- -
GPGQTFYAT XDVIGDIRKA YCXVSRAAWX STLQKISTQL R--K-YFN-- NKT-IXFKNS S-GGDLEVTT HSFNCGGEFF YCNTTDLFNS TW----
DGXG TXT------- XITXA----N GT--ITLPCR IKQIINMWQR VGQAMYAPPIKGSIRCESNI TGLXLTRDGG GGT--NXX-- ----XETFRP
IGGNMRDNWR SELYKYKVVK IEPIGVAPTR AKRRVV-ERE KR [redacted] AFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQSNLLR
AIEAQQHLLK LTVWGIKQLQ ARVLAVERYL KDQQLLGIWG CSGKLICTTN VPWNSSWSNK SQSEIWDNMT WMQWDKEVIN
YTXIIYDLIE KSQNQQEKNE QDLLALDKWA SLWXWFDISN WLWYIKIFII IVGSLIGLRI XFAVLYIINR ARQGYSPLSL QTLTPHPEGP
DRPGRIKEEG GEQGRDRSIR LVSGFLALAW DDLRSLCLFS YHRLRDFIXI AARTVELLGR SSLKGLRLGW EGLKYLGNLL GYWGQELKSS
AINLIDTIAI

120 #6 There are 493 Amino acids, and MW is 55328.29 Daltons, or 55.33 KiloDaltons

MKAKGMQRNY QHLWRWG--- -XMLFWXIIM CKAA-ENLWV TVYYGVPVWR DAETTLFCAS DAKAYDKEVH NVWATHACVP
TDPNPQEIAL ENVTEKFDMW KNNMVEQMQT DIISLWDQSL KPCVKLTPLC VTLNCAEPNS TSSNNNS--- ---------- ---VNSNSSD
SVFEEMKNCT FNMTTELRDK RKTVHSLFYK LDIVSTGS-- --NGS----- -------GQY RLINCNTSAM TQACPKVTFE PIPIHYCAPA
GFAILKCKDT NFTGTGPCKN VSTVQCTHGI KPVVSTQLLL NGSLAE-KEV MXRSENITDN GKXIIVQLTE PVNITCIRPG NNTRTSIRI- -
GPGQTFYAT XDVIGDIRKA YCXVSRAAWX STLQKISTQL R--K-YFN-- NKT-IXFKNS S-GGDLEVTT HSFNCGGEFF YCNTTDLFNS TW----
DGXG TXT------- XITXA----N GT--ITLPCR IKQIINMWQR VGQAMYAPPIKGSIRCESNI TGLXLTRDGG GGT--NXX-- ----XETFRP
IGGNMRDNWR SELYKYKVVK IEPIGVAPTR AKRRVV-ERE KR

41#1 There are 346 Amino acids, and MW is 39333.71 Daltons, or 39.33 KiloDaltons

AI-G-LGA AFLGFLGAAG STMGAASMTL TVQARQLLSG IVQQQSNLLR AIEAQQHLLK LTVWGIKQLQ ARVLAVERYL KDQQLLGIWG
CSGKLICTTN VPWNSSWSNK SQSEIWDNMT WMQWDKEVIN YTXIIYDLIE KSQNQQEKNE QDLLALDKWA SLWXWFDISN WLWYIKIFII
IVGSLIGLRI XFAVLYIINR ARQGYSPLSL QTLTPHPEGP DRPGRIKEEG GEQGRDRSIR LVSGFLALAW DDLRSLCLFS YHRLRDFIXI
AARTVELLGR SSLKGLRLGW EGLKYLGNLL GYWGQELKSS AINLIDTIAI AVARWTDXVI EIGQRLCRAI RNIPRRIRQG XEKALQ

Continuation of Figure 24

160 #26 (C.ZA.01.01ZATM45_AY228557) There are 878 Amino acids, and MW is 99122.18 Daltons, or 99.12 KiloDaltons; Charge is 15.5 assuming N and C termini are not blocked

MRVRGIPRNW QQWWIWI--- -ILGFWMLLI CNVG-GNSWV TIYYGVPVWR EAKTTLFCAS DAKAHETEVH NVWATHACVP TDPNPQEIEL ENVTENFNMW KNDMVDQMHE DIISLWDQSL KPCVKLTPLC VTLNCTNATR PVTRTNTTAT GTNNTVTNCS GSASTNNTCM ENIEGMKNCS FNITTELRDK KKKEYALFYR LDIVPLNETR D-NSS----- ---------Y RLINCNTSTI TQACPKVSFD PIPIHYCAPA GYAILKCNNK TFSGTGPCNN VSTVQCTHGI MPVVSTQLLL NGSLAE-EMV IIRSDNMTNA ATTIIVHLKD PVEIVCTRPN NTTRREVGI- -GPGQTFYTT GQIIGDIRQA HCNITGKEWN KTLRQVGAEL E--K-HFP-- NKT-IQFKPH S-GGDLEITT HSFTCSGEFF YCNTSKLFNI SMS---NLT- ---Y------ NNTDNTD--N PTQ-ITLPCR IKQIINMWQE VGRAIYAPPI AGNITCKSNI TGLLLTWDGG SGE--NNT-- -----ETFRP GGGDMRDNWR SELYKYKVVE IKPLGIAPTE AKRRVV-ERE KR [redacted] VFLGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ LTVWGIKQLQ ARVLAIERYL KDQQLLGIWG CSGKLICTTS VPWNSSWSNK TLGEIWNNMT WMEWDKEISN YTHTIYQLLE ESQIQQEQNE KELLALDSWK NLWNWFDISN WLWYIKIFIM IVGGLIGLRI VFAVLSIVNR VRQGYSPLSF QTLTPSPREP DRLRGIEEEG GEQDKGRSIR LVQGFLALAW DDLRSLCLFS YHRLRDFISI AARVVEVLGH SSLRGLQRGW EALKYLKSLV QYWGLELKKS AVSLLDTLAI AVGEGTDRII ELIQGICRAI RNIPRRIRQG FEAALL

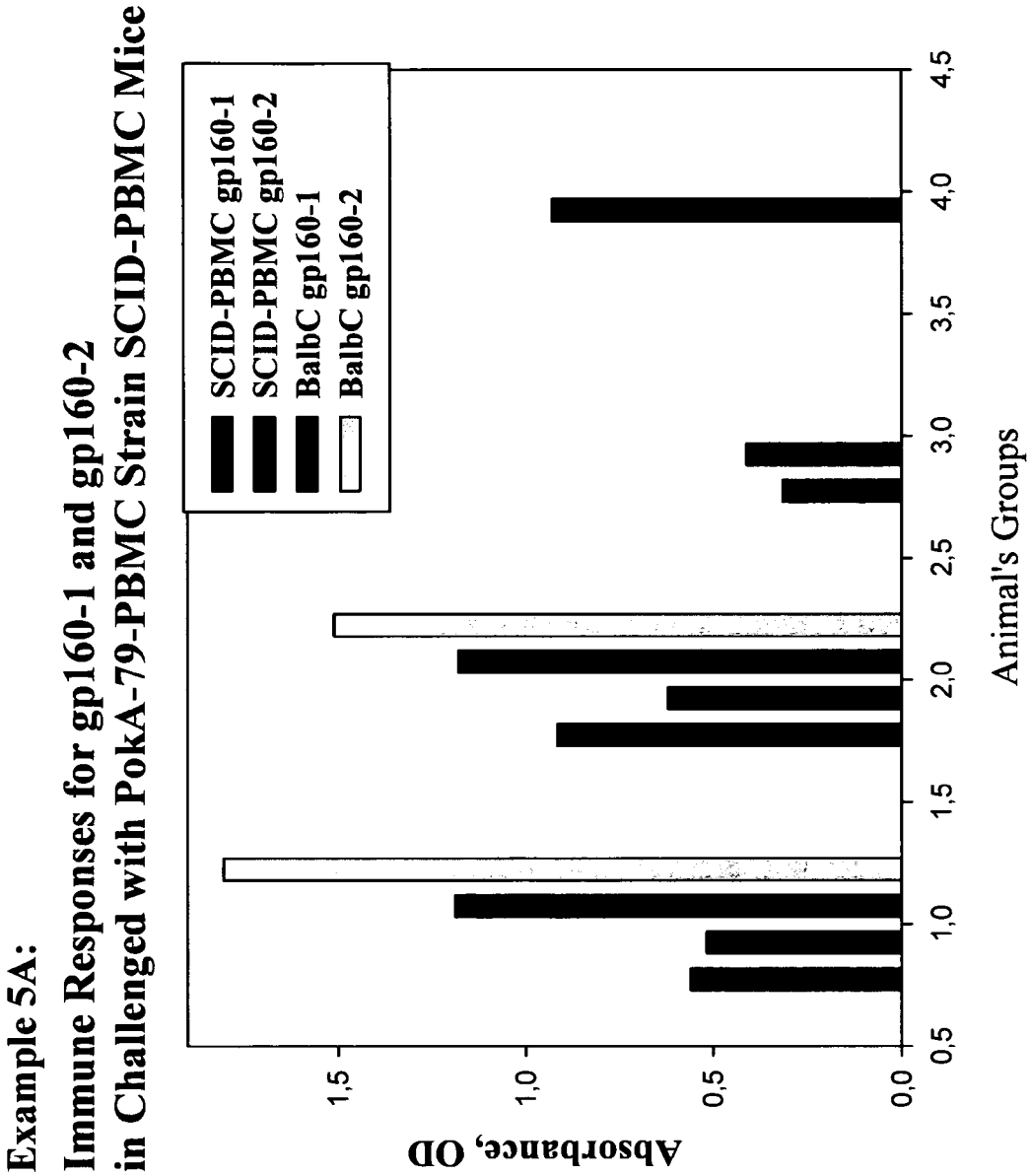
120 #26 (C.ZA.01.01ZATM45_AY228557) There are 526 Amino acids, and MW is 59182.32 Daltons, or 59.18 KiloDaltons

MRVRGIPRNW QQWWIWI--- -ILGFWMLLI CNVG-GNSWV TIYYGVPVWR EAKTTLFCAS DAKAHETEVH NVWATHACVP TDPNPQEIEL ENVTENFNMW KNDMVDQMHE DIISLWDQSL KPCVKLTPLC VTLNCTNATR PVTRTNTTAT GTNNTVTNCS GSASTNNTCM ENIEGMKNCS FNITTELRDK KKKEYALFYR LDIVPLNETR D-NSS----- ---------Y RLINCNTSTI TQACPKVSFD PIPIHYCAPA GYAILKCNNK TFSGTGPCNN VSTVQCTHGI MPVVSTQLLL NGSLAE-EMV IIRSDNMTNA ATTIIVHLKD PVEIVCTRPN NTTRREVGI- -GPGQTFYTT GQIIGDIRQA HCNITGKEWN KTLRQVGAEL E--K-HFP-- NKT-IQFKPH S-GGDLEITT HSFTCSGEFF YCNTSKLFNI SMS---NLT- ---Y------ NNTDNTD--N PTQ-ITLPCR IKQIINMWQE VGRAIYAPPI AGNITCKSNI TGLLLTWDGG SGE--NNT-- -----ETFRP GGGDMRDNWR SELYKYKVVE IKPLGIAPTE AKRRVV-ERE KR

41 #26 (C.ZA.01.01ZATM45_AY228557) There are 352 Amino acids, and MW is 39957.88 Daltons, or 39.96 KiloDaltons

AI-G-IGA VFLGFLGAAG STMGAASITL TVQARQLLSG IVQQQSNLLR AIEAQQHLLQ LTVWGIKQLQ ARVLAIERYL KDQQLLGIWG CSGKLICTTS VPWNSSWSNK TLGEIWNNMT WMEWDKEISN YTHTIYQLLE ESQIQQEQNE KELLALDSWK NLWNWFDISN WLWYIKIFIM IVGGLIGLRI VFAVLSIVNR VRQGYSPLSF QTLTPSPREP DRLRGIEEEG GEQDKGRSIR LVQGFLALAW DDLRSLCLFS YHRLRDFISI AARVVEVLGH SSLRGLQRGW EALKYLKSLV QYWGLELKKS AVSLLDTLAI AVGEGTDRII ELIQGICRAI RNIPRRIRQG FEAALL

Figure 25
A)

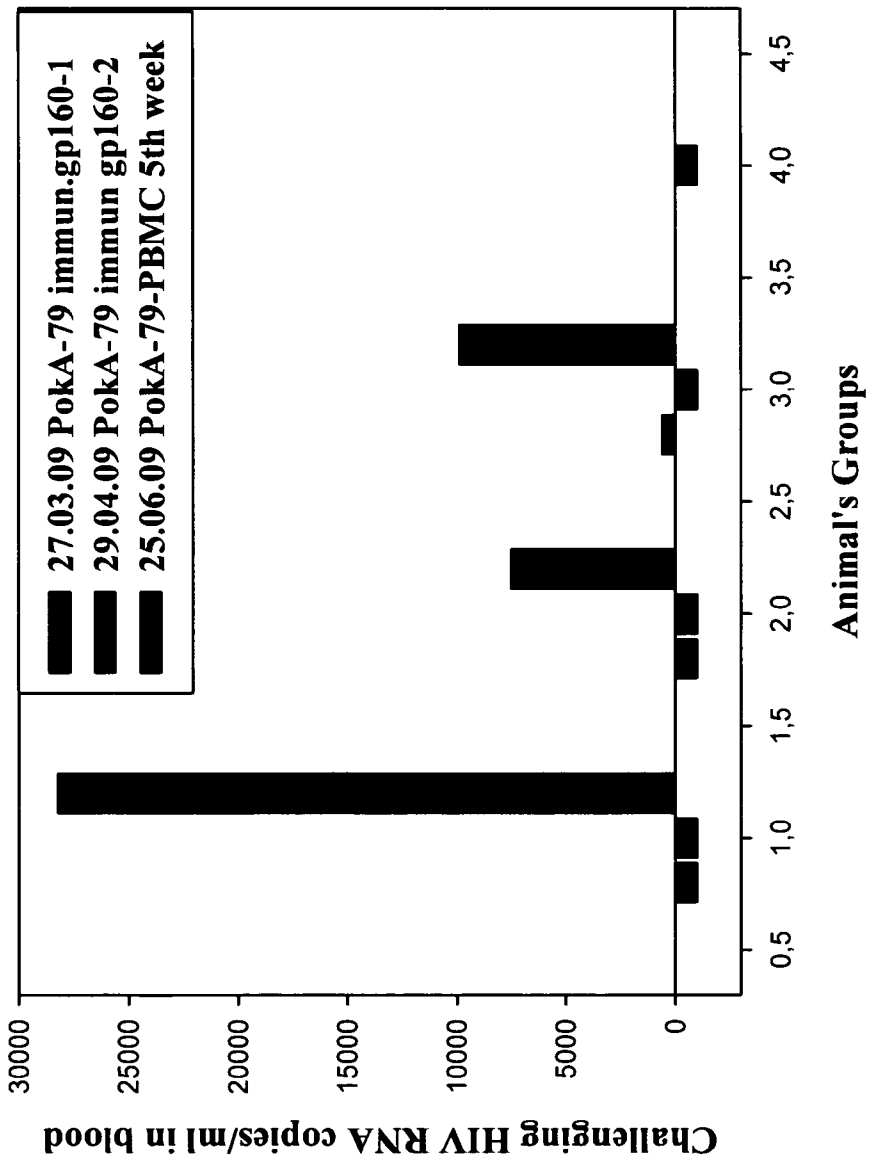

Example 5A:
Immune Responses for gp160-1 and gp160-2
in Challenged with PokA-79-PBMC Strain SCID-PBMC Mice
SCID-PBMC gp160-1
SCID-PBMC gp160-2
BalbC gp160-1
BalbC gp160-2
Absorbance, OD
0,0
0,5
1,0
1,5
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
Animal's Groups

Continuation of Figure 25

B)

Example 5B:

Recombinant gp160-1 and gp160-2 Immunization
Effect for SCID-PBMC Challenging with PokA-79-PBMC
6th passage Strain



Continuation of Figure 25

C)

Example 5C:

Recombinant gp160-PokA-PBMC and gp160-U455-PBMC Immunization Effect for Challenging with PokA79-PBMC and U455-PBMC 6-8 Weeks Strains

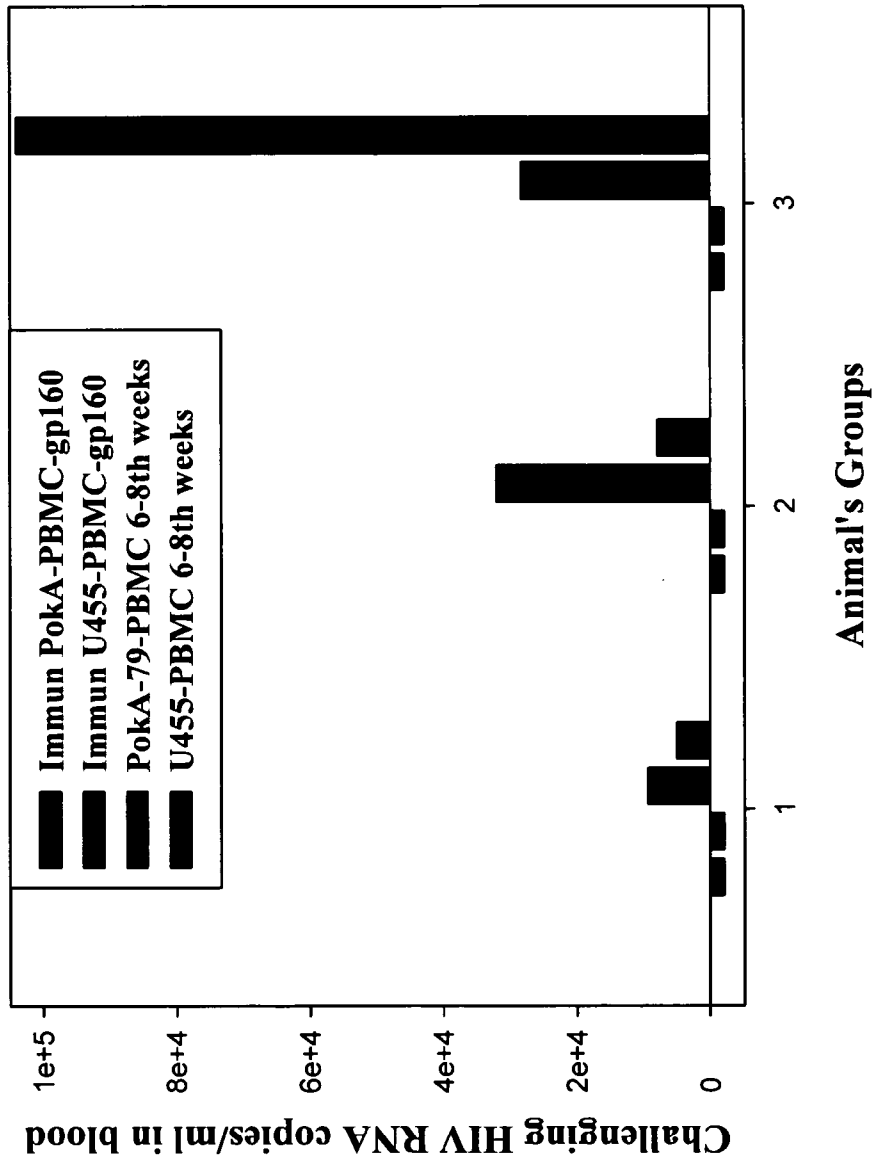
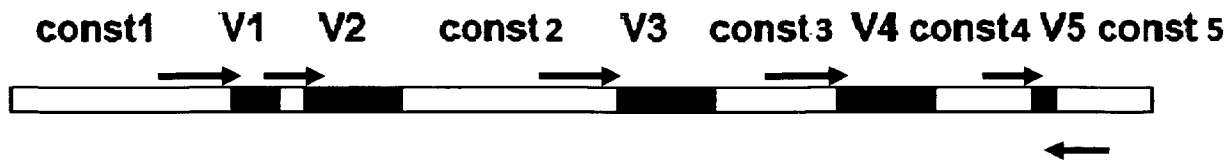
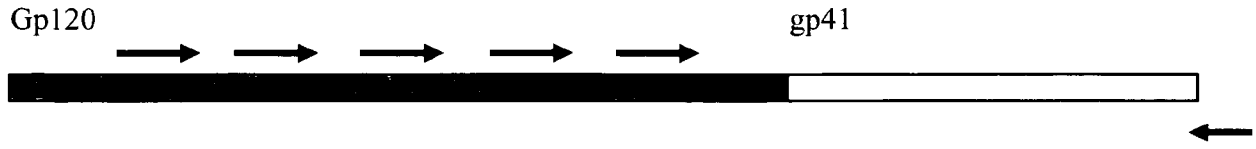


Figure 26
A)



B)



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/002737

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027 A61K49/00 A61K39/21
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009/046984 A1 (TECHNOLOGIE INTEGRALE LTD [AT]; FILINOVA ELENA YU [RU]) 16 April 2009 (2009-04-16) cited in the application page 20 - page 46 page 61 - page 62 claims 1-18; figure 11; example 3 ----- -/--	1-13

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 August 2011	30/08/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Deleu, Laurent

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AZIZI ALI ET AL: "Induction of broad cross-subtype-specific HIV-1 immune responses by a novel multivalent HIV-1 peptide vaccine in cynomolgus macaques", JOURNAL OF IMMUNOLOGY, AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 180, no. 4, 1 February 2008 (2008-02-01), pages 2174-2186, XP002507433, ISSN: 0022-1767 page 2178 - page 2179	1-13
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/002737

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	VAN DUYNE RACHEL ET AL: "The utilization of humanized mouse models for the study of human retroviral infections", RETROVIROLOGY, BIOMED CENTRAL LTD., LONDON, GB LNKD- DOI:10.1186/1742-4690-6-76, vol. 6, no. 1, 12 August 2009 (2009-08-12) , page 76, XP021059355, ISSN: 1742-4690 page 6 - page 11	14-34
A	SHULTZ L D ET AL: "Humanized mice in translational biomedical research", NATURE REVIEWS. IMMUNOLOGY, NATURE PUBLISHING GROUP, GB LNKD- DOI:10.1038/NRI2017, vol. 7, no. 2, 1 February 2007 (2007-02-01), pages 118-130, XP002493022, ISSN: 1474-1733 page 124	14-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2011/002737

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-13

A method for producing an HIV vaccine composition, comprising the steps of:
a) creation of a library comprising HIV-1 specific antibodies,
b) enrichment for HIV-1-specific antibodies in the library by panning with HIV- peptides, in particular native and/or recombinant HIV-1 peptides,
c) multiplying HIV-1 material comprising HIV-1 peptides, polypeptides or proteins,
d) collecting HIV-1 peptides of the multiplied HIV-1 material using HIV-1- specific antibodies of step b) bound to a support,
e) identification and characterization of the HIV-1 peptides obtained in step d) by mass spectrometry, in particular by MS-MS,
f) cloning of fragments of the gp120 gp160 genes encoding the peptides identified in step e),
g) expressing glycosylated env HIV-1 peptides using the results of step f) in a eukaryotic expression system,
h) purification of the glycosylated env HIV-1 peptides, and
i) production of a vaccine composition. A composition comprising nucleic acids for use in steps f) and/or g) of said method. A HIV vaccine composition comprising at least 50, preferably at least 70, more preferably at least 100, even more preferably at least 150 different HIV- envelop peptides, characterized in that:
(i) the gpI20 peptides have a length of 441-541 amino acids and/or a glycosylated protein molecular weight of about 90 - 115kDa, and
(ii) the gp 160 peptides have a length of 816-923 amino acids, and/or a glycosylated protein molecular weight of about 145-175kDa.

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2. claims: 14-34

The use of a Severe Combined T-B-Immune Deficient (SCID) mouse engrafted with human immunocompetent cells (Hu-SCID-mouse) as an animal model for the evaluation of the effectiveness of an HIV vaccine. A method for the evaluation of an HIV vaccine, wherein said Hu-SCID-mouse is inoculated with the HIV vaccine and thereafter challenged with HI-virus.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/002737

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