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(54) **VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNIZATION AGAINST HIV**

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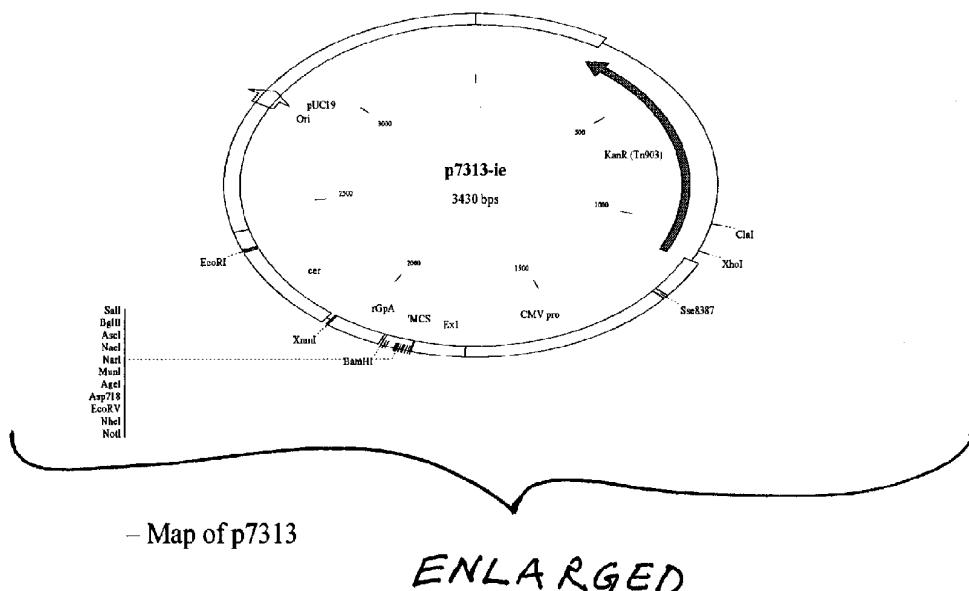
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(52) **U.S. Cl.** ..... **424/208.1; 514/44; 536/23.4**

(57) **ABSTRACT**

Use of a) an HIV Tat protein or polynucleotide; or  
b) an HIV Nef protein or polynucleotide; or  
c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide; and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV, wherein the protein or polynucleotide is delivered via a bombardment approach.



**FIGURE 1**

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

**Pichia-expressed constructs (plain constructs)**

⇒ Nef - HIS

**DNA sequence (Seq. ID. No. 8)**

```
ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAAGTAGCAATAACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAACAGCACAAGAGGAGGAGGAGGTGGGTTTCCAGTCACACCTCAGGTACCTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCACGAAGACAAGATATCCTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGCCAGGGTC
AGATATCCACTGACCTTGGATGGTGCTACAAGCTAGTACCAAGCTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTACACCCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTAGAGTGGAGGTTGACAGCCGCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC
CACCACACCACATCACCATTAA
```

**Protein sequence (Seq. ID. No. 9)**

```
MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEVEGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLIHSQRQDILDLWI
YHTQGYFPDWQNYTPGPVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHzHHHHH.
```

⇒ Tat - HIS

**DNA sequence (Seq. ID. No. 10)**

```
ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATTCCAGGAAGTCAGCCTAAA
ACTGCTTGTACCAATTGCTATTGTAAAAAGTGTGCTTTCATTGCCAAGTTGTTTC
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTCTCTATCAAAGCAACCCACCTCCAA
```

TCCCGAGGGGACCCGACAGGCCGAAGGAAACTAGTGGCCACCATCACCATCACCAT  
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNCYCKCCFHQCQVCFITKALGISYGRKKRRQRRR  
PPQGSQTHQVSLSKQPTSQSREGDPTGPKETSGHHHHHH.

⇒ Nef - Tat - HIS

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAAGGGAAAGA  
ATGAGACGAGCTGAGCCAGCAGCAGATGGGTGGGAGCAGCATCTCGAGACCTGGAA  
AAACATGGAGCAATACAAGTAGCAATAACAGCAGCTACCAATGCTGCTTGTGCCTGG  
CTAGAACACAAGAGGAGGAGGAGGTGGTTTCCAGTCACACCTCAGGTACCTTA  
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTAAAAGAAAAGGGG  
GGACTGGAAGGGCTAATTCACTCCCACGAAGACAAGATATCCTGATCTGTGGATC  
TACCACACACAAGGCTACTTCCTGATTGGCAGAACTACACACCAGGGCCAGGGTC  
AGATATCCACTGACCTTGGATGGTGCTACAAGCTAGTACCAAGTTGAGCCAGATAAG  
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTACACCCCTGTGAGCCTGCAT  
GGAATGGATGACCTTGAGAGAGAAGTGTAGAGTGGAGGTTGACAGCCGCCTAGCA  
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTCAAGAACTGCACACTAGTGAG  
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAACTGCT  
TGTACCAATTGCTATTGTAAAAAGTGTGCTTCAATTGCAAGTTGTTCTATAACA  
AAAGCCTTAGGCATCTCTATGGCAGGAAGAACGGAGACAGCGACGAAGACCTCCT  
CAAGGCAGTCAGACTCATCAAGTTCTATCAAAGCAACCCACCTCCCAATCCCGA  
GGGGACCCGACAGGCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence (Seq. ID. No. 13)

'MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGAITSNTAATNAACAW  
LEAQEEEEVGFPVTQPQVPLRPMTYKAAVDSLHFLKEKGGLIHSQRQDIILDLWI  
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH  
GMDDPEREVLEWRFDSSLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA  
CTNCYCKCCFHQCQVCFITKALGISYGRKKRRQRRPQQGSQTHQVSLSKQPTSQSR  
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ LipoD-Nef-HIS

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.  
 The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

\*

```

ATGGATCCAAAACTTTAGCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT
```

```

AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAATCATTA
```

```

GCTCACCGTGGTGCTAGCGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
```

```

GCTTTTGCACAACACAGGCTGATTAGAGCAAGATTAGCAATGACTAAAGGATGGT
```

```

CGTTTAGGTTATTCACGATCACTTTAGATGGCTTGACTGATGTTGCAAAAAA
```

```

TTCCCACATCGTCATCGAAAGATGGCGTTACTATGTCATCGACTTTACCTTAAAA
```

```

GAAATTCAAAGTTAGAAATGACAGAAAACTTGAAAACATGGGTGGCAAGGTGGTCA
```

```

AAAAGTAGTGTGGTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
```

```

GCAGCAGATGGGTGGGAGCAGCATCTCGAGACCTGGAAAACATGGAGCAATCACA
```

```

AGTAGCAATACAGCAGCTACCAATGGCTGCTTGGCCTGGCTAGAAGCACAAGAGGAG
```

```

GAGGAGGTGGGTTTCCAGTCACACCTCAGGTACCTTAAGACCAATGACITACAAG
```

```

GCAGCTGTAGATCTTAGCCACTTTTAAAGAAAAGGGGGACTGGAAGGGCTAATT
```

```

CACTCCCACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
```

```

TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTT
```

```

GGATGGTGTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
```

```

GGAGAGAACACCAGTTGTTACACCCTGTGAGCCTGCATGGAAATGGATGACCCTGAG
```

```

AGAGAAGTGTAGAGTGGAGGTTGACAGCCGCTAGCATTICATCACGTGGCCACCACCATCACC
```

```

TAA
```

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

```

CSSHSSNMANTQMKSDKIIIIARGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
```

```

GRLVVIHDHFLDGLTDVAKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
```

```

SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
```

```

EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
```

```

YFPDWQNYTPGPGVRYPLTFGWCYKLVEPDKVEEANKGENTSLLHPVSLHGMDDP
```

```

EREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHH.
```

⇒ LipoD-Nef-Tat-HIS

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.  
 The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

\*

```

ATGGATCCAAAAACTTAGCCTTCTTATTAGCAGCTGGCGTACTAGCAGGTTGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTACCAAGAGCATACGTTAGAATCTAAAGCACTT
GCGTTTGCAACACAGGCTGATTATTAGAGCAAGATTAGCAATGACTAAAGGATGGT
CGTTAGTGGTTATTACGATCACTTTTAGATGGCTTACTATGTCATCGACTTACCTAAAA
TTCCCACATCGTCATCGTAAAGATGGCGTTACTATGTCATCGACTTACCTAAAA
GAAATTCAAAGTTAGAAATGACAGAAAACTTGAAACCATTGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAACATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAACCACAAGAGGAG
GAGGAGGTGGGTTTCAGTCACACCTCAGGTACCTTAAGACCAATGACTTACAAG
GCAGCTGAGATCTTAGCCACTTTAAAAGAAAAGGGGGACTGGAAGGGCTAATT
CACTCCAACGAAGACAAGATATCCTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCAGGGGTACGATATCCACTGACCTT
GGATGGTGCTACAAGCTAGTACCAAGCTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTGTTACACCCGTGAGCCTGCATGGAATGGATGACCTGAG
AGAGAAGTGTAGAGTGGAGGTTGACAGCCGCTAGCATTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA
GAGCCCTGGAAAGCATCAGGAAGTCAGCTAAAACTGCTTGTACCAATTGCTATTGT
AAAAAGTGTGCTTCATTGCCAAGTTGTTCATAACAAAAGCCTTAGGCATCTCC
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT
CAAGTTCTATCAAAGCACCCACCTCCAATCCGAGGGACCCGACAGGCCG
AAGGAAACTAGTGGCCACCATCACCATCACCATTAA

```

Protein sequence of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)

(Amino-acids corresponding to Prot D fusion partner are in bold)

```

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKGHGAITSSNTAATNAACAWLEAQE
EEEVGFPTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY
CKKCCFHCQVCFITKALGISYGRKRRQRPPQGSQTHQVSLSKQPTSQSRGDPTG
PKETSGHHHHHH.

```

**⇒ ProtD-Nef -HIS****DNA sequence (Seq. ID. No. 18)**

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCAAAATATGGCGAATACCCAAATGAAATCAGACAAA  
ATCATTATTGCTCACCGTGGTCTAGCGGTTATTCACAGAGCATACGTTAGAATCT  
AAAGCACTTGCCTTGACAAACAGGCTGATTATTAAGAGCAAGATTTAGGAATGACT  
AAGGATGGTCGTTAGTGGTTATTCACGATCACTTTAGATGGCTTGACTGATGTT  
GCGAAAAAAATTCCCACATCGTCATCGTAAAGATGGCGTTACTATGTCATCGACTTT  
ACCTTAAAAGAAATTCAAAGTTAGAAATGACAGAAAACTTGAAACCATGGGTGGC  
AAGTGGTCAAAAGTAGTGTGGTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA  
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTGAGACCTGGAAAAACATGGA  
GCAATCACAAGTAGCAATAACAGCAGCTACCAATGTCCTTGCTGGCTAGAACGA  
CAAGAGGAGGAGGAGGTGGGTTTCCAGTCACACCTCAGGTACCTTAAGACCAATG  
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTAAAAGAAAAGGGGGACTGGAA  
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTGATCTGTGGATCTACCACACA  
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCAGATATCCA  
CTGACCTTGGATGGTGTACAAGCTAGTACCGAGTTGAGCCAGATAAGGTAGAAGAG  
GCCAATAAAGGAGAGAACACCAGCTGTACACCCCTGTGAGCCTGCATGGAATGGAT  
GACCCCTGAGAGAGAGAGTGTAGAGTGGAGGTTGACAGCCGCTAGCATTTCATCAC  
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC  
CATCACCATTAA

**Protein sequence (Seq. ID. No. 19)**

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDFKIIIAHRGASGYLPEHTLESKALAFQQADYL  
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK  
EIQSLEMTEFETMGGKWSKSSVVGWPTVRERMRAEAADGVGAASRDL  
EKHGAITSSNTAATNAACA WLEAQEEEVGFPVTQVPLRPMTYKAADVLSH  
FLKEKGGLEGLIHSQRQRDILDLWIYHTQGYFPDWQNYTPGPVRYPLTFGW  
CYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDRLAFAH  
HVARELHPEYFKNCTS GHHHHHH .

**⇒ ProtD-Nef-Tat-HIS****DNA sequence (Seq. ID. No. 20)**

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCAAAATATGGCGAATACCCAAATGAAATCAGACAAA  
 ATCATTATTGCTCACCGTGGTGTAGCGTTATTTACCAGAGCATACTGTTAGAATCT  
 AAAGCACTTGCCTTGACAAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT  
 AAGGATGGTCGTTAGTGGTTATTACGATCACTTTAGATGGCTTGACTGATGTT  
 GCGAAAAAAATTCCCACATCGTCACTCGTAAAGATGGCCGTTACTATGTCATCGACTTT  
 ACCTTAAAGAAATTCAAAGTTAGAAATGACAGAAAACTTGAAACCATGGGTGGC  
 AAGTGGTCAAAAAGTAGTGTGGTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA  
 GCTGAGCCAGCAGCAGATGGGTGGGAGCAGCAGCTCGAGACCTGGAAAAACATGGA  
 GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA  
 CAAGAGGAGGAGGAGGAGGTGGTTTCCAGTCACACCTCAGGTACCTTAAGACCAATG  
 ACTTACAAGGCAGCTGTAGATCTAGCCACTTTAAAAGAAAAGGGGGACTGGAA  
 GGGCTAATTCACTCCCAACGAAGACAAGATATCCTGATCTGTGGATCTACCACACA  
 CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCAGATATCCA  
 CTGACCTTGGATGGTGTACAAGCTAGTACCACTGAGCCAGATAAGGTAGAAGAG  
 GCCAATAAAGGAGAGAACACCAAGCTTGTACACCCCTGTGAGCCTGCATGGAATGGAT  
 GACCCCTGAGAGAGAAGTGTAGTGGAGGTTGACAGCCGCTAGCATTTCATCAC  
 GTGGCCCCAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT  
 CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAACTGCTTGTACCAAT  
 TGCTATTGAAAAAGTGTGCTTCATTGCCAAGTTGTTCTATAACAAAAGCCTTA  
 GGCACTCCTATGGCAGGAAGAACGGAGACAGCGACGAAGACCTCCTCAAGGCAGT  
 CAGACTCATCAAGTTCTCTATCAAAGCAACCCACCTCCCAATCCGAGGGGACCCG  
 ACAGGCCCGAAGGAAACTAGTGGCCACCATCACCACCATCACCATTAA

Protein sequence (Seq. ID. No. 21)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQQADYLEQDLAMT  
 KDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMENFETMGG  
 KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSNTAATNAACAWLEA  
 QEEEVGFPVTPQVPLRPMTYKAADVLSHFLKEKGGLEGLIHSQRQRQDILDLWIYHT  
 QGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD  
 DPEREVLEWRFDSDLAFHHVARELHPEYFKNCITSEPVDPRLEPWKHPGSQPKTACTN  
 CYCKKCCFHQCVCFITKALGISYGRKKRQRRPPQGSQTHQVSLSKQPTSQRGDPT  
 TGPKETSGHHHHHH.

⇒ Tat-MUTANT-HIS

DNA sequence (Seq. ID. No. 22)

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC	40
CAGGAAGTCAGCCTAAAACGTGTTTACCAATTGCTATTG	80
TAAAAAGTGTGCTTCATTGCCAAGTTGTTCATACAA	120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAACGGAGAC	160
AGCGACGAAGACCTCCTCAAGGCAGTCAGA <b>CT</b> CATCAAGT	200
TTCTCTATCAAAGCAACCCACCTCCAAATCCAAGGGAG	240
CCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATC	280
ACCATTA	288

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

MEPVDPRLPWKHPGSQPKTACTNCYCKKCCFHQCQVCFIT	40
<b>AALGISYGRKKRRQRRPPQGSQTHQVSLSKQPTSQSKE</b>	80
PTGPKETSGHHHHHH.	95

⇒*Nef-Tat-Mutant-HIS*DNA sequence(Seq. ID. No. 24)

ATGGGTGGCAAGTGGTCAAAAGTAGTGTGGTGGATGGC	40
CTACTGTAAGGGAAAGAACGAGACGAGCTGAGCCAGCAGC	80
AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACAT	120
GGAGCAATCACAAAGTAGCAATACAGCAGCTACCAATGCTG	160
CTTGTGCCTGGCTAGAACGACAAGAGGAGGAGGAGGTGGG	200
TTTTCCAGTCACACCTCAGGTACCTTAAGACCAATGACT	240
TACAAGGCAGCTGTAGATCTTAGCCACTTTAAAAGAAA	280
AGGGGGACTGGAAGGGCTAATTCACTCCAACGAAGACA	320
AGATATCCTGATCTGGATCTACCAACACACAAGGCTAC	360
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGTCA	400
GATATCCACTGACCTTGGATGGCTACAAGCTAGTACC	440
AGTTGAGCCAGATAAGGTAGAACGAGGCCATAAAGGAGAG	480
AACACCAGCTTGTACACCCCTGTGAGCCTGCATGGAATGG	520
ATGACCCCTGAGAGAGAACGTGTTAGAGTGGAGGTTGACAG	560
CCGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCG	600
GAGTACTTCAAGAACTGCACACTAGTGAGCCAGTAGATCCTA	640
GAATAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAC	680
TGCTTGTACCAATTGCTATTGAAAAAGTGTGCTTTCAT	720
TGCCAAGTTGTTCTACACAGCTGCCTTAGGCATCTCCT	760
ATGGCAGGAAGAACGGAGACAGCGACGAAGACCTCCTCA	800
AGGCAGTCAGACTCATCAAGTTCTATCAAAGCAACCC	840
ACCTCCCAATCCAAGGGAGCCACAGGCCGAAGGAAA	880
CTAGTGGCCACCACATCACCATCACCATTAA	909

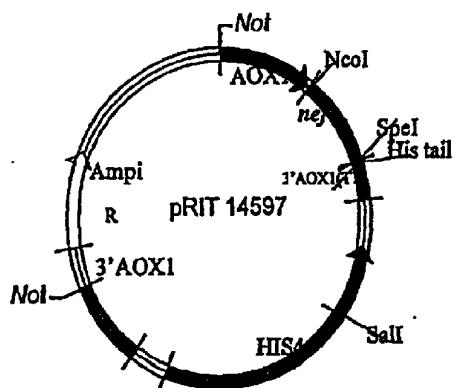
Protein sequence (Seq. ID. No. 25)

Mutated amino-acids in Tat sequence are in bold.

MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKH 40  
GAITSSNTAATNAACAWLEAQEEEVGFVTPQVPLRPMT 80  
**YKAAVDL**SHFLKEKGGLIHSQRQDILD**LW**IYHTQGY 120  
FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE 160  
NTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHP 200  
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH 240  
CQVCFITAALGISYGRKKRRQRRPPQGSQTHQVSLSKQP 280  
TSQSKGEPTGPKETSGHHHHHH . 302

Figure 2

## Map of pRIT14597 integrative vector

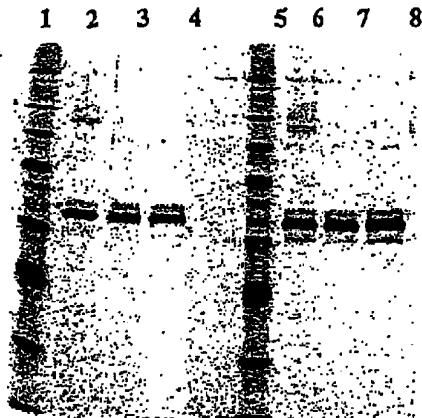


MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

<i>Asn II</i>	<i>Nco I</i>	<i>Spe I</i>	<i>Eco RI</i>
TTCGAA	<u>ACC</u>	<u>ATGGCCGCGGACTAGTGGC</u>	CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGGAATTTC
	Ser.	Gly.	His . His . His . His . His

The amino acid sequence of Figure 3 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No. 26.

Figure 3 : SDS-PAGE: Nef-Tat-his fusion protein



- 1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
2: TNH/23 SP eluate (250 ng)  
3: TNH/23 Purified bulk (250 ng)  
4: TNH/22 Purified bulk (250 ng)  
5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)  
6: TNH/23 SP eluate (400 ng)  
7: TNH/23 Purified bulk (400 ng)  
8: TNH/22 Purified bulk (400 ng)

Daiichi Silver Staining

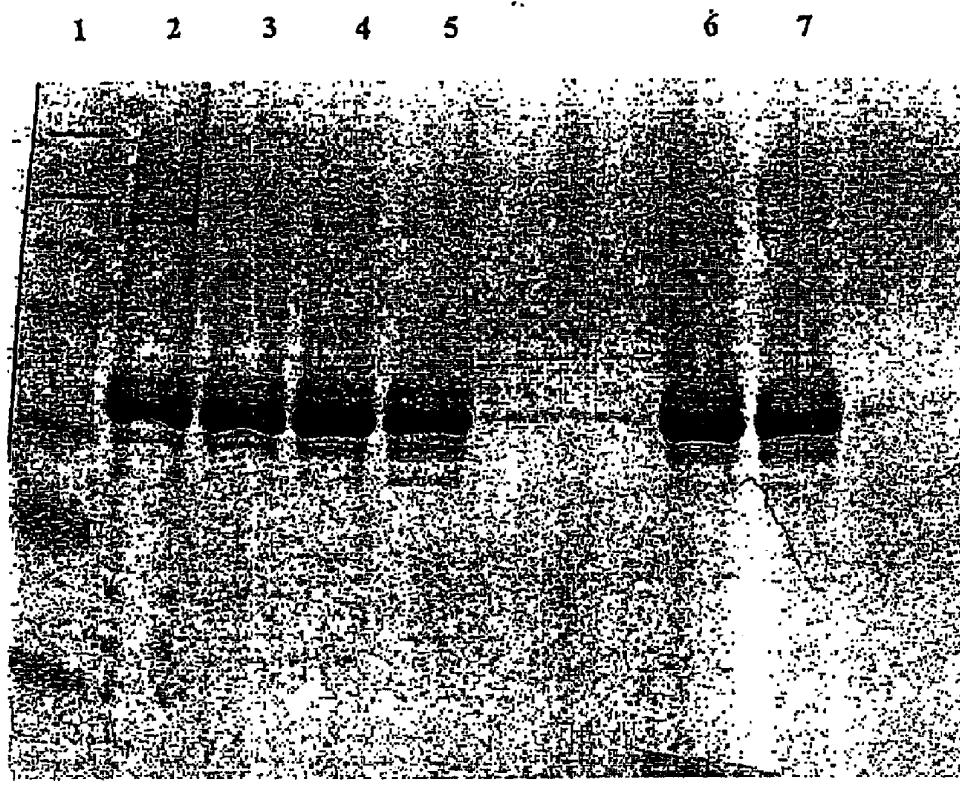


Blot  $\alpha$  Nef-Tat (LAS 97340)



Blot Tat2

**Figure 4 : SDS-PAGE: Nef-Tat-his fusion protein**



**Coomassie blue G250**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)

2: TNH/23 SP eluate (4 µg)

3: TNH/23 Superdex200 eluate (4 µg)

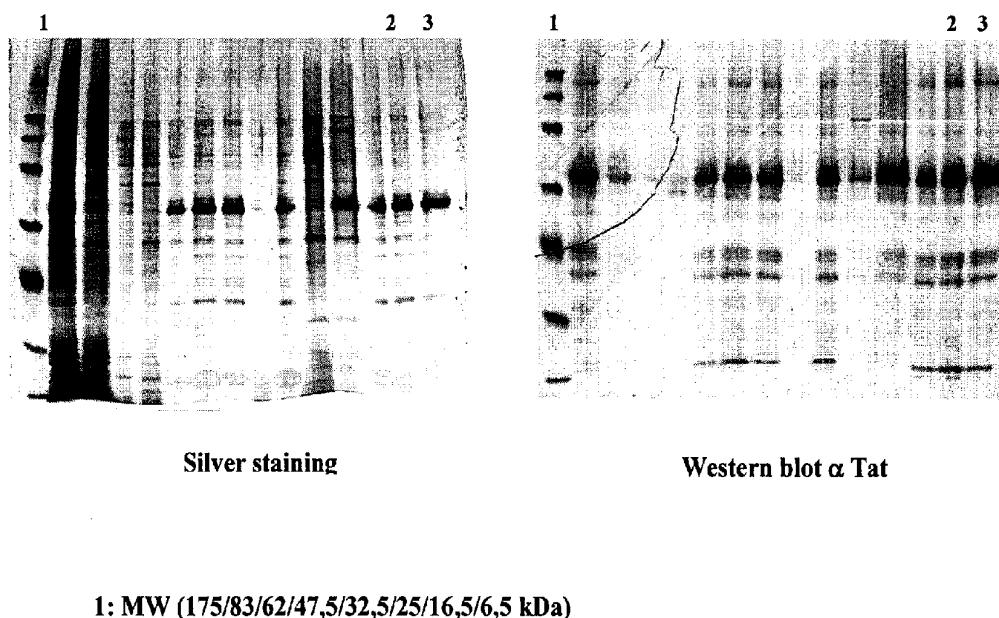
4: TNH/23 Purified bulk (4 µg)

5: TNH/22 Purified bulk (4 µg)

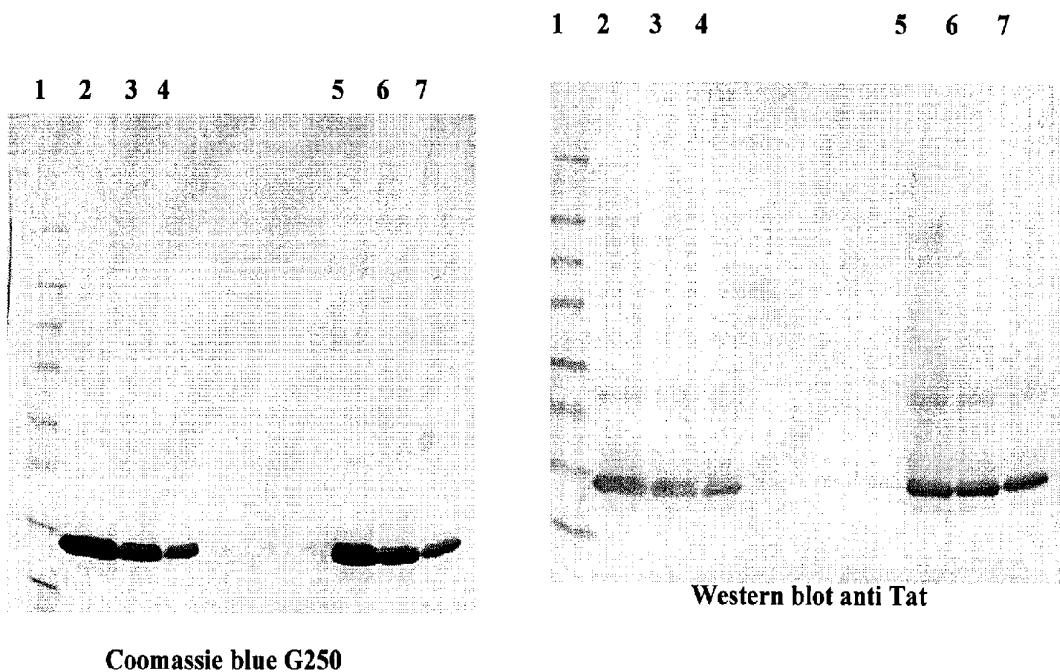
6: TNH/23 Purified bulk (4 µg) / non reducing conditions

7: TNH/22 Purified bulk (4 µg) / non reducing conditions

**Figure 5: SDS-PAGE ANALYSIS – reducing conditions  
(14% polyacrylamide precasted gels - Novex) See example 5**



**Figure 6 (relating to Example 6): SDS-PAGE ANALYSIS:**  
(4-20% polyacrylamide precasted gels - Novex)



1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)

2: Purified bulk (reducing conditions)

3: Purified bulk (reducing conditions)

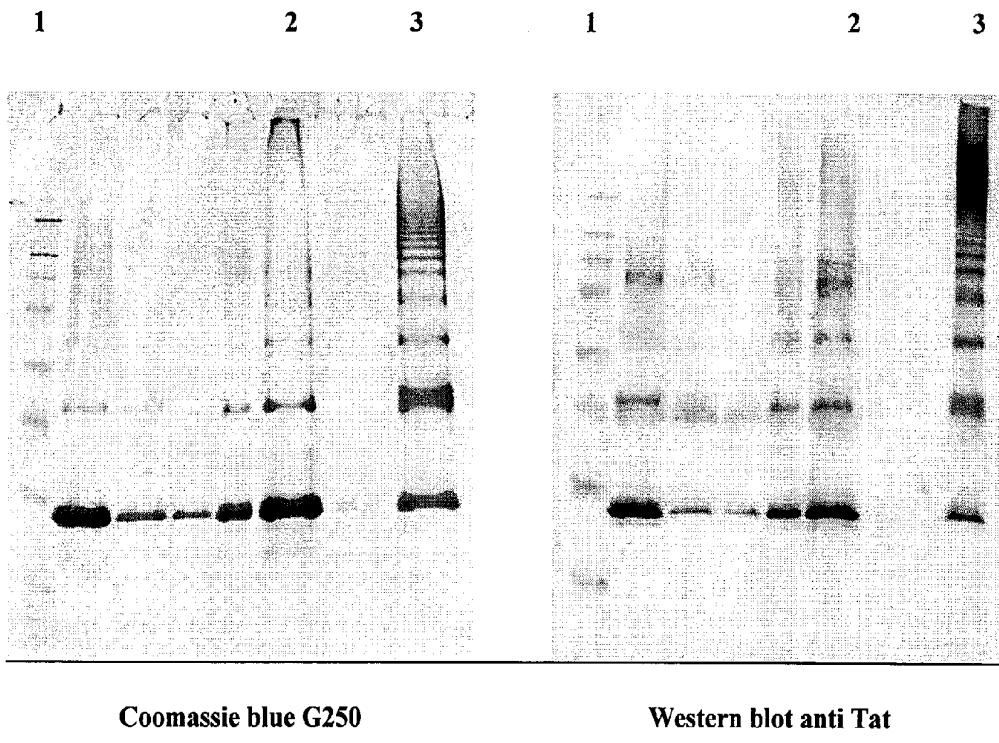
4: Purified bulk (reducing conditions)

5: Purified bulk (non reducing conditions)

6: Purified bulk (non reducing conditions)

7: Purified bulk (non reducing conditions)

**Figure 7 (relating to Example 7): SDS-PAGE ANALYSIS:**  
(4-20% polyacrylamide precasted gels - Novex)



Coomassie blue G250

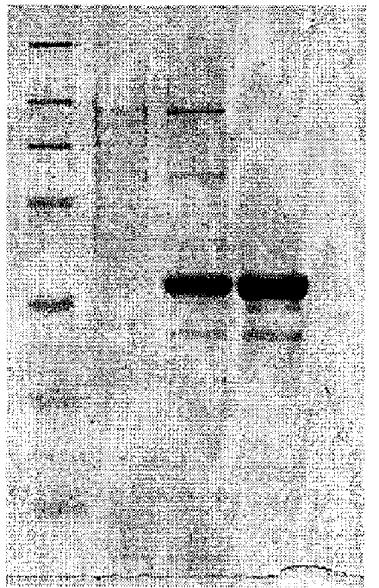
Western blot anti Tat

- 1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
- 2: Purified bulk (reducing conditions)
- 3: Purified bulk (non reducing conditions)

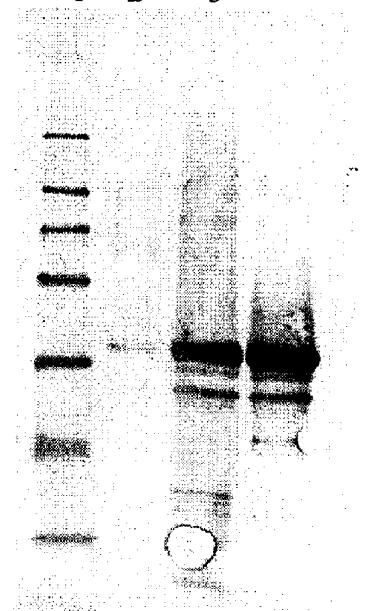
**FIGURE 8: SDS-PAGE ANALYSIS - REDUCING CONDITIONS**

(14% polyacrylamide precasted gels - Novex) see Example 8

1 2 3

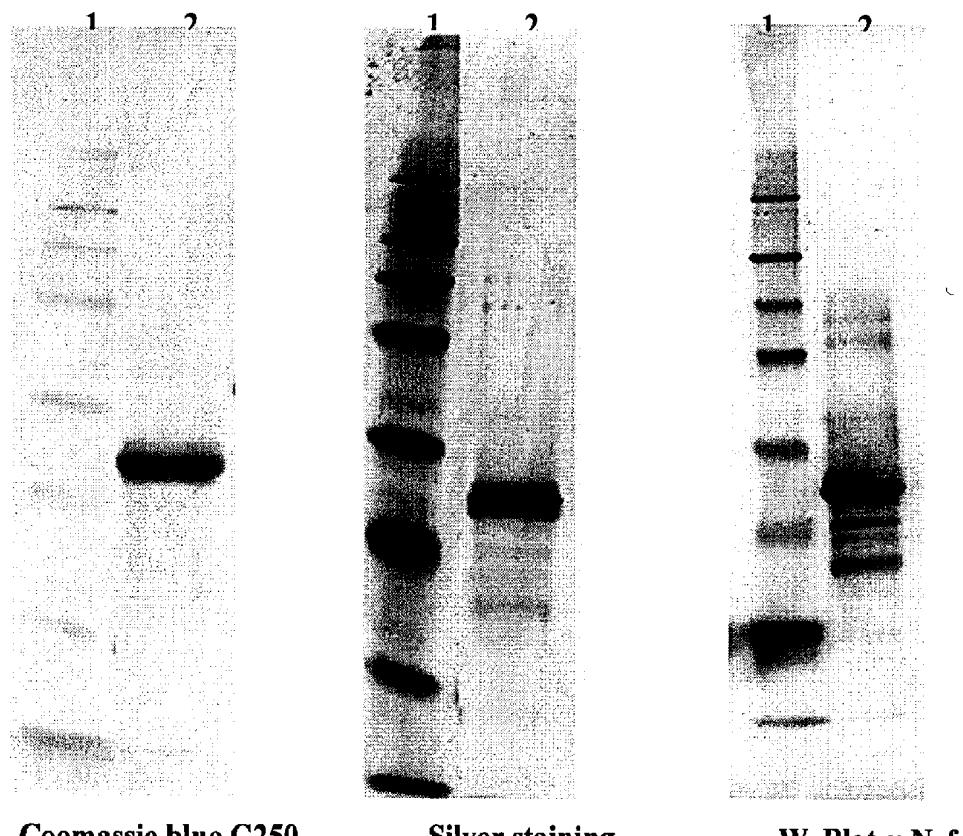
**Coomassie blue R250**

1 2 3

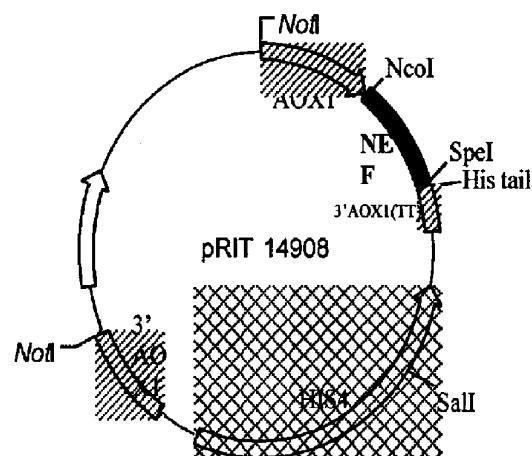
**Silver staining**

**Figure 9: SDS-PAGE ANALYSIS – reducing conditions**

(14% polyacrylamide precasted gels - Novex) See Example 9



**1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)**  
**2: Purified bulk**

**Figure 10****Map of pRIT14908 integrative vector**

MCS POLYLINKER : NEF gene inserted between *Nco*I and *Spe*I sites.

*Asu* II      *Nco* I      *Spe* I      *Eco* RI  
TTCGAA.A CC.ATGGCCGGCGG ACTAGT .GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGC    GAATTG  
Thr .Ser . Gly. His . His . His . His . His . His .

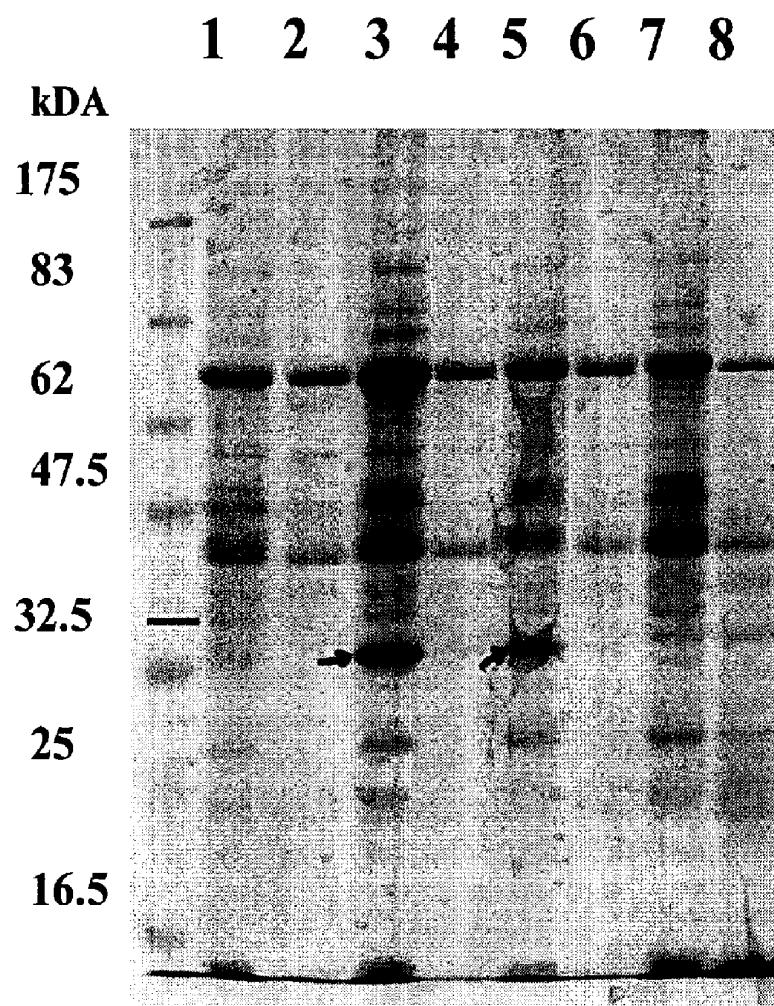
Figure 11**Sequences of Pichia-expressed SIV-NEF-His protein****DNA SEQUENCE ID No 30:**

atgggtggagctattccatgaggcggtccaggccgtctggagatctgcg	50
acagagactcttgcggcgctgggagacttatggagactcttaggag	100
aggtggaaagatgatactcgcaatccccaggaggattagacaaggcctg	150
agctcaactcttgtgagggacagaatacatcaggacagtatatgaa	200
tactccatggagaaacccagctgaagagagagaaaaattacatcagaa	250
aacaaaatatggatgatatacatgaggaagatgatgacttggtagggta	300
tcagtgaggccaaaagttcccctaagaacaatgagttacaaattggcaat	350
agacatgtctcatttataaaaagaaaaggggactggaaggattatt	400
acagtgcagaagacatagaatcttagacatatacttagaaaaaggaagaa	450
ggcatcataccagattggcaggattcacacccctcaggaccaggaattagata	500
cccaaagacattggctgctatggaaattagtccctgtaaatgtatcag	550
atgaggcacaggaggatgaggagcattatthaatgcattccagctcaaact	600
tcccagtggatgacccttgggagaggttctagcatgaaagttgatcc	650
aactctggcctacacttatgaggcatatgttagataaccagaagagtttg	700
gaagcaagtgcaggcctgtcagaggaagaggttagaagaaggctaaccgca	750
agaggccttcttaacatggctgacaagaaggaaactcgcactagtggcca	800
ccatcaccatcaccattaa.	819

**PROTEIN SEQUENCE ID No 31:**

MGGAISMRRSRPSGDLRQLLRARGETYGRLLGEVEDGYSQSPGLDKGL	50
SSLSCEGQKYNQQYQMNTPWRNPAEEREKLAIRKQNMDIDEEDDDLVGV	100
SVRPKVPLRTMSYKLAIDMSHFIKEKGLEGIYYSARRHRILDIYLEKEE	150
GIIPDWQDYTSGPGIRYPKTFGLWKLVPVNVSDEAQEDEEHYLMHPAQT	200
SQWDDPWGEVLAWKFDPTLAYTYEAYVRYPEEFGSKSGLSEEVRRRLTA	250
RGLLNMAKKETR <b>TSGHHHHHH</b> .	272

**Figure 12**  
**Coomassie Blue Stained SDS-PAGE of recombinant  
Pichia pastoris SIV/NEF expressing strains**



- lane 1: P- Y1752 strain  
lane 2: S- " "  
lane 3: P- Y1772 strain  
lane 4: S- " " "  
lane 7: P- GS115 strain ( negative control)  
lane 8: S- " "

Figure 13, Monkey study 1. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

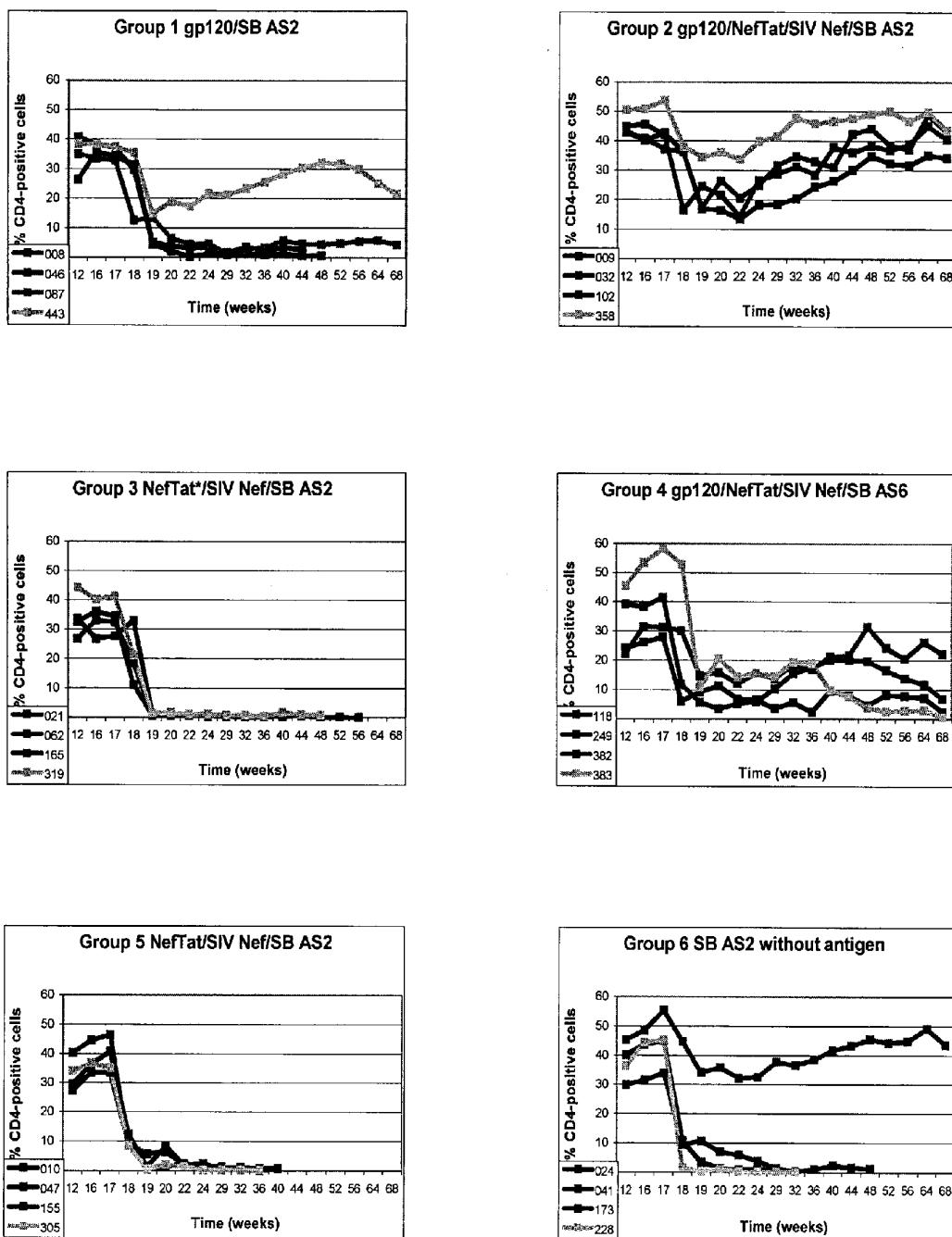


Figure 14. Monkey study 1. Analysis of SHIV plasma virus load after challenge with SHIV

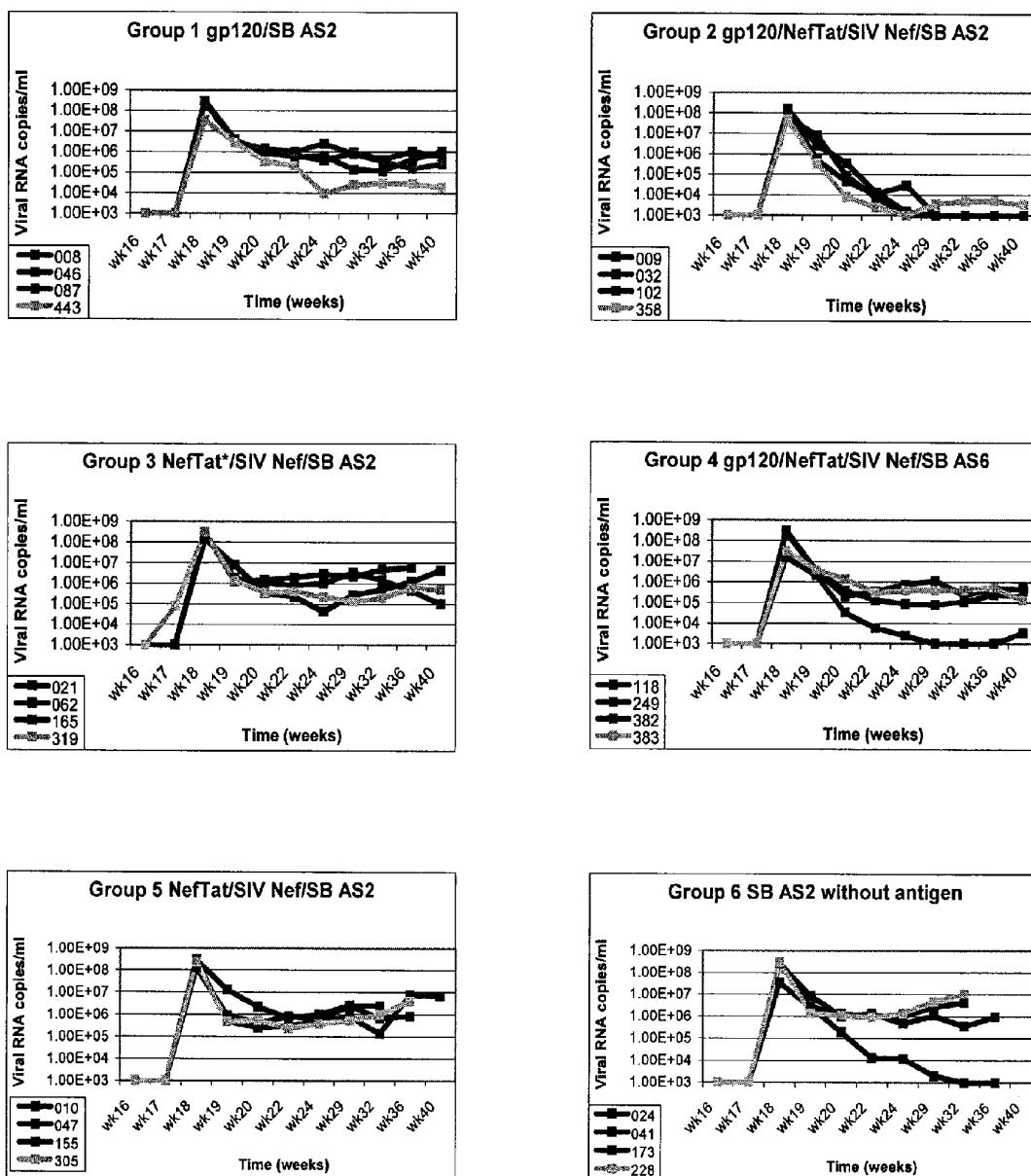


Figure 15. Monkey study 2. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

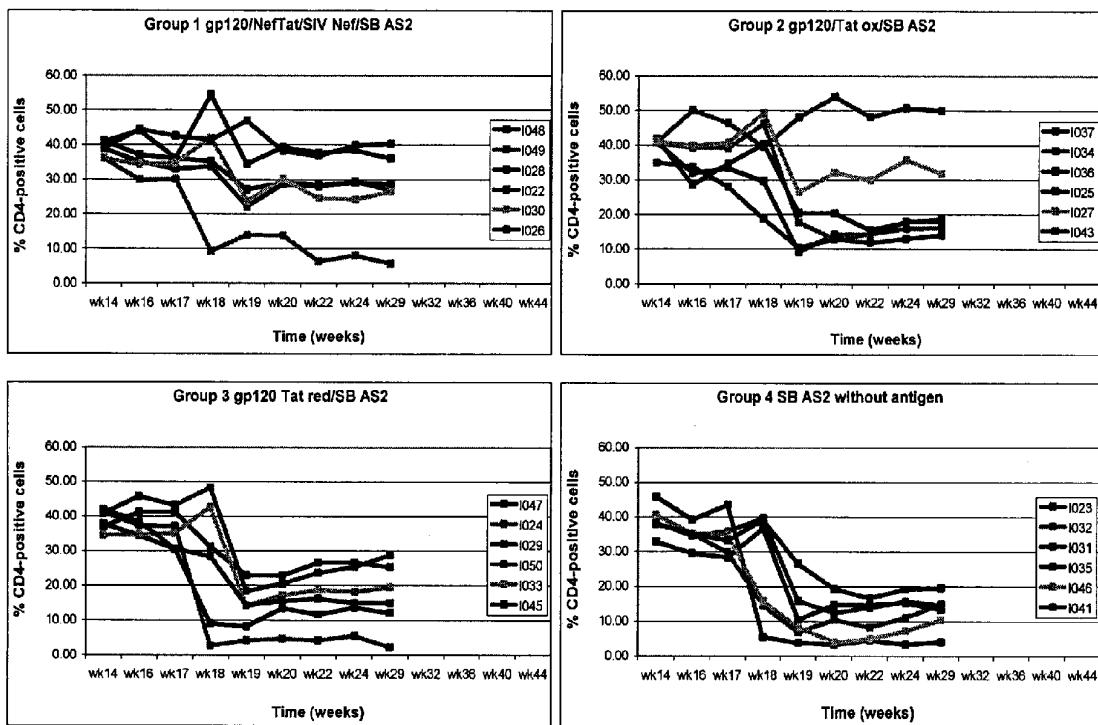
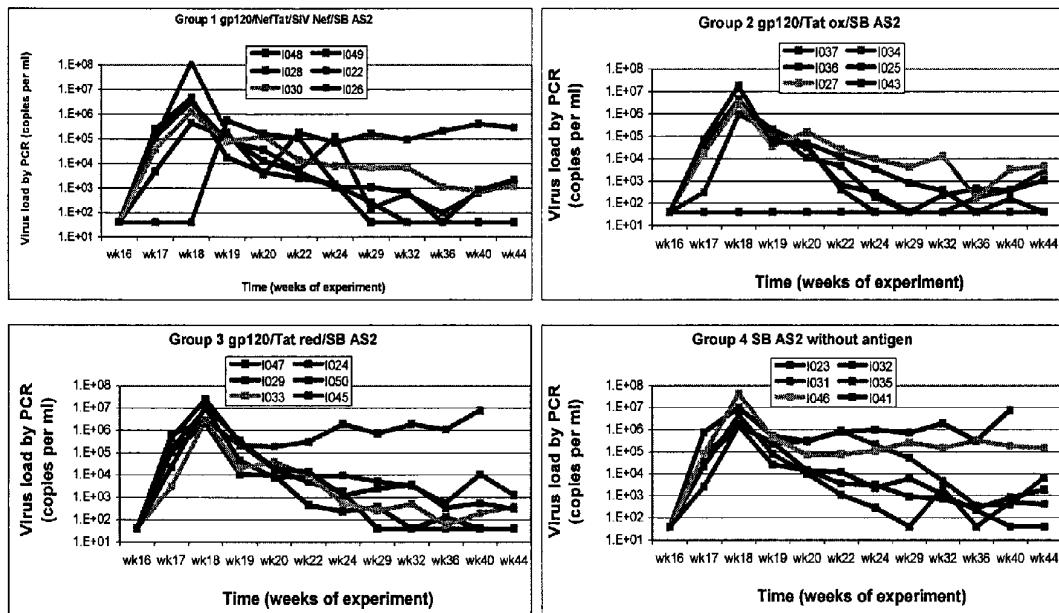


Figure 16. Monkey study 2. Analysis of SHIV plasma virus load after challenge with SHIV



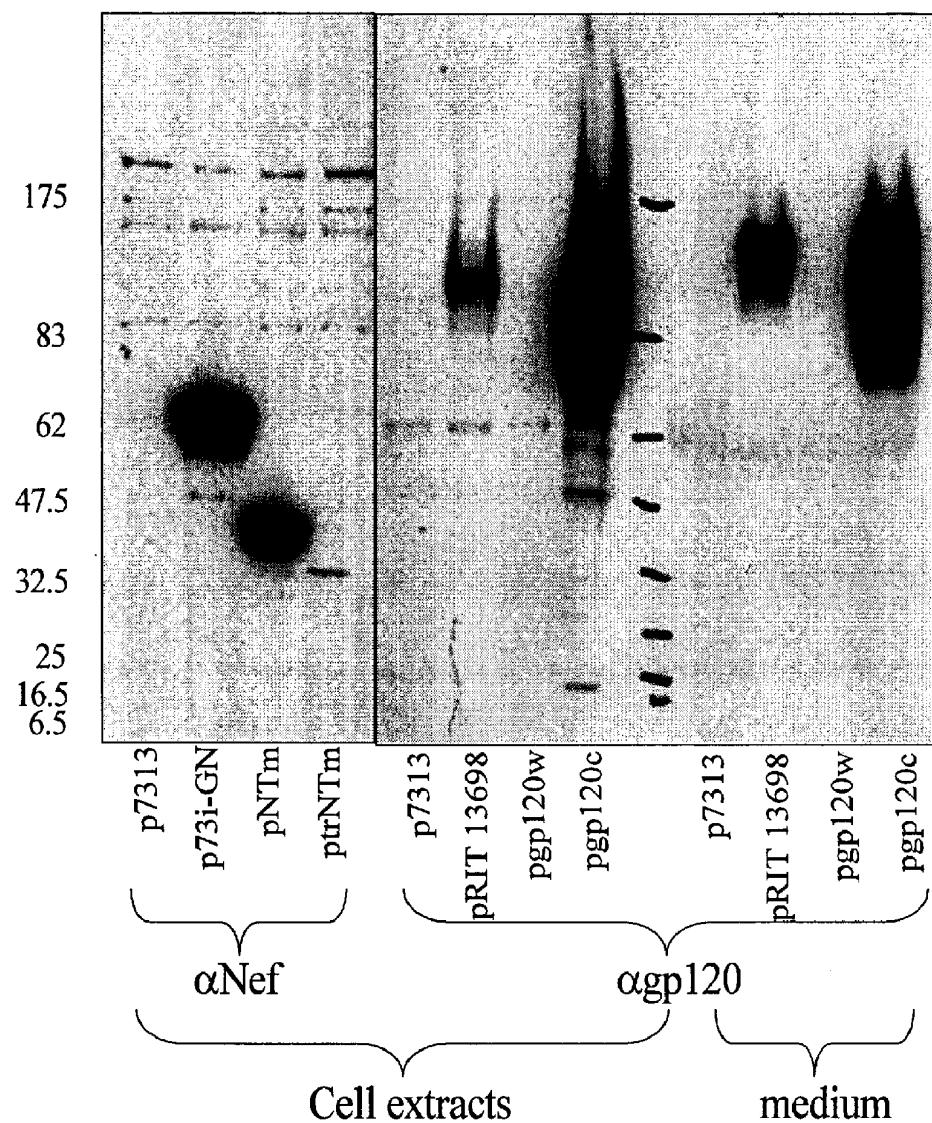


Fig. 17. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in cell extracts and supernatant medium, 24hr post transfection.

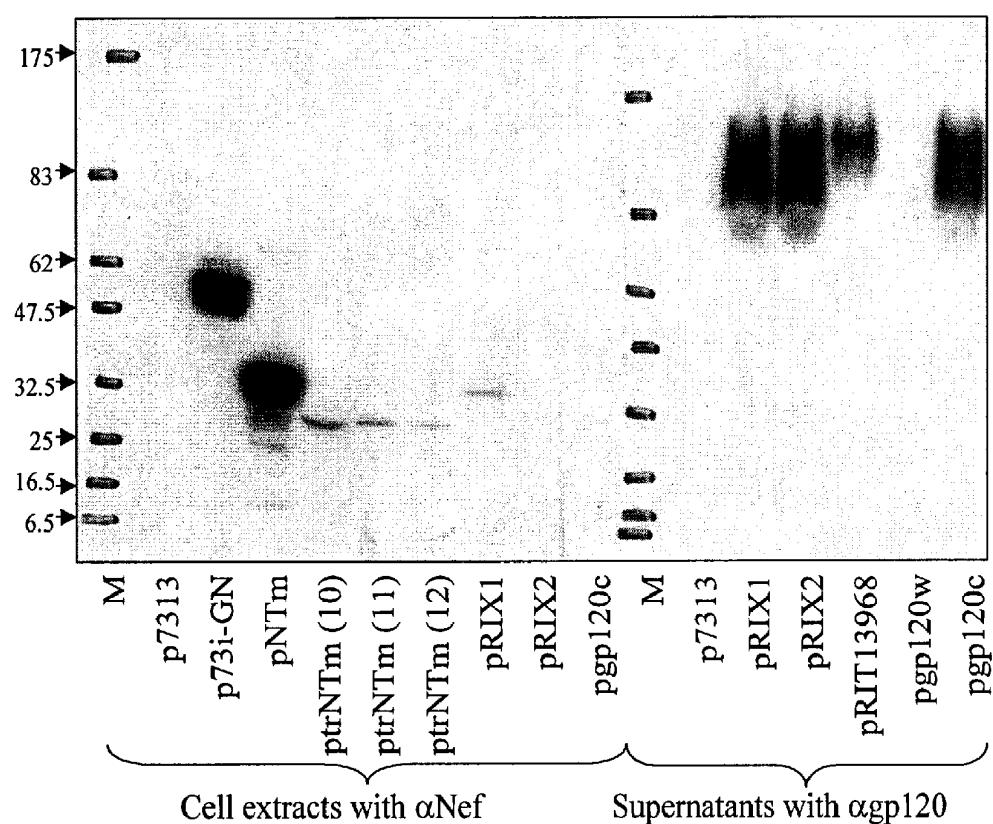


Fig. 18. Western blot showing the level of Nef detected in 293T cell extracts, and gp120 in supernatants, 24hr post transfection.

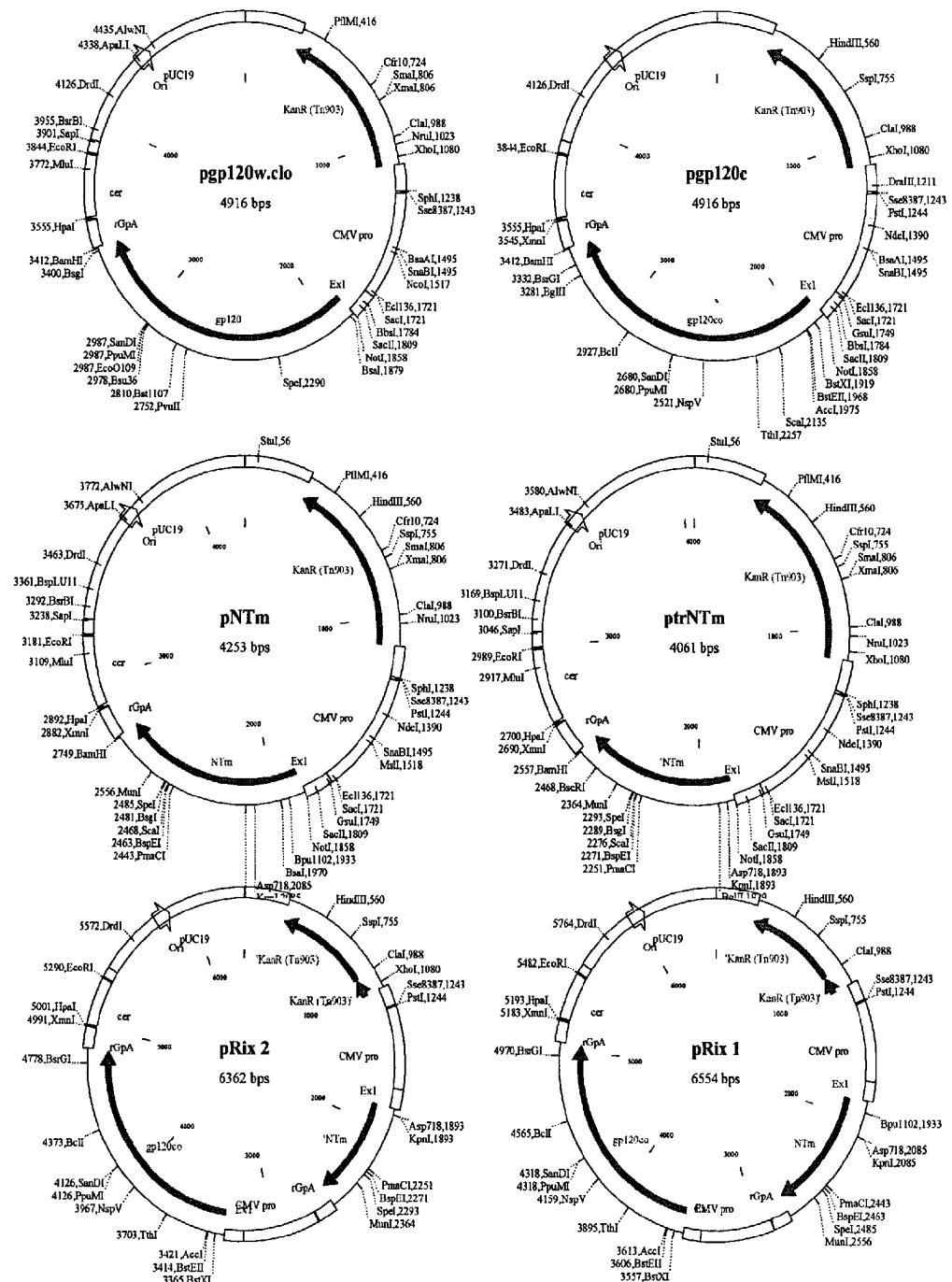


Fig.19. Plasmid Maps:

Fig. 20. Oligos for codon optimisation and sequencing of gp120: Seq ID No 43 to 82

g120-u:1 GAATTCGCGGCCAATGAAGGTCAAGGAGACCAGAAAAGAACTACCAGCATCTGTG  
 g120-u:2 CCTCGGAATGCTGATGATCTGCTCCGCCGAGCAGCTGTGGGTACCGTCTACTACGG  
 g120-u:3 GGCCACGACGACCCCTTCCTGCGCAGGACGCCAACGGCTACGACACGGAAAGTGCTATAA  
 g120-u:4 TTGCGTGCCTACGGACCCCAACCCCCAGGGAGGTGGTGTGGGAAACGTGACCGAGTA  
 g120-u:5 CATGCTGGATCACATGACGAGGACATCATCTCTGTGGACCAACTCCCTGAAGCCCTG  
 g120-u:6 CTGGTGACACTGGACTGTGACGACGTCACACCAACAGCACTACCACCAAGCAA  
 g120-u:7 TCGGAAGGGCAGATCAAGAACTGCTCTTCATATCACGACCTCGATCAGAGACAAGGT  
 g120-u:8 GTTTTATAATCTCGATGTTGCCCCATCGACGACGAAATGCCACCCAAGAACAGAC  
 g120-u:9 CATTCACTGACACAGCAGCGTCAATGACGAGGCCCTGACGGGAAAGGTGCTCTCGAACCA  
 g120-u:10 CCCCTGCCGGATTCGGATCTCAAGTGTAAACAAGAACCTTCAGCTGACGGGAAGGGCTGTG  
 g120-u:11 GCACTGCACCCATGGCATCCGCCGGTGTGAGCACCCAGCTGCTGAAACGGTCCCT  
 g120-u:12 GATCCGGTCGGACAACCTCATGGACAACACCAAGAACATCATGTCAGCTAACAGAGTC  
 g120-u:13 CCGGCTAACAAACAACACCCGTAAGGGCATCCACATCGGGCTGGACGGGCTTATGC  
 g120-u:14 CGACATCCGGCAGGCCATTGCAACCTCTCCCGGCCAGTGGATAAACACCCGAGCA  
 g120-u:15 AGAGCACTTGGAAAGAACATCAAGTCAATCAGACTCTGGCGAGAACCCCAGAT  
 g120-u:16 CTGGGGGGGAGTTCTTACTCGCGATACGACAGCTCTCAACTCCACCTGGAAACGG  
 g120-u:17 AGAGGGAAACTCCACTATCACCTCCCTTGCGCATCAAGCAGATCATCAACATGTTG  
 g120-u:18 GTATGCCCCCCCATCGGGGCGAGATCCGTGCTCTTCACATCAGGGCTGCTGCT  
 g120-u:19 CGAGGGCAACGGCACGGAGAACGAGACGGAGATCTCAGGCCGGCGGCGACATGAG  
 g120-u:20 GCTGTACAAGTACAAGGTGTGAAGGTGGAGCCGCTGGCGTGGCCCCCACCGGGCAA  
 g120-l:1 GAATTCGGAATCTCATCTGCAACGACGCGCGTGGCCCGGGTGGGGCACG  
 g120-l:2 ACCACCTTGACTGTACGCTCGCTCCGCCAGTTACCTCATGTCGCCGCCGGG  
 g120-l:3 TTCTCGTGGCGTTGCCCTGGTACGGCCCTCTGGTGTGAGCAGCAGGGCTGATGTTG  
 g120-l:4 CCCCCGATGGGGGGGATACATGCCCTTCCCACCTCTGCCACATGTTGATGATCTG  
 g120-l:5 GTGATAGTGGAGTTCCCTGTGTTGTTGCCCCCTGGTCCGGTGGAGTTGAAG  
 g120-l:6 TAGAAGAACTCGCCCCCGCAGTTGAAGGAGTGCACGACGATCTCGGGTCTCCAGAA  
 g120-l:7 GTCTTGTTTCAAAGGTCTCTCAGCTGATCAGATCTGCTTCAAGGTGTTATTCCAC  
 g120-l:8 CAATGGCCCTGGGATGTGCCGATGATCTGGGGGGCATAGAAGGCCGTCCAGGC  
 g120-l:9 CGGGTGTGTTGTTAGGGGGTACAGTTAATGCCACAGACTGTTCAAGTGGAGAT  
 g120-l:10 ATGAGTTGTCGACCGCATCACCCCTCTCGACGGGACCGTTCACCGAGCAGC  
 g120-l:11 CGGATGCCATGGGTGCACTGCACCGTGTGACGTTGGTGCACAGGCCCTCCGTGAG  
 g120-l:12 AGGATCGCGAATCCGGCAGGGGCAACTAATGGATGGGATTGGTCAAGGACACCTG  
 g120-l:13 ACCTGCTGTGCACTGAAATGAGTCTGAAATTACGCGTGTCTGTTGTTGGCA  
 g120-l:14 ACCACATCGAGATTATAAAACAGCGCTATCCCTCGACCTGTCCTGATCGAGGTC  
 g120-l:15 TTCTGATCTGCCCTCCGAATCTCCGGTCCAGGGTTGCTGGGGTGGTAGTGTG  
 g120-l:16 TCACAGTCAGTGTCAACCGAGAGGGCTGAGCTTCAACCGAGGGCTCAGGGACTGGTCC  
 g120-l:17 TCGTGCATCTGATCCACCATGTTATTCTCCACATGTTGAAGTACTCGGTACGTTCCC  
 g120-l:18 TTGGGGTCCGTAGGCACGCAAGCATGCGTGTGCCCCACACGTTATGCACTCCGTGCTG  
 g120-l:19 CAGAAGAGGGTGTGTCGGCTCTTCCACACAGGCACGCCGTAGTAGACGGTGACCCAC  
 g120-l:20 CAGATCATCAGCATTCCAGGAGCATGGTGGCCCCAGCCACAGATGCTGGTAGTTCTT

#### Sequencing primers for optimised gp120 (Seq. ID No 83 to 90)

g120c-SF1	cggcgtgcctgtgtggaggc	Seq ID No 83
g120c-SF2	gacaagggtcagaaggaaatacgcg	Seq ID No 84
g120c-SF3	cgtccagctgaaacgactgtggc	Seq ID No 85
g120c-SF4	catgtggcaggagggtggaaaggcc	Seq ID No 86
g120c-SR1	gtgagcagcaggccgtgttgttgg	Seq ID No 87
g120c-SR2	cggcatagaaggcccgtccaggcc	Seq ID No 88
g120c-SR3	catgaccgtctgttgtcaagtga	Seq ID No 89
g120c-SR4	aggcacgcacgcgtcgccccac	Seq ID No 90

#### Sequencing primers for wild type gp120 (Seq. ID No 91-98)

g120w-SF1	ccactctattctgtgcatacgatgc	Seq ID No 91
g120w-SF2	ctttgatgttagtaccaaatacgatgtgt	Seq ID No 92
g120w-SF3	ggtatacatataggaccaggagac	Seq ID No 93
g120w-SF4	agatgttcataatatacgaggcc	Seq ID No 94
g120w-SR1	catatctccctccagggtctgaag	Seq ID No 95
g120w-SR2	gttattccattgtgtctactaa	Seq ID No 96
g120w-SR3	ggacaggccgtgtcatgactgag	Seq ID No 97
g120w-SR4	ctacttcttgggtgggtctg	Seq ID No 98

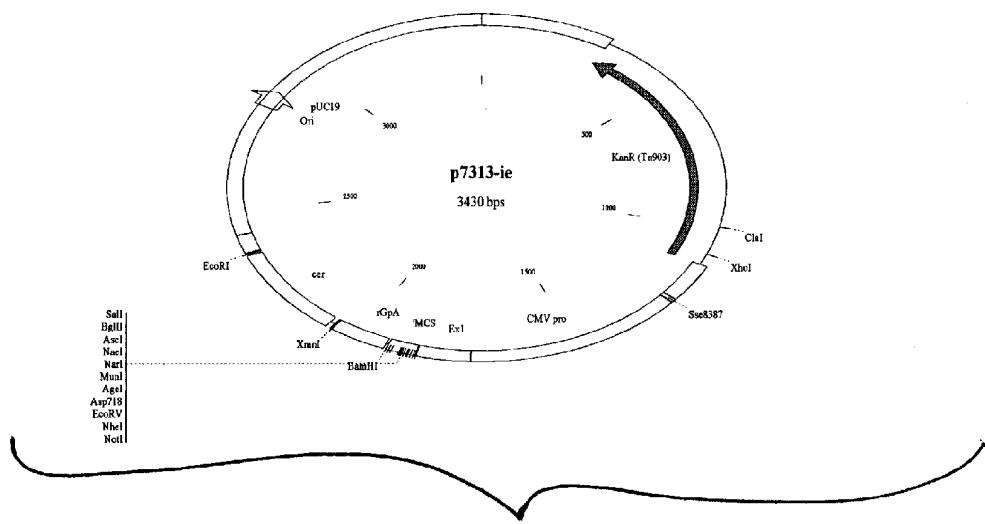
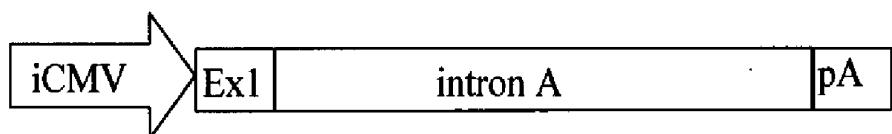
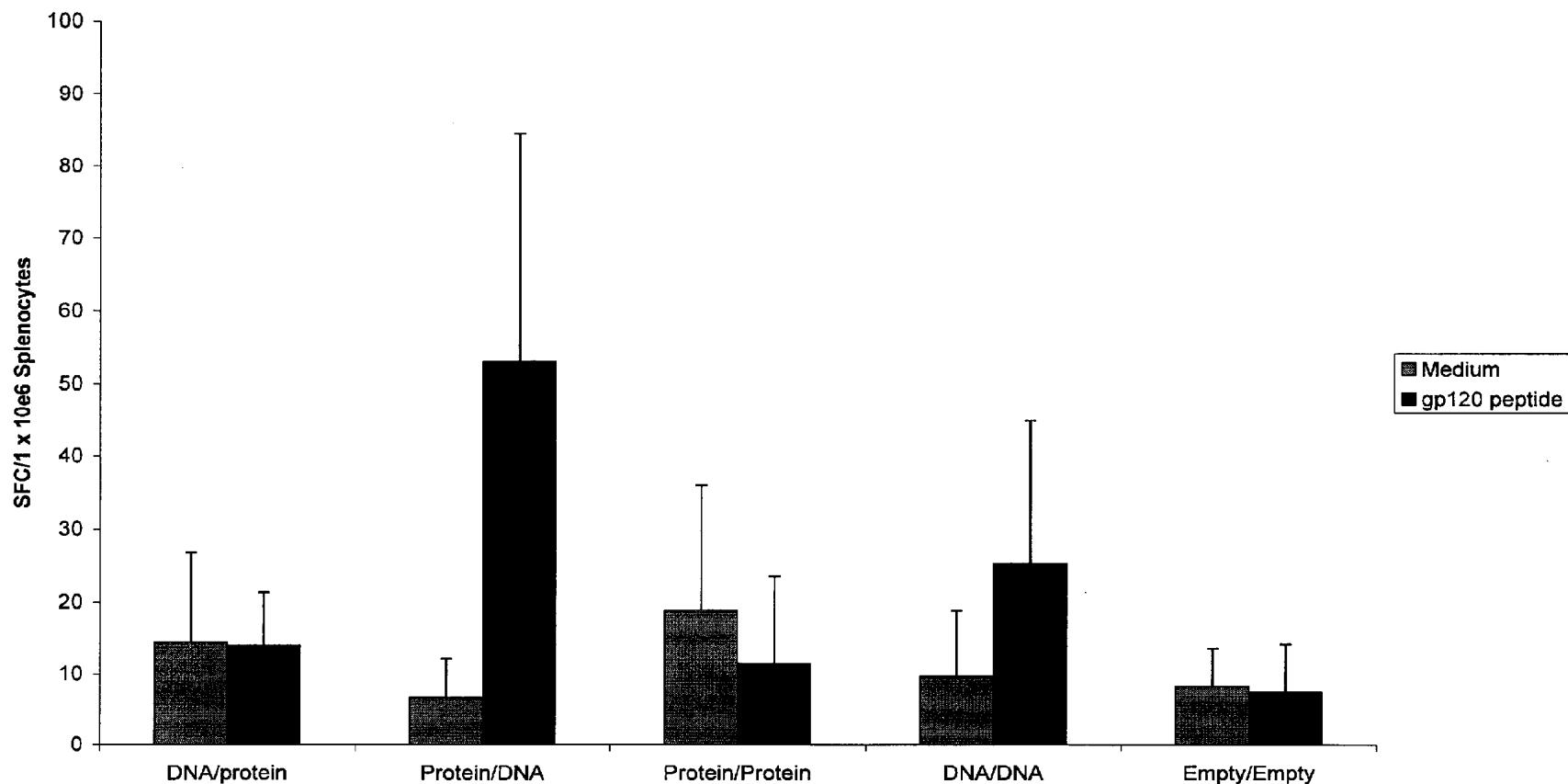


Figure 21 – Map of p7313

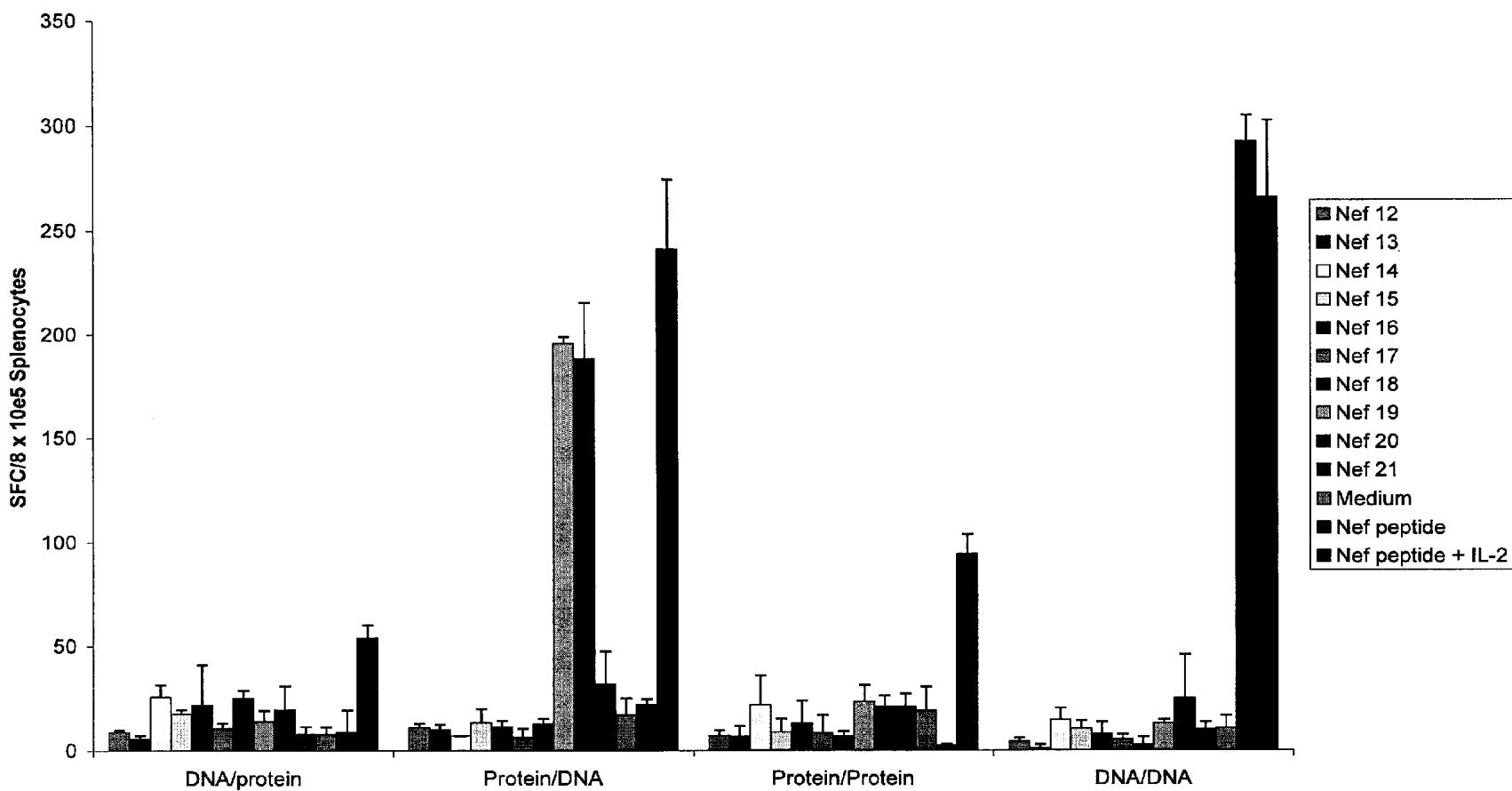
Figure 22: Typical expression plasmid for antigens



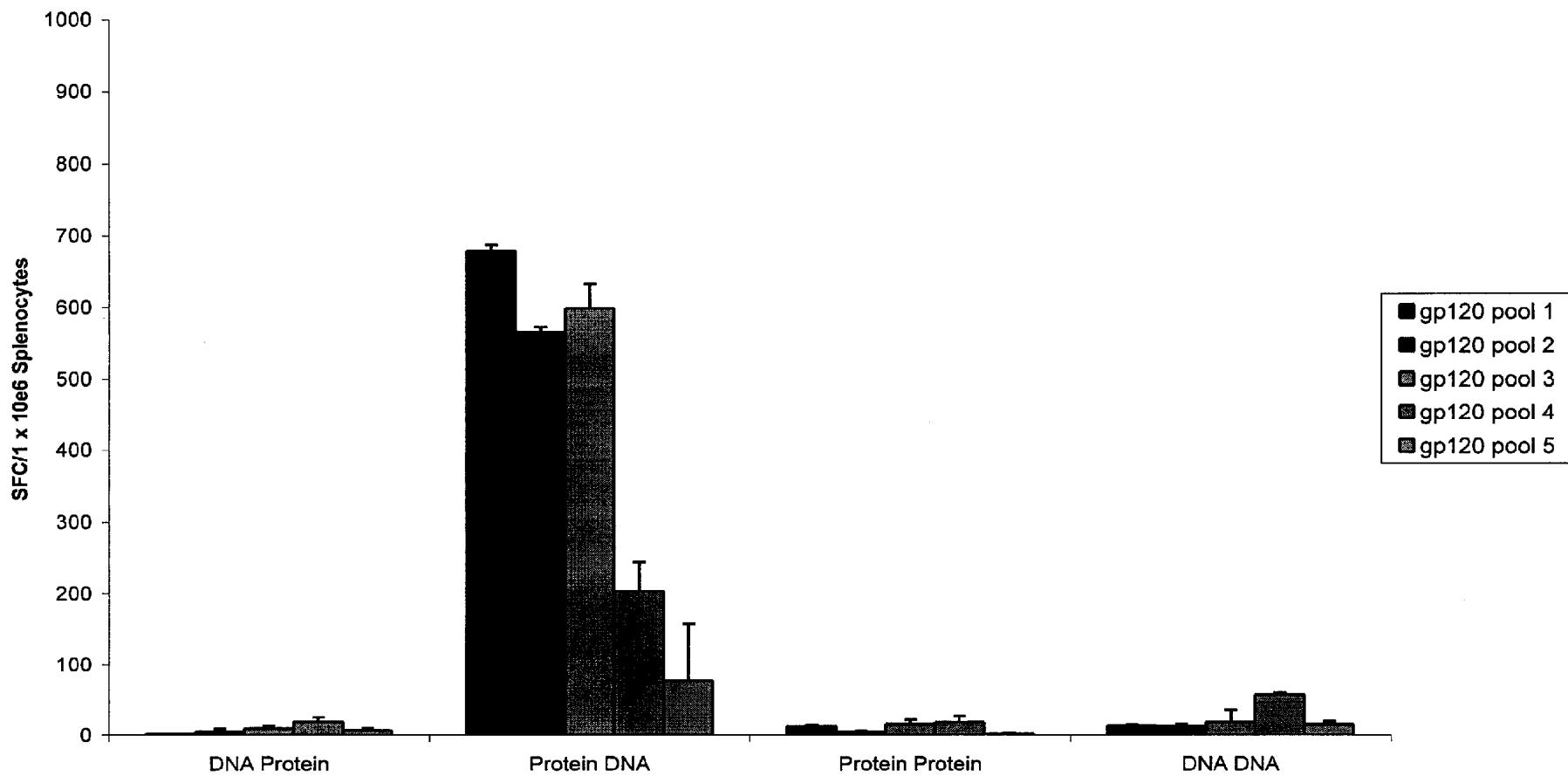
**Figure 23: Responses of immunised F1 (Balb/c x C3H) mice to gp120 peptide by IFN-gamma ELispot at 5 days post-boost**



**Figure 24: Responses to 20-mer Nef peptide library by IFN-gamma ELIspot at 5 days post-boost**



**Figure 25: Responses of immunised Balb/c mice to gp120 peptide pools by IFN-gamma ELIspot at 7 days post-boost**



**VACCINE COMPRISING GP120 AND NEF AND/OR TAT FOR THE IMMUNIZATION AGAINST HIV****CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application is a divisional of U.S. Ser. No. 10/485,048 filed 22 Sep. 2004, which is a 371 application of International Application No. PCT/EP02/08343 filed 26 Jul. 2002, which are incorporated herein by reference.

**DESCRIPTION**

[0002] The present invention relates to novel uses of HIV proteins in medicine and vaccine compositions containing such HIV proteins. In particular, the invention relates to the use of HIV Tat and HIV gp120 proteins in combination. Furthermore, the invention relates to the use of HIV Nef and HIV gp120 proteins in combination. The invention also relates to DNA encoding HIV Tat and/or Nef (hereinafter Tat DNA and/or Nef DNA) and DNA encoding HIV gp120 (hereinafter gp120 DNA) and vectors comprising such DNAs. The invention relates in particular to administering the proteins and/or DNAs in a prime-boost schedule via a particle bombardment approach.

[0003] HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts thus far have not been successful.

[0004] The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by the binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger precursor molecule (gp160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.

[0005] The gp120 protein is the principal target of neutralizing antibodies, but unfortunately the most immunogenic regions of the proteins (V3 loop) are also the most variable parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly protective vaccine. The gp120 protein does also contain epitopes that are recognized by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

[0006] Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the gag and pol genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, 324, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., 11, 5, 390 et seq (1992).

[0007] HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

[0008] In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12<sup>th</sup> Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and one dose recombinant protein). However, the results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

[0009] However, we have found that a Tat- and/or Nef-containing immunogen (especially a Nef-Tat fusion protein) acts synergistically with gp120 in protecting rhesus monkeys from a pathogenic challenge with chimeric human-simian immunodeficiency virus (SHIV). To date the SHIV infection of rhesus macaques is considered to be the most relevant animal model for human AIDS. Therefore, we have used this preclinical model to evaluate the protective efficacy of vaccines containing a gp120 antigen and a Nef- and Tat-containing antigen either alone or in combination. Analysis of two markers of viral infection and pathogenicity, the percentage of CD4-positive cells in the peripheral blood and the concentration of free SHIV RNA genomes in the plasma of the monkeys, indicated that the two antigens acted in synergy. Immunization with either gp120 or Neffat+SIV Nef alone did not result in any difference compared to immunization with an adjuvant alone. In contrast, the administration of the combination of gp120 and Neffat+SIV Nef, antigens resulted in a marked improvement of the two above-mentioned parameters in all animals of those particular experimental group.

[0010] As described above, the Neffat protein, the SIV Nef protein and gp120 protein together give an enhanced response over that which is observed when either Neffat+SIV Nef, or gp120 are used alone. This enhanced response, or synergy can be seen in a decrease in viral load as a result of vaccination with these combined proteins. Alternatively, or additionally the enhanced response manifests itself by a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV Neffat, SIV Nef and HIV gp120. The synergistic effect is attributed to the combination of gp120 and Tat, or gp120 and Nef, or gp120 and both Nef and Tat.

[0011] It has been found that not only the Nef, Tat or Neffat proteins are advantageously combined or administered with the gp120 protein. The same advantages are seen when DNA encoding Nef, Tat or Neffat is administered with gp120 (protein or corresponding DNA).

[0012] It has been found that the proteins above, or DNA encoding the proteins, may advantageously be administered via a prime-boost strategy. In one aspect the present invention relates to such administration via a bombardment approach. Accordingly the invention provides the use of

a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

[0013] c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide; and an HIV gp120 pro-

tein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV, wherein the protein or polynucleotide is delivered via a bombardment approach.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1 Provides a list of various DNA and protein sequences, labelled Seq. ID No. 8 to 25

[0015] FIG. 2 Show a plasmid map of Prit14597

[0016] FIG. 3 Shows an SDS-Page analysis of a Nef-Tat-His fusion protein

[0017] FIG. 4 Also shows an SDS-Page analysis of a Nef-Tat-His fusion protein

[0018] FIG. 5 Shows an SDS-Page analysis relevant to Example 5

[0019] FIG. 6 Shows an SDS-Page analysis relevant to Example 6

[0020] FIG. 7 Shows an SDS-Page analysis relevant to Example 7

[0021] FIG. 8 Shows an SDS-Page analysis under reducing conditions

[0022] FIG. 9 Also shows an SDS-Page analysis under reducing conditions

[0023] FIG. 10 Shows a plasmid map for pRIT14908

[0024] FIG. 11 Shows Seq ID Nos 30 and 31

[0025] FIG. 12 Shows a coomassie blue stained SDS-Page analysis

[0026] FIG. 13 Shows results of analysis of CD4-positive cells for monkey study 1

[0027] FIG. 14 Shows analysis of SHIV plasma virus load for monkey study 1

[0028] FIG. 15 Shows results of analysis of CD4-positive cells for monkey study 2

[0029] FIG. 16 Shows analysis of SHIV plasma virus load for monkey study 2

[0030] FIG. 17 Shows a Western Blot analysis of levels of Nef detected

[0031] FIG. 18 Also shows a Western Blot analysis of levels of Nef detected

[0032] FIG. 19 Shows various plasmid maps

[0033] FIG. 20 Shows certain sequences for codon optimization and sequencing of gp120

[0034] FIG. 21 Shows a plasmid map of p7313-ie

[0035] FIG. 22 Shows a schematic representation of the standard expression cassette

[0036] FIG. 23 Shows murine response to gp120 peptide by IFN-gamma ELIspot

[0037] FIG. 24 Shows responses to Nef peptide library by IFN-gamma ELIspot

[0038] FIG. 25 Also shows murine response to gp120 peptide by IFN-gamma ELIspot

[0039] Numerous methods of carrying out a particle bombardment approach are known. See for example WO 91/07487. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, Wis.), some examples of which are described in U.S. Pat. Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, coated with a substance such as polynucleotide, is accelerated to high speed within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest, typically the skin. The particles are preferably gold beads of a 0.4-4.0  $\mu\text{m}$ , more preferably 0.6-2.0  $\mu\text{m}$  diameter and the polynucleotide, preferably DNA is coated onto these and then encased in a cartridge for placing into the “gene gun”.

[0040] The addition of other HIV proteins or DNA encoding them may further enhance the synergistic effect, which was observed between gp120 and Tat and/or Nef. These other proteins may also act synergistically with individual components of the gp120, Tat and/or Nef-containing vaccine, not requiring the presence of the full original antigen combination. The additional proteins may be regulatory proteins of HIV such as Rev, Vif, Vpu, and Vpr. They may also be structural proteins derived from the HIV gag or pol genes.

[0041] The HIV gag gene encodes a precursor protein p55, which can assemble spontaneously into immature virus-like particles (VLPs). The precursor is then proteolytically cleaved into the major structural proteins p24 (capsid) and p18 (matrix), and into several smaller proteins. Both the precursor protein p55 and its major derivatives p24 and p18 may be considered as appropriate vaccine antigens which may further enhance the synergistic effect observed between gp120 and Tat and/or Nef. The precursor p55 and the capsid protein p24 may be used as VLPs or as monomeric proteins.

[0042] The HIV Tat protein for use in the present invention may, optionally, be linked to an HIV Nef protein, for example as a fusion protein.

[0043] The HIV Tat protein, the HIV Nef protein or the Nef-Tat fusion protein for use in the present invention may have a C terminal Histidine tail which preferably comprises between 5-10 Histidine residues. The presence of an histidine (or ‘His’) tail aids purification.

[0044] In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (*Saccharomyces cerevisiae*), of Nef (Macreadie I. G. et al., 1993, Yeast 9 (6) 565-573) and Tat (Braddock M et al., 1989, Cell 58 (2) 269-79) has been reported. Nef protein and the Gag proteins p55 and p18 are myristoylated. The expression of Nef and Tat separately in a *Pichia* expression system (Nef-His and Tat-His constructs), and the expression of a fusion construct Nef-Tat-His have been described previously in WO99/16884.

[0045] The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-H is (Seq. ID. No.s 10 and 11) and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13) are set forth in FIG. 1.

[0046] The HIV proteins may be used in their native conformation, or more preferably, may be modified for vaccine

use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the Tat or Nef protein. Thus the invention encompasses the use of derivatives of HIV proteins or polynucleotides, particularly DNAs, which may be, for example, mutated. The term 'mutated' is used herein to mean a DNA or protein molecule which has undergone deletion, addition or substitution of one or more nucleotides or amino acids using well known techniques for site directed mutagenesis or any other conventional method.

[0047] For example, a mutant Tat protein may be mutated so that it is biologically inactive whilst still maintaining its immunogenic epitopes. One possible mutated tat gene, constructed by D. Clements (Tulane University), (originating from BH 10 molecular clone) bears mutations in the active site region (Lys41→Ala) and in RGD motif (Arg78→Lys and Asp800→Glu) (Virology 235: 48-64, 1997).

[0048] A mutated Tat is illustrated in FIG. 1 (Seq. ID. No.s 22 and 23) as is a Nef-Tat Mutant-His (Seq. ID. No.s 24 and 25).

[0049] The HIV Tat or Nef proteins for use in the present invention may be modified by chemical methods during the purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a protein such as Tat or Nef is the use of chemical modifications of the protein's thiol groups. In a first step the disulphide bridges are reduced by treatment with a reducing agent such as DTT, beta-mercaptoethanol, or glutathione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (for example, the protein can be caused to react with iodoacetamide). Such chemical modification does not modify functional properties of Tat or Nef as assessed by cell binding assays and inhibition of lymphoproliferation of human peripheral blood mononuclear cells.

[0050] It will be understood that the invention also encompasses the use of fragments of the full length proteins provided that the fragments comprise at least one immunogenic epitope.

[0051] The HIV Tat protein and HIV gp120 proteins can be purified by the methods outlined in the attached examples.

[0052] An immunoprotective or immunotherapeutic quantity of the Tat and/or Nef or Neffat and gp120 components (protein or DNA) for use in the invention may be prepared by conventional techniques.

[0053] Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Md., U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fulerton, U.S. Pat. No. 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Pat. No. 4,372,945 and by Armor et al., U.S. Pat. No. 4,474,757.

[0054] The amount of protein in a vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 [[□]] $\mu$ g of each protein, preferably 2-200 [[□]] $\mu$ g, most preferably 4-40 [[□]] $\mu$ g of Tat or Nef or

Neffat and preferably 1-150  $\mu$ g, most preferably 2-25 [[□]] $\mu$ g of gp120. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. One particular example of a vaccine dose will comprise 20 [[□]] $\mu$ g of Neffat and 5 or 20 [[□]] $\mu$ g of gp120. Following an initial vaccination, subjects may receive a boost in about 4 weeks, and a subsequent second booster immunisation.

[0055] The proteins of the present invention are preferably adjuvanted in a vaccine formulation of the invention. The polynucleotides used in the present invention are optionally adjuvanted, and may be delivered in a formulation with an adjuvant or separately from the adjuvant, either simultaneously or sequentially. Adjuvants are described in general in Vaccine Design—the Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

[0056] Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

[0057] In the formulation of vaccines for use in the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

[0058] An immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. The resultant immune response may be broadly distinguished into two extreme categories, being humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

[0059] Extreme Th1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice Th1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. Th2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

[0060] It can be considered that the driving force behind the development of these two types of immune responses are cytokines, a number of identified protein messengers which serve to help the cells of the immune system and steer the eventual immune response to either a Th1 or Th2 response. Thus high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

[0061] It is important to remember that the distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4+ ve T cell

clones by Mosmann and Coffman (Mosmann, T R. and Coffman, R. L. (1989) *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology*, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF- $\gamma$  and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor- $\beta$ (TNF- $\beta$ ).

[0062] It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2-type cytokine responses. Traditionally the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection includes direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes in vitro after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

[0063] Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen in vitro, and induces antigen specific immunoglobulin responses associated with Th1-type isotype.

[0064] Preferred Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

[0065] Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (U.S. Pat. No. 6,005,099 and EP 0 729 473 B1; Hilgers et al., 1986, *IntArch.Allergy.Immunol.*, 79(4):392-6; Hilgers et al., 1987, *Immunology*, 60(1):141-6; and EP 0 549 074 B1). A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2  $\mu\text{m}$  in diameter, and its method of manufacture is disclosed in EP 0 689 454.

[0066] Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. *Phytomedicine* vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree *Quillaja Saponaria Molina*), and fractions thereof, are described in U.S. Pat. No. 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit. Rev Ther Drug Carrier Syst.*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in U.S. Pat. No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil et al.

(1991. *J. Immunology* vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

[0067] Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis et al., *J.Immunol.*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect.

[0068] In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, *Nature* 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

[0069] In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon  $\gamma$  and have cytolytic activity) and macrophages (Wooldridge et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

[0070] CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, supra) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis et al. supra; Brazolot-Millan et al., *Proc.Natl.Acad.Sci., USA*, 1998, 95(26), 15553-8).

[0071] Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis et al supra; Brazolot-Millan supra) or with other cationic carriers.

[0072] Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid

A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

[0073] Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.

[0074] An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

[0075] A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

[0076] Another preferred formulation comprises a CpG oligonucleotide alone or together with an aluminium salt.

[0077] Particularly preferred adjuvant and/or carrier combinations are as follows:

- i) 3D-MPL+QS21 in DQ
- ii) Alum+3D-MPL
- iii) Alum+QS21 in DQ+3D-MPL
- iv) Alum+CpG
- v) 3D-MPL+QS21 in DQ+oil in water emulsion
- vi) CpG

[0078] As already noted, the vaccine may contain polynucleotide, preferably DNA, encoding one or more of the Tat, Nef and gp120 polypeptides, such that the polypeptide is generated in situ.

[0079] The DNA constructs per se, especially those described herein, also form part of the invention.

[0080] The polynucleotide may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems such as plasmid DNA, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, Crit. Rev. Therap. Drug Carrier Systems 15:143-198, 1998 and references cited therein, incorporated herein by reference.

[0081] Plasmid based delivery of genes, particularly for immunisation or gene therapy purposes is known. For example, administration of naked DNA by injection into mouse muscle is outlined by Vical in International Patent Application WO90/11092.

[0082] Johnston et al WO 91/07487 describe methods of transferring a gene to vertebrate cells, by the use of micro-projectiles that have been coated with a polynucleotide encoding a gene of interest, and accelerating the microparticles such that the microparticles can penetrate the target cell.

[0083] DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong viral promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequences. The gene of interest may encode a full protein, a fusion protein

comprising different antigens, or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which is intended to be protected against. Thus the plasmid may encode a fragment of a full protein, provided that the fragment comprises at least one immunogenic epitope of the full protein. The plasmid can be grown in bacteria, such as for example *E. coli* and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the plasmid is taken up by cells of the host where the encoded peptide is produced. The plasmid vector will preferably be made without an origin of replication which is functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned. All of these features may apply singly or in combination to the present invention.

[0084] There are a number of advantages of DNA vaccination relative to traditional vaccination techniques. First, it is predicted that because the proteins which are encoded by the DNA sequence are synthesised in the host, the structure or conformation of the protein will be similar to the native protein associated with the disease state. It is also likely that DNA vaccination will offer protection against different strains of a virus, by generating a cytotoxic T lymphocyte response that recognises epitopes from conserved proteins. Furthermore, because the plasmids are taken up by the host cells where antigenic protein can be produced, a long-lasting immune response will be elicited. The technology also offers the possibility of combining diverse immunogens into a single preparation to facilitate simultaneous immunisation in relation to a number of disease states.

[0085] Helpful background information in relation to DNA vaccination is provided in Donnelly et al "DNA vaccines" Ann. Rev Immunol. 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

[0086] In one preferred embodiment the DNA can be delivered via a particle bombardment approach e.g. a "gene gun" approach as described hereinabove.

[0087] Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). When the expression system is a recombinant live microorganism, such as a virus or bacterium, the gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and in vivo infection with this live vector will lead to in vivo expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox, modified poxviruses e.g. Modified Virus Ankara (MVA)), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelan Equine Encephalitis Virus), flaviviruses (yellow fever virus, Dengue virus, Japanese encephalitis virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), *Listeria*, *Salmonella*, *Shigella*, *Neisseria*, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines.

[0088] Thus, the Nef, Tat and gp120 components of a preferred vaccine according to the invention may be provided in the form of polynucleotides or recombinant DNA encoding the desired proteins. The polynucleotides employed in the invention may encode a full protein, a fusion protein comprising different antigens, or one or more antigenic peptide sequences. Thus the polynucleotides may encode a fragment

of a full protein, provided that the fragment comprises at least one immunogenic epitope of the full protein.

[0089] At least one of the DNAs for Nef, Tat, Neffat or gp120 may preferably be codon optimised as described, for example, in Andre S. Seed B. Eberle J. Schraut W. Bultmann A. Haas J. (1998): Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage, Journal of Virology. 72(2):1497-503. In one preferred aspect the DNA encoding gp120 is codon optimised.

[0090] Codon optimisation is used to optimise the polynucleotide sequences for expression in mammalian cells. That is the sequence is optimised to resemble the codon usage of genes in mammalian cells.

[0091] In one embodiment of the present invention the Nef, Tat, Neffat or gp120 polynucleotide sequence has a codon usage pattern which resembles that of highly expressed mammalian genes, particularly human genes. Preferably the polynucleotide sequence is a DNA sequence. Desirably the codon usage pattern of the polynucleotide sequence is typical of highly expressed human genes.

[0092] The DNA code has 4 letters (A, T, C and G) and uses these to spell three letter "codons" which represent the amino acids the proteins encode in an organism's genes. The linear sequence of codons along the DNA molecule is translated into the linear sequence of amino acids in the protein(s) encoded by those genes. The code is highly degenerate, with 61 codons coding for the 20 natural amino acids and 3 codons representing "stop" signals. Thus, most amino acids are coded for by more than one codon—in fact several are coded for by four or more different codons.

[0093] Where more than one codon is available to code for a given amino acid, it has been observed that the codon usage patterns of organisms are highly non-random. Different species show a different bias in their codon selection and, furthermore, utilisation of codons may be markedly different in a single species between genes which are expressed at high and low levels. This bias is different in viruses, plants, bacteria and mammalian cells, and some species show a stronger bias away from a random codon selection than others. For example, humans and other mammals are less strongly biased than certain bacteria or viruses. For these reasons, there is a significant probability that a mammalian gene expressed in *E. coli* or a viral gene expressed in mammalian cells will have an inappropriate distribution of codons for efficient expression. It is believed that the presence in a heterologous DNA sequence of clusters of codons which are rarely observed in

the host in which expression is to occur, is predictive of low heterologous expression levels in that host.

[0094] In the polynucleotides of the present invention, the codon usage pattern is altered from that typical of human immunodeficiency viruses to more closely represent the codon bias of the target organism, e.g. a mammal, especially a human. The "codon usage coefficient" is a measure of how closely the codon pattern of a given polynucleotide sequence resembles that of a target species. Codon frequencies can be derived from literature sources for the highly expressed genes of many species (see e.g. Nakamura et al. Nucleic Acids Research 1996, 24:214-215). The codon frequencies for each of the 61 codons (expressed as the number of occurrences occurrence per 1000 codons of the selected class of genes) are normalised for each of the twenty natural amino acids, so that the value for the most frequently used codon for each amino acid is set to 1 and the frequencies for the less common codons are scaled to lie between zero and 1. Thus each of the 61 codons is assigned a value of 1 or lower for the highly expressed genes of the target species. In order to calculate a codon usage coefficient for a specific polynucleotide, relative to the highly expressed genes of that species, the scaled value for each codon of the specific polynucleotide are noted and the geometric mean of all these values is taken (by dividing the sum of the natural logs of these values by the total number of codons and take the anti-log). The coefficient will have a value between zero and 1 and the higher the coefficient the more codons in the polynucleotide are frequently used codons. If a polynucleotide sequence has a codon usage coefficient of 1, all of the codons are "most frequent" codons for highly expressed genes of the target species.

[0095] According to the present invention, the codon usage pattern of the polynucleotide will preferably exclude rare codons representing less than 10% of the codon use for an amino acid in highly expressed genes of the target organism. In an alternative preferred embodiment, the polynucleotide will exclude codons with an RSCU value of less than 0.2 in highly expressed genes of the target organism. A relative synonymous codon usage (RSCU) value is the observed number of codons divided by the number expected if all codons for that amino acid were used equally frequently. A polynucleotide of the present invention will generally have a codon usage coefficient for highly expressed human genes of greater than 0.3, preferably greater than 0.4, most preferably greater than 0.5. Codon usage tables for human can also be found in Genebank.

[0096] In comparison, a highly expressed beta action gene has a RSCU of 0.747. The codon usage table for a *homo sapiens* is set out below:

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Codon Usage Table:

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*Homo sapiens* [gbpri]: 27143 CDS's (12816923 codons) Standard Codon Usage Table

fields: [triplet] [frequency: per thousand] ([number])

UUU	17.0	(217684)	UCU	14.8	(189419)	UAU	12.1	(155645)	UGU	10.0	(127719)
UUC	20.5	(262753)	UCC	17.5	(224470)	UAC	15.8	(202481)	UGC	12.3	(157257)
UUA	7.3	(93924)	UCA	11.9	(152074)	UAA	0.7	(9195)	UGA	1.3	(16025)
UUG	12.5	(159611)	UCG	4.5	(57572)	UAG	0.5	(6789)	UGG	12.9	(165930)

- continued

Codon Usage Table:

CUU 12.8 (163707)	CCU 17.3 (222146)	CAU 10.5 (134186)	CGU 4.6 (59454)
CUC 19.3 (247391)	CCC 20.0 (256235)	CAC 14.9 (190928)	CGC 10.8 (137865)
CUA 7.0 (89078)	CCA 16.7 (214583)	CAA 12.0 (153590)	CGA 6.3 (80709)
CUG 39.7 (509096)	CCG 7.0 (89619)	CAG 34.5 (441727)	CGG 11.6 (148666)
AUU 15.8 (202844)	ACU 12.9 (165392)	AAU 17.0 (218508)	AGU 12.0 (154442)
AUC 21.6 (277066)	ACC 19.3 (247805)	AAC 19.8 (253475)	AGC 19.3 (247583)
AUA 7.2 (92133)	ACA 14.9 (191518)	AAA 24.0 (308123)	AGA 11.5 (147264)
AUG 22.3 (285776)	ACG 6.3 (80369)	AAG 32.6 (418141)	AGG 11.3 (145276)
GUU 10.9 (139611)	GCU 18.5 (236639)	GAU 22.4 (286742)	GGU 10.8 (138606)
GUC 14.6 (187333)	GCC 28.3 (362086)	GAC 26.1 (334158)	GGC 22.7 (290904)
GUA 7.0 (89644)	GCA 15.9 (203310)	GAA 29.1 (373151)	GGA 16.4 (210643)
GUG 28.8 (369006)	GCG 7.5 (96455)	GAG 40.2 (515485)	GGG 16.4 (209907)

Coding GC 52.51% 1st letter GC 56.04% 2nd letter GC 42.35% 3rd letter GC 59.13%.

[0097] According to a further aspect of the invention, an expression vector is provided which comprises and is capable of directing the expression of a Nef and/or Tat or NefTat, and a gp120 polynucleotide sequence according to the first aspect of the invention, in particular where the codon usage pattern of at least one of the Nef, Tat, NefTat or gp120 polynucleotide sequences, particularly the gp120 sequence, is typical of highly expressed mammalian genes, preferably highly expressed human genes. The vector is suitable for driving expression of heterologous DNA in mammalian cells, particularly human cells. In one embodiment, the expression vector is p7313 (see FIGS. 21 and 22).

[0098] In a further aspect the invention provides a plurality of particles, preferably gold particles, coated with DNA comprising one or more vectors encoding gp120 and nef and/or tat or neftat. Preferably the particles are coated with a single vector which encodes gp120 and nef and tat, the latter most preferably in the form of a NefTat fusion protein. Most preferably one or more of the sequences are codon optimised for expression in human cells.

[0099] In a preferred aspect the DNA encoding the nef, tat and gp120 is present on a single vector.

[0100] Preferably the vector comprises the nef, tat and gp120 sequences inserted 3' to an enhanced HCMV IE1 promoter for efficient expression. This is preferably the HCMV immediate early promoter devoid of intron A, but including exon 1.

[0101] One suitable vector according to the invention is that denoted as p7313, further described below.

[0102] The vectors which comprise the nucleotide sequences described herein are administered in such amount as will be prophylactically or therapeutically effective. The quantity to be administered is generally in the range of one picogram to 1 milligram, preferably 1 picogram to 10 micrograms for particle-mediated delivery as described herein. The exact quantity may vary depending on the weight of the patient being immunised and the precise route of administration.

[0103] Immunisations according to the invention may be performed with a combination of protein and DNA-based formulations. Adjuvanted protein vaccines induce mainly antibodies and T helper immune responses, while delivery of

DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL) responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses. This is particularly relevant in the context of HIV, since both neutralising antibodies and CTL are thought to be important for the immune defense against HIV.

[0104] The DNA may be delivered as plasmid DNA or in the form of a recombinant live vector, e.g. a poxvirus vector or any other suitable live vector such as those described herein. Protein antigens may be injected once or several times followed by one or more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations.

[0105] A particular example of prime-boost immunisation according to the invention involves priming with DNA in the form of a recombinant live vector such as a modified poxvirus vector, for example Modified Virus Ankara (MVA) or a derivative thereof e.g through passaging or genetic manipulation, or an alphavirus vector for example Venezuelan Equine Encephalitis Virus, followed by boosting with a protein, preferably an adjuvanted protein. Optionally the DNA is adjuvanted with a suitable DNA vaccine adjuvant known in the art.

[0106] The invention further provides a method of prophylactic or therapeutic immunisation which method comprises administering to a subject in need thereof a composition comprising

a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;

and an HIV gp120 protein or polynucleotide, in a prime-boost delivery wherein the protein or polynucleotide is delivered via a bombardment approach.

[0107] In a further aspect the invention provides a kit comprising at least two different vaccine compositions including a) a composition comprising a plurality of particles coated with DNA encoding gp120 and nef and/or tat or neftat as described herein and b) a composition comprising gp120 and nef and/or tat or neftat DNA or proteins as described herein wherein the DNA or proteins in b) are not coated onto particles.

[0108] The invention is illustrated in the accompanying examples and Figures:

## EXAMPLES

### General

[0109] The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs of these experiments since this gene is among those that are most closely related to the consensus Nef.

[0110] The starting material for the Bru/Lai Nef gene was a 1170 bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

[0111] The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985.

[0112] The expression of the Nef and Tat genes could be in *Pichia* or any other host.

### Example 1

#### Expression of HIV-1 nef and tat Sequences in *Pichia Pastoris*

[0113] Nef protein, Tat protein and the fusion Nef-Tat were expressed in the methylotrophic yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1) promoter.

[0114] To express these HIV-1 genes a modified version of the integrative vector PHL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues. This PHL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent AsuII and EcoRI sites of PHL-D2 vector (see FIG. 2 which includes Seq ID Nos: 26 and 27). In addition to the H is tail, this linker carries NcoI, SpeI and XbaI restriction sites between which nef, tat and nef-tat fusion were inserted.

1.1 Construction of the Integrative Vectors pRIT14597 (Encoding Nef-His Protein), pRIT14598 (Encoding Tat-His Protein) and pRIT14599 (Encoding Fusion Nef-Tat-His)

[0115] The nef gene was amplified by PCR from the pcDNA3/Nef plasmid with primers O1 and O<sub>2</sub>.

PRIMER 01 (Seq ID NO 1):  
NcoI  
5'ATCGTCC**ATG**.GGT.GGC.AAG.TGG.T 3'

PRIMER 02 (Seq ID NO 2):  
SpeI  
5' CGGGTACTAGTG**CAG**TCTTGAA 3'

[0116] The PCR fragment obtained and the integrative PHL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14597 (see FIG. 2).

[0117] The tat gene was amplified by PCR from a derivative of the pCV1 plasmid with primers 05 and 04:

PRIMER 04 (Seq ID NO 4):  
SpeI  
5' CGGCTACTAGTTCCCTCGGGCCT 3'

PRIMER 05 (Seq ID NO 5):  
NcoI  
5' ATCGTCC**ATG**GAGGCCAGTAGATC 3'

[0118] An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14598.

[0119] To construct pRIT14599, a 910 bp DNA fragment corresponding to the nef-tat-His coding sequence was ligated between the EcoRI blunted (T4 polymerase)

[0120] and NcoI sites of the PHL-D2-MOD vector. The nef-tat-His coding fragment was obtained by XbaI blunted (T4 polymerase) and NcoI digestions of pRIT14596.

1.2 Transformation of *Pichia Pastoris* Strain GS115(his4).

[0121] To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-His, strain GS115 was transformed with linear NotI fragments carrying the respective expression cassettes plus the HIS4 gene to complement his4 in the host genome. Transformation of GS115 with NotI-linear fragments favors recombination at the AOX1 locus.

[0122] Multicopy integrant clones were selected by quantitative dot blot analysis and the type of integration, insertion (Mutphenotype) or transplacement (Mut<sup>S</sup> phenotype), was determined.

[0123] From each transformation, one transformant showing a high production level for the recombinant protein was selected:

[0124] Strain Y 1738 (Mut<sup>+</sup> phenotype) producing the recombinant Nef-His protein, a myristylated 215 amino acids protein which is composed of:

[0125] Myristic acid

[0126] A methionine, created by the use of NcoI cloning site of PHL-D2-MOD vector

[0127] 205 a.a. of Nef protein (starting at a.a.2 and extending to a.a.206)

[0128] A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHL-D2-MOD vector)

[0129] One glycine and six histidines.

[0130] Strain Y1739 (Mut<sup>+</sup> phenotype) producing the Tat-His protein, a 95 amino acid protein which is composed of:

[0131] A methionine created by the use of NcoI cloning site

[0132] 85 a.a. of the Tat protein (starting at a.a.2 and extending to a.a.86)

[0133] A threonine and a serine introduced by cloning procedure

[0134] One glycine and six histidines

[0135] Strain Y 1737(Mut<sup>S</sup> phenotype) producing the recombinant Nef-Tat-His fusion protein, a myristylated 302 amino acids protein which is composed of:

[0136] Myristic acid

[0137] A methionine, created by the use of NcoI cloning site

[0138] 205.a. of Nef protein (starting at a.a.2 and extending to a.a.206)

[0139] A threonine and a serine created by the cloning procedure

[0140] 85.a. of the Tat protein (starting at a.a.2 and extending to a.a.86)

[0141] A threonine and a serine introduced by the cloning procedure

[0142] One glycine and six histidines

### Example 2

#### Expression of HIV-1 Tat-Mutant in *Pichia Pastoris*

[0143] A mutant recombinant Tat protein has also been expressed. The mutant Tat protein must be biologically inactive while maintaining its immunogenic epitopes.

[0144] A double mutant tat gene, constructed by D. Clements (Tulane University) was selected for these constructs.

[0145] This tat gene (originates from BH±0 molecular clone) bears mutations in the active site region (Lys41Ala) and in RGD motif (Arg78-Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

[0146] The mutant tat gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

2.1 Construction of the Integrative Vectors pRIT14912(Encoding Tat Mutant-His Protein) and pRIT14913(Encoding Fusion Nef-Tat Mutant-His).

[0147] The tat mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 05 and 04 (see section 1.1construction of pRIT14598)

[0148] An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14912

[0149] To construct pRIT14913, the tat mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04.

PRIMER 03 (Seq ID NO 3):  
SpeI  
5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'

PRIMER 04 (Seq ID NO 4):  
SpeI  
5' CGGCTACTAGTTCCCTCGGGCCT 3'

[0150] The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913

#### 2.2 Transformation of *Pichia Pastoris* Strain GS115.

[0151] *Pichia pastoris* strains expressing Tat mutant-His protein and the fusion Nef-Tat mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 1.2.

[0152] Two recombinant strains producing Tat mutant-His protein, a 95 amino-acids protein, were selected: Y1775 (Mut<sup>+</sup> phenotype) and Y1776(Mut<sup>S</sup> phenotype).

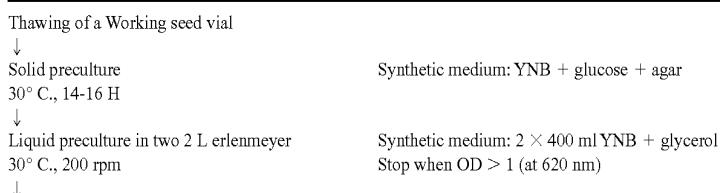
[0153] One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut<sup>+</sup> phenotype).

### Example 3

#### Fermentation of *Pichia Pastoris* Producing Recombinant TAT-HIS

[0154] A typical process is described in the table hereafter.

[0155] Fermentation includes a growth phase (feeding with a glycerol-based medium according to an appropriate curve) leading to a high cell density culture and an induction phase (feeding with a methanol and a salts/micro-elements solution). During fermentation the growth is followed by taking samples and measuring their absorbance at 620 nm. During the induction phase methanol was added via a pump and its concentration monitored by Gas chromatography (on culture samples) and by on-line gas analysis with a Mass spectrometer. After fermentation the cells were recovered by centrifugation at 5020 g during 30' at 2-8° C. and the cell paste stored at -20° C. For further work cell paste was thawed, resuspended at an OD (at 620 nm) of 150 in a buffer (Na<sub>2</sub>HPO<sub>4</sub> pH750 mM, PMSF 5%, Isopropanol 4 mM) and disrupted by 4 passages in a DynoMill (room 0.6 L, 3000 rpm, 6 L/H, beads diameter of 0.40-0.70 mm). For evaluation of the expression samples were removed during the induction, disrupted and analyzed by SDS-Page or Western blot. On Coomassie blue stained SDS-gels the recombinant Tat-his was clearly identified as an intense band presenting a maximal intensity after around 72-96H induction.



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Inoculation of a 20 L fermentor	5 L initial medium (FSC006AA) 3 ml antifoam SAG471 (from Witco) Set-points: Temperature: 30° C. Overpressure: 0.3 barg Air flow: 20 NL/min Dissolved O <sub>2</sub> : regulated >40% pH: regulated at 5 by NH <sub>4</sub> OH
↓	
Fed-batch fermentation: growth phase	Feeding with glycerol-based medium FFB005AA
Duration around 40 H	Final OD between 200-500 OD (620 nm)
Fed-batch fermentation: induction phase	Feeding with methanol and with a salt/micro-elements solution (FSE021AB).
Duration: up to 97 H	
↓	
Centrifugation	5020 g/30 min/2-8° C.
↓	
Recover cell paste and store at -20° C.	
↓	
Thaw cells and resuspend at OD150 (620 nm) in buffer	Buffer: Na <sub>2</sub> HPO <sub>4</sub> pH7 50 mM, PMSF 5%, Isopropanol 4 mM
↓	
Cell disruption in Dyno-mill 4 passages	Dyno-mill: (room 0.6 L, 3000 rpm, 6 L/H, beads diameter of 0.40-0.70 mm).
↓	
Transfer for extraction/purification	

[0156] Media used for Fermentation:

Solid preculture: (YNB + glucose + agar)				
Glucose:	10 g/l	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O:	0.0002 g/l	Acide folique: 0.000064 g/l
KH <sub>2</sub> PO <sub>4</sub> :	1 g/l	MnSO <sub>4</sub> •H <sub>2</sub> O:	0.0004 g/l	Inositol: 0.064 g/l
MgSO <sub>4</sub> •7H <sub>2</sub> O:	0.5 g/l	H <sub>3</sub> BO <sub>3</sub> :	0.0005 g/l	Pyridoxine: 0.008 g/l
CaCl <sub>2</sub> •2H <sub>2</sub> O:	0.1 g/l	KI:	0.0001 g/l	Thiamine: 0.008 g/l
NaCl:	0.1 g/l	CoCl <sub>2</sub> •6H <sub>2</sub> O:	0.00009 g/l	Niacine: 0.000032 g/l
FeCl <sub>3</sub> •6H <sub>2</sub> O:	0.0002 g/l	Riboflavin:	0.000016 g/l	Panthotenate Ca: 0.008 g/l
CuSO <sub>4</sub> •5H <sub>2</sub> O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid: 0.008 g/l
ZnSO <sub>4</sub> •7H <sub>2</sub> O:	0.0004 g/l	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	5 g/l	Agar 18 g/l

[0157]

Liquid preculture, (YNB + glycerol)				
Glycerol:	2% (v/v)	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O:	0.0002 g/l	Acide folique: 0.000064 g/l
KH <sub>2</sub> PO <sub>4</sub> :	1 g/l	MnSO <sub>4</sub> •H <sub>2</sub> O:	0.0004 g/l	Inositol: 0.064 g/l
MgSO <sub>4</sub> •7H <sub>2</sub> O:	0.5 g/l	H <sub>3</sub> BO <sub>3</sub> :	0.0005 g/l	Pyridoxine: 0.008 g/l
CaCl <sub>2</sub> •2H <sub>2</sub> O:	0.1 g/l	KI:	0.0001 g/l	Thiamine: 0.008 g/l
NaCl:	0.1 g/l	CoCl <sub>2</sub> •6H <sub>2</sub> O:	0.00009 g/l	Niacine: 0.000032 g/l
FeCl <sub>3</sub> •6H <sub>2</sub> O:	0.0002 g/l	Riboflavin:	0.000016 g/l	Panthotenate Ca: 0.008 g/l
CuSO <sub>4</sub> •5H <sub>2</sub> O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid: 0.008 g/l
ZnSO <sub>4</sub> •7H <sub>2</sub> O:	0.0004 g/l	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	5 g/l	

[0158]

-continued

Initial fermentor charge: (FSC006AA)				Initial fermentor charge: (FSC006AA)			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> :	6.4 g/l	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O:	2.04 mg/l	FeCl <sub>3</sub> •6H <sub>2</sub> O:	10 mg/l	CoCl <sub>2</sub> •6H <sub>2</sub> O:	0.91 mg/l
KH <sub>2</sub> PO <sub>4</sub> :	9 g/l	MnSO <sub>4</sub> •H <sub>2</sub> O:	4.08 mg/l	HCl:	1.67 ml/l	NaCl:	0.06 g/l
MgSO <sub>4</sub> •7H <sub>2</sub> O:	4.7 g/l	H <sub>3</sub> BO <sub>3</sub> :	5.1 mg/l	CuSO <sub>4</sub> •5H <sub>2</sub> O:	0.408 mg/l	Biotine:	0.534 mg/l
CaCl <sub>2</sub> •2H <sub>2</sub> O:	0.94 g/l	KI:	1.022 mg/l	ZnSO <sub>4</sub> •7H <sub>2</sub> O:	4.08 mg/l		

[0159]

Feeding solution used for growth phase (FFB005AA)			
Glycérol:	38.7% v/v	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O:	5.7 mg/l
MgSO <sub>4</sub> •7H <sub>2</sub> O:	13 g/l	CuSO <sub>4</sub> •5H <sub>2</sub> O:	1.13 mg/l
CaCl <sub>2</sub> •2H <sub>2</sub> O:	2.6 g/l	CoCl <sub>2</sub> •6H <sub>2</sub> O:	2.5 mg/l
FeCl <sub>3</sub> •6H <sub>2</sub> O:	27.8 mg/l	H <sub>3</sub> BO <sub>3</sub> :	14.2 mg/l
ZnSO <sub>4</sub> •7H <sub>2</sub> O	11.3 mg/l	Biotine:	1.5 mg/l
MnSO <sub>4</sub> •H <sub>2</sub> O:	11.3 mg/l	KI:	2.84 mg/l
KH <sub>2</sub> PO <sub>4</sub> :	24.93 g/l	NaCl:	0.167 g/l

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Feeding solution of salts and micro-elements used during induction (FSE021AB):			
NaCl:	0.3 g/l	KI:	5.11 mg/l
HCl:	8.3 ml/l	CoCl <sub>2</sub> •6H <sub>2</sub> O:	4.55 mg/l
CuSO <sub>4</sub> •5H <sub>2</sub> O:	2.04 mg/l	FeCl <sub>3</sub> •6H <sub>2</sub> O:	50.0 mg/l
ZnSO <sub>4</sub> •7H <sub>2</sub> O:	20.4 mg/l	Biotine:	2.70 mg/l

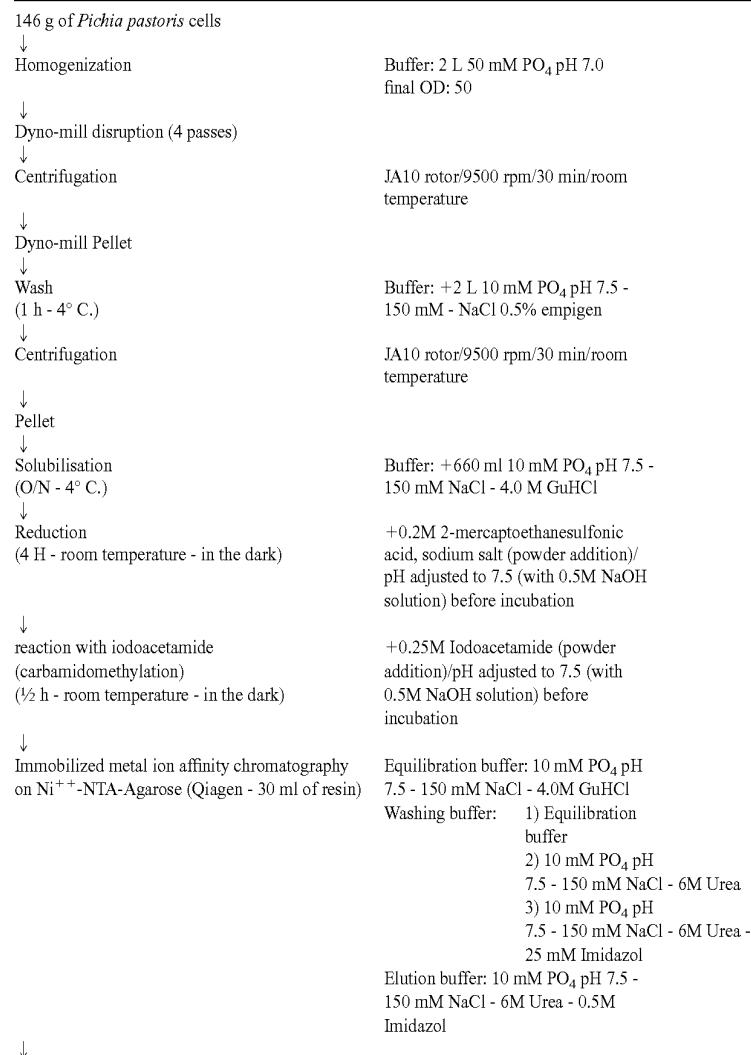
[0160]

Feeding solution of salts and micro-elements used during induction (FSE021AB):			
KH <sub>2</sub> PO <sub>4</sub> :	45 g/l	Na <sub>2</sub> MoO <sub>4</sub> •2H <sub>2</sub> O:	10.2 mg/l
MgSO <sub>4</sub> •7H <sub>2</sub> O:	23.5 g/l	MnSO <sub>4</sub> •H <sub>2</sub> O:	20.4 mg/l
CaCl <sub>2</sub> •2H <sub>2</sub> O:	4.70 g/l	H <sub>3</sub> BO <sub>3</sub> :	25.5 mg/l

## Example 4

Purification of Nef-Tat-His Fusion Protein (*Pichia pastoris*)

[0161] The purification scheme has been developed from 146 g of recombinant *Pichia pastoris* cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps, Nef-Tat positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.



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Dilution	Down to an ionic strength of 18 mS/cm <sup>2</sup> Dilution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 6M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 30 ml of resin)	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6.0M Urea Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 250 mM NaCl - 6M Urea Elution buffer: 10 mM Borate pH 9.0 - 2M NaCl - 6M Urea
↓	
Concentration	up to 5 mg/ml 10 kDa Omega membrane (Filtron)
↓	
Gel filtration chromatography on Superdex200 XK 16/60 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6M Urea 5 ml of sample/injection \$5 injections
↓	
Dialysis (O/N - 4° C.)	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - 0.5M Arginin*
↓	
Sterile filtration	Millex GV 0.22 µm

---

\*ratio: 0.5M Arginin for a protein concentration of 1600 µg/ml.

#### Purity

[0162] The level of purity as estimated by SDS-PAGE is shown in FIG. 3 by Daiichi Silver Staining and in FIG. 4 by Coomassie blue G250.

---

After Superdex200 step:	>95%
After dialysis and sterile filtration steps:	>95%

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#### Recovery

[0163] 51 mg of Nef-Tat-his protein are purified from 146 g of recombinant *Pichia pastoris* cells (=2 L of Dyno-mill homogenate OD 55)

#### Example 5

##### Purification of Oxidized NEF-TAT-HIS Fusion Protein in *Pichia Pastoris*

[0164] The purification scheme has been developed from 73 g of recombinant *Pichia pastoris* cells (wet weight) or 1 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps, Nef-Tat positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.

---

73 g of <i>Pichia pastoris</i> cells	
↓	
Homogenization	Buffer: 1 L 50 mM PO <sub>4</sub> pH 7.0 - Pefabloc 5 mM final OD: 50
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (2 h - 4° C.)	Buffer: +1 L 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 0.5% Empigen
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4° C.)	Buffer: +330 ml 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl
↓	

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-continued

↓	Dilution	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea 3) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 0.5 M Imidazol
↓	Cation exchange chromatography on SP Sepharose FF (Pharmacia - 7 ml of resin)	Down to an ionic strength of 18 mS/cm <sup>2</sup> Dilution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 6 M Urea
↓	Concentration	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 250 mM NaCl - 6 M Urea Elution buffer: 10 mM Borate pH 9.0 - 2 M NaCl - 6 M Urea
↓	Dialysis (O/N - 4° C.)	up to 0.8 mg/ml 10 kDa Omega membrane (Filtron)
↓	Sterile filtration	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - 0.5 M Arginin
→	Level of purity estimated by SDS-PAGE is shown in FIG. 6 (Daichi Silver Staining, Coomassie blue G250, Western blotting): After dialysis and sterile filtration steps:	>95%
→	Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA) 2.8 mg of oxidized Nef-Tat-his protein are purified from 73 g of recombinant <i>Pichia pastoris</i> cells (wet weight) or 1 L of Dyno-mill homogenate OD 50.	

#### Example 6

##### Purification of Reduced TAT-HIS Protein (*Pichia pastoris*)

[0165] The purification scheme has been developed from 160 g of recombinant *Pichia pastoris* cells (wet weight) or 2L Dyno-mill homogenate OD 66. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.

160 g of <i>Pichia pastoris</i> cells	
↓	Homogenization
	Buffer: +2 L 50 mM PO <sub>4</sub> pH 7.0 - 4 mM PMSF final OD: 66
↓	Dyno-mill disruption (4 passes)
↓	Centrifugation
	JA10 rotor/9500 rpm/30 min/room temperature
↓	Dyno-mill Pellet
↓	Wash (1 h - 4° C.)
	Buffer: +2 L 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 1% Empigen
↓	Centrifugation
↓	JA10 rotor/9500 rpm/30 min/room temperature
↓	Pellet
↓	

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Solubilisation (O/N - 4° C.)	Buffer: + 660 ml 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Reduction (4H - room temperature - in the dark)	+0.2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
reaction with iodoacetamide (½ h - room temperature - in the dark)	+0.25 M Iodoacetamide (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni <sup>++</sup> -NTA-Agarose (Qiagen - 60 ml of resin)	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea 3) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 35 mM Imidazol Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 0.5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm Dilution buffer: 20 mM Borate pH 8.5 - 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 30 ml of resin)	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: Equilibration buffer Elution buffer: 20 mM Borate pH 8.5 - 400 mM NaCl - 6.0 M Urea
↓	
Concentration	up to 1.5 mg/ml 10 kDa Omega membrane (Filtron)
↓	
Dialysis (O/N - 4° C.)	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - 0.5 M Arginin
↓	
Sterile filtration	Millex GV 0.22 µm → Level of purity estimated by SDS-PAGE as shown in FIG. 7 (Daiichi Silver Staining, Coomassie blue G250, Western blotting): After dialysis and sterile filtration steps: >95% → Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA) 48 mg of reduced Tat-his protein are purified from 160 g of recombinant <i>Pichia pastoris</i> cells (wet weight) or 2 L of Dyno-mill homogenate OD 66.

### Example 7

#### Purification of oxidized Tat-his protein (*Pichia pastoris*)

[0166] The purification scheme has been developed from 74 g of recombinant *Pichia pastoris* cells (wet weight) or 1 L Dyno-mill homogenate OD60. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.

---

74 g of <i>Pichia pastoris</i> cells	
↓	
Homogenization	Buffer: +1 L 50 mM PO <sub>4</sub> pH 7.0 - 5 mM Pefabloc final OD: 60
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	

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Dyno-mill Pellet	
↓	
Wash (1 h - 4° C.)	Buffer: +1 L 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 1% Empigen
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4° C.)	Buffer: +330 ml 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Immobilized metal ion affinity chromatography on Ni <sup>++</sup> -NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea 3) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 35 mM Imidazol Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 0.5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm Dilution buffer: 20 mM Borate pH 8.5 - 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 15 ml of resin)	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 20 mM Borate pH 8.5 - 400 mM NaCl - 6.0 M Urea Elution buffer: 20 mM Piperazine pH 11.0 - 2 M NaCl - 6 M Urea
↓	
Concentration	up to 1.5 mg/ml 10 kDa Omega membrane (Filtron)
↓	
Dialysis (O/N - 4° C.)	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - 0.5 M Arginin
↓	
Sterile filtration	Millex GV 0.22 µm
→ Level of purity estimated by SDS-PAGE as shown in FIG. 8 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):	
After dialysis and sterile filtration steps:	>95%
→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)	
19 mg of oxidized Tat-his protein are purified from 74 g of recombinant <i>Pichia</i> pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 60.	

---

### Example 8

#### Purification of SIV Reduced NEF-HIS Protein (*Pichia Pastoris*)

[0167] The purification scheme has been developed from 340 g of recombinant *Pichia pastoris* cells (wet weight) or 4 L Dyno-mill homogenate OD 100. The chromatographic steps are performed at room temperature. Between steps, Nef positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.

---

340 g of <i>Pichia pastoris</i> cells	
↓	
Homogenization	Buffer: 4 L 50 mM PO <sub>4</sub> pH 7.0 - PMSF 4 mM final OD: 100
↓	
Dyno-mill disruption (4 passes)	
↓	

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Centrifugation	JA10 rotor/9500 rpm/60 min/room temperature
↓	
Dyno-mill Pellet	
↓	
Solubilisation (O/N - 4° C.)	Buffer: +2.6 L 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor/9500 rpm/30 min/room temperature
↓	
Reduction (4 H - room temperature - in the dark)	+0.2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Reaction with iodoacetamide (½ h - room temperature - in the dark)	+0.25 M Iodoacetamide (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni <sup>++</sup> -NTA-Agarose (Qiagen - 40 ml of resin)	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 0.5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10 kDa Omega membrane (Filtron)
↓	
Gel filtration chromatography on Superdex 200 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea
↓	
Concentration	up to 1.5 mg/ml 10 kDa Omega membrane (Filtron)
↓	
Dialysis (O/N - 4° C.)	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - Empigen 0.3%
↓	
Sterile filtration	Millex GV 0.22 µm
→ Level of purity estimated by SDS-PAGE as shown in FIG. 9 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):	
After dialysis and sterile filtration steps:	>95%
→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)	
20 mg of SIV reduced Nef-his protein are purified from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L of Dyno-mill homogenate OD 100.	

---

### Example 9

#### Purification of HIV Reduced NEF-HIS Protein (*Pichia Pastoris*)

[0168] The purification scheme has been developed from 160 g of recombinant *Pichia pastoris* cells (wet weight) or 3 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps, Nef positive fractions are kept overnight in the cold room (+4° C.); for longer time, samples are frozen at -20° C.

---

160 g of <i>Pichia pastoris</i> cells	
↓	
Homogenization	Buffer: 3 L 50 mM PO <sub>4</sub> pH 7.0 - Pefabloc 5 mM final OD: 50
↓	
Dyno-mill disruption (4 passes)	
↓	

-continued

Freezing/Thawing	
↓	
Centrifugation	JA10 rotor/9500 rpm/60 min/room temperature
↓	
Dyno-mill Pellet	
↓	
Solubilisation (O/N - 4° C.)	Buffer: +1 L 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0M GuHCl
↓	
Centrifugation	JA10 rotor/9500 rpm/60 min/room temperature
↓	
Reduction (3 H - room temperature - in the dark)	+0.1 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Reaction with iodoacetamide (½ h - room temperature - in the dark)	+0.15 M Iodoacetamide (powder addition)/pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni <sup>++</sup> -NTA-Agarose (Qiagen - 10 ml of resin)	Equilibration buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea 3) 10 mM PO <sub>4</sub> pH 7.5 - 150 mM NaCl - 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM Citrate pH 6.0 - 150 mM NaCl - 6 M Urea - 0.5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10 kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex 200 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO <sub>4</sub> pH 7.5 - 150 nM NaCl - 6 M Urea
↓	
Dialysis (O/N - 4° C.)	Buffer: 10 mM PO <sub>4</sub> pH 6.8 - 150 mM NaCl - 0.5M Arginin
↓	
Sterile filtration	Millex GV 0.22 µm
→ Level of purity estimated by SDS-PAGE as shown in FIG. 10 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):	
After dialysis and sterile filtration steps:	>95%
→ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)	
20 mg of HIV reduced Nef-his protein are purified from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L of Dyno-mill homogenate OD 50.	

#### Example 10

##### Expression of SIV nef Sequence in *Pichia Pastoris*

[0169] In order to evaluate Nef and Tat antigens in the pathogenic SHIV challenge model, we have expressed the Nef protein of simian immunodeficiency virus (SIV) of macaques, SIV mac239 (Aids Research and Human Retroviruses, 6:1221-1231, 1990).

[0170] In the Nef coding region, SIV mac 239 has an in-frame stop codon after 92aa predicting a truncated product of only 10 kD. The remainder of the Nef reading frame is open and would be predicted to encode a protein of 263aa (30 kD) in its fully open form.

[0171] Our starting material for SIV mac239 nef gene was a DNA fragment corresponding to the complete coding sequence, cloned on the LX5N plasmid (received from Dr R. C. Desrosiers, Southborough, Mass., USA).

[0172] This SIV nef gene is mutated at the premature stop codon (nucleotide G at position 9353 replaces the original T nucleotide) in order to express the full-length SIV mac239 Nef protein.

[0173] To express this SIV nef gene in *Pichia pastoris*, the PHIL-D2-MOD

[0174] Vector (previously used for the expression of HIV-1 nef and tat sequences) was used. The recombinant protein is expressed under the control of the inducible alcohol oxidase (AOX1) promoter and the c-terminus of the protein is elongated by a Histidine affinity tail that will facilitate the purification.

##### 10.1 Construction of the Integrative Vector PRIT 14908

[0175] To construct pRIT 14908, the SIV nef gene was amplified by PCR from the pLX5N/SIV-NEF plasmid with primers SNEF1 and SNEF2.

## Solubility

PRIMER SNEF1:  
 (Seq ID No: 6)  
 5' ATCGTCCATG.GGTGGAGCTATT 3'  
NcoI

PRIMER SNEF2:  
 (Seq ID No: 7)  
 5' CGGCTACTAGTGCGAGTTCCCT 3'  
SpeI

[0176] The SIV nef DNA region amplified starts at nucleotide 9077 and terminates at nucleotide 9865 (Aids Research and Human Retroviruses, 6:1221-1231, 1990).

[0177] An NcoI restriction site (with carries the ATG codon of the nef gene) was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end.

[0178] The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI. Since one NcoI restriction site is present on the SIV nef amplified sequence (at position 9286), two fragments of respectively +200 bp and

[0179] ±600 bp were obtained, purified on agarose gel and ligated to PHIL-D2-MOD vector.

[0180] The resulting recombinant plasmid received, after verification of the nef amplified region by automated sequencing, the pRIT 14908 denomination.

10.2 Transformation of *Pichia Pastoris* Strain GS115(his4).

[0181] To obtain *Pichia pastoris* strain expressing SIV nef-His, strain GS115 was transformed with a linear NotI fragment carrying only the expression cassette and the HIS4 gene (FIG. 11). This linear NotI DNA fragment, with homologies at both ends with AOX1 resident *P.pastoris* gene, favors recombination at the AOX1 locus.

[0182] Multicopy integrant clones were selected by quantitative dot blot analysis.

[0183] One transformant showing the best production level for the recombinant protein was selected and received the Y1772 denomination.

[0184] Strain Y1772 produces the recombinant SIV Nef-His protein, a 272 amino acids protein which would be composed of:

[0185] Myristic acid

[0186] A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector.

[0187] 262 amino acids (aa) of Nef protein (starting at aa 2 and extending to aa 263, see FIG. 12)

[0188] A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector (FIG. 11—includes Seq ID No: 29).

[0189] One glycine and six histidines.

[0190] Nucleic and Protein sequences are shown in FIG. 12 (Seq ID Nos: 30 and 31).

## 10.3 Characterization of the Expressed Product of Strain Y1772.

## Expression Level

[0191] After 16 hours induction in medium containing 1% methanol as carbon source, abundance of the recombinant Nef-His protein, was estimated at 10% of total protein (FIG. 13, lanes 3-4).

[0192] Induced cultures of recombinant strain Y 1772 producing the Nef-His protein were centrifuged. Cell pellets were resuspended in breaking buffer, disrupted with 0.5 mm glass beads and the cell extracts were centrifuged. The proteins contained in the insoluble pellet (P) and in the soluble supernatant (S) were compared on a Coomassie Blue stained SDS-PAGE10%.

[0193] As shown in FIG. 13, the majority of the recombinant protein from strain Y1772 (lanes 3-4) is associated with the insoluble fraction.

[0194] Strain Y1772 which presents a satisfactory recombinant protein expression level is used for the production and purification of SIV Nef-His protein.

## Example 11

## Expression of GP120 in CHO

[0195] A stable CHO-K1 cell line which produces a recombinant gp120 glycoprotein has been established. Recombinant gp120 glycoprotein is a recombinant truncated form of the gp120 envelope protein of HIV-1 isolate W61D. The protein is excreted into the cell culture medium, from which it is subsequently purified.

## Construction of gp120 Transfection Plasmid pRIT13968

[0196] The envelope DNA coding sequence (including the 5' exon of tat and rev) of HIV-1 isolate W61D was obtained (Dr. Tersmette, CCB, Amsterdam) as a genomic gp160 envelope containing plasmid W61D (Nco-XbaI). The plasmid was designated pRIT13965.

[0197] In order to construct a gp120 expression cassette a stop codon had to be inserted at the amino acid glu 515 codon of the gp160 encoding sequence in pRIT13965 using a primer oligonucleotide sequence (DIR 131) and PCR technology. Primer DIR 131 contains three stop codons (in all open reading frames) and a Sall restriction site.

[0198] The complete gp120 envelope sequence was then reconstituted from the N-terminal BamH1-DraI fragment (170 bp) of a gp160 plasmid subclone pW61d env (pRIT13966) derived from pRIT13965, and the DraI-Sall fragment (510 bp) generated by PCR from pRIT13965. Both fragments were gel purified and ligated together into the *E. coli* plasmid pUC18, cut first by Sall (klenow treated), and then by BamH1. This resulted in plasmid pRIT13967. The gene sequence of the XmaI-Sall fragment (1580 bp) containing the gp120 coding cassette was sequenced and found to be identical to the predicted sequence. Plasmid RIT13967 was ligated into the CHO GS-expression vector pEE14 (Celltech Ltd., UK) by cutting first with Bell (klenow treated) and then by XmaI. The resulting plasmid was designated pRIT13968.

## Preparation of Master Cell Bank

[0199] The gp120-construct (pRIT13968) was transfected into CHO cells by the classical CaPO<sub>4</sub>-precipitation/glycerol shock procedure. Two days later the CHOK1 cells were subjected to selective growth medium (GMEM+methionine sulfoximine (MSX) 25 μM+Glutamate+asparagine+10% Foetal calf serum). Three chosen transfectant clones were further amplified in 175 m<sup>2</sup> flasks and few cell vials were stored at -80° C. C-env 23,9 was selected for further expansion.

[0200] A small prebank of cells was prepared and 20 ampoules were frozen. For preparation of the prebank and the MCB, cells were grown in GMEM culture medium, supplemented with 7.5% fetal calf serum and containing 50  $\mu$ M MSX. These cell cultures were tested for sterility and mycoplasma and proved to be negative.

[0201] The Master Cell Bank CHOK1 env 23.9 (at passage 12) was prepared using cells derived from the premaster cell bank. Briefly, two ampoules of the premaster seed were seeded in medium supplemented with 7.5% dialysed foetal bovine serum. The cells were distributed in four culture flasks and cultured at 37° C. After cell attachment the culture medium was changed with fresh medium supplemented with 50  $\mu$ M MSX. At confluence, cells were collected by trypsinisation and subcultured with a 1/8 split ratio in T-flasks—roller bottle—cell factory units. Cells were collected from cell factory units by trypsinisation and centrifugation. The cell pellet was resuspended in culture medium supplemented with DMSO as cryogenic preservative. Ampoules were prelabelled, autoclaved and heat-sealed (250 vials). They were checked for leaks and stored overnight at -70° C. before storage in liquid nitrogen.

#### Cell Culture and Production of Crude Harvest

[0202] Two vials from a master cell bank are thawed rapidly. Cells are pooled and inoculated in two T-flasks at 37° $\pm$ 1° C. with an appropriate culture medium supplemented with 7.5% dialysed foetal bovine (FBS) serum. When reaching confluence (passage 13), cells are collected by trypsinisation, pooled and expanded in 10 T-flasks as above. Confluent cells (passage 14) are trypsinised and expanded serially in 2 cell factory units (each 6000 cm<sup>2</sup>; passage 15), then in 10 cell factories (passage 16). The growth culture medium is supplemented with 7.5% dialysed foetal bovine (FBS) serum and 1% MSX. When cells reach confluence, the growth culture medium is discarded and replaced by “production medium” containing only 1% dialysed foetal bovine serum and no MSX. Supernatant is collected every two days (48 hrs-interval) for up to 32 days. The harvested culture fluids are clarified immediately through a 1.2-0.22  $\mu$ m filter unit and kept at -20° C. before purification.

#### Example 12

##### Purification of HIV GP 120 (W61D CHO) from Cell Culture Fluid

[0203] All purification steps are performed in a cold room at 2-8° C. pH of buffers are adjusted at this temperature and are filtered on 0.2  $\mu$ m filter. They are tested for pyrogen content (LAL assay). Optical density at 280 nm, pH and conductivity of column eluates are continuously monitored.

###### (i) Clarified Culture Fluid

[0204] The harvested clarified cell culture fluid (CCF) is filter-sterilized and Tris buffer, pH 8.0 is added to 30 mM final concentration. CCF is stored frozen at -20° C. until purification.

###### (ii) Hydrophobic Interaction Chromatography

[0205] After thawing, ammonium sulphate is added to the clarified culture fluid up to 1 M. The solution is passed overnight on a TSK/TOYOPEARL-BUTYL 650 M (TOSOHAS) column, equilibrated in 30 mM Tris buffer-pH 8.0-1

M ammonium sulphate. Under these conditions, the antigen binds to the gel matrix. The column is washed with a decreasing stepwise ammonium sulphate gradient. The antigen is eluted at 30 mM Tris buffer-pH 8.0-0.25 M ammonium sulphate.

###### (iii) Anion-Exchange Chromatography

[0206] After reducing the conductivity of the solution between 5 and 6 mS/cm, the gP120 pool of fractions is loaded onto a Q-sepharose Fast Flow (Pharmacia) column, equilibrated in Tris-saline buffer—pH 8.0. The column is operated on a negative mode, i.e. gP120 does not bind to the gel, while most of the impurities are retained.

###### (iv) Concentration and Diafiltration by Ultrafiltration

[0207] In order to increase the protein concentration, the gP120 pool is loaded on a FILTRON membrane “Omega Screen Channel”, with a 50 kDa cut-off. At the end of the concentration, the buffer is exchanged by diafiltration with 5 mM phosphate buffer containing CaCl<sub>2</sub> 0.3 mM, pH 7.0. If further processing is not performed immediately, the gP120 pool is stored frozen at -20° C. After thawing the solution is filtered onto a 0.2  $\mu$ M membrane in order to remove insoluble material.

###### (v) Chromatography on Hydroxyapatite

[0208] The gP120 UF pool is loaded onto a macro-Prep Ceramic Hydroxyapatite, type II (Biorad) column equilibrated in 5 mM phosphate buffer+CaCl<sub>2</sub> 0.3 mM, pH 7.0. The column is washed with the same buffer. The antigen passes through the column and impurities bind to the column.

###### (vi) Cation Exchange Chromatography

[0209] The gP120 pool is loaded on a CM/TOYOPEARL-650 S (TOSOHAS) column equilibrated in acetate buffer 20 mM, pH 5.0. The column is washed with the same buffer, then acetate 20 mM, pH 5.0 and NaCl 10 mM. The antigen is then eluted by the same buffer containing 80 mM NaCl.

###### (vii) Ultrafiltration

[0210] In order to augment the virus clearance capacity of the purification process, an additional ultrafiltration step is carried out. The gP120 pool is subjected to ultrafiltration onto a FILTRON membrane “Omega Screen Channel”, cut-off 150 kDa. This pore-size membrane does not retain the antigen. After the process, the diluted antigen is concentrated on the same type of membrane (Filtron) but with a cut-off of 50 kDa.

###### (viii) Size Exclusion Gel Chromatography

[0211] The gP120 pool is applied to a SUPERDEX 200 (PHARMACIA) column in order to exchange the buffer and to eliminate residual contaminants. The column is eluted with phosphate buffer saline (PBS).

###### (ix) Sterile Filtration and Storage

[0212] Fractions are sterilized by filtration on a 0.2  $\mu$ M PVDF membrane (Millipore). After sterile filtration, the purified bulk is stored frozen at -20° C. up to formulation. The purification scheme is summarized by the flow sheet below.

⇒ Level of purity of the purified bulk estimated by SDS-PAGE analysis (Silver staining/Coomassie Blue/Western Blotting) is ≥95%.
⇒ Production yield is around 2.5 mg/L CCF (according to Lowry assay) - Global purification yield is around 25% (according to Elisa assay)
⇒ Purified material is stable 1 week at 37° C. (according to WB analysis)
Purification of gp120 from culture fluid
Mark ✓ indicate steps that are critical for virus removal.
CLARIFIED CULTURE FLUID
↓
HYDROPHOBIC INTERACTION CHROMATOGRAPHY (BUTYL-TOYOPEARL 650 M)
↓
ANION EXCHANGE CHROMATOGRAPHY (NEGATIVE MODE) ✓ (Q-SEPHAROSE)
↓
50 KD ULTRAFILTRATION (CONCENTRATION AND BUFFER EXCHANGE)
↓
(STORAGE -20° C.)
↓
HYDROXYAPATITE CHROMATOGRAPHY (NEGATIVE MODE) (MACROPREP CERAMIC HYDROXYAPATITE II)
↓
CATION EXCHANGE CHROMATOGRAPHY (CM-TOYOPEARL 650 S)
↓
150 KD ULTRAFILTRATION (OMEGA MEMBRANES/FILTRON) ✓
↓
50 KD ULTRAFILTRATION (CONCENTRATION)
↓
SIZE EXCLUSION CHROMATOGRAPHY (SUPERDEX 200) ✓
STERILE FILTRATION
↓
PURIFIED BULK
STORAGE -20° C.

## Example 13

## Vaccine Preparation

[0213] A vaccine prepared in accordance with the invention comprises the expression products of one or more DNA recombinants encoding an antigen. Furthermore, the formulations comprise a mixture of 3 de-O-acylated monophosphoryl lipid A 3D-MPL and QS21 in an oil/water emulsion or an oligonucleotide containing unmethylated CpG dinucleotide motifs and aluminium hydroxide as carrier.

[0214] 3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria *Salmonella minnesota*.

[0215] Experiments have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral immunity and a T<sub>H1</sub> type of cellular immunity.

[0216] QS21: is a saponin purified from a crude extract of the bark of the Quillaja *Saponaria Molina* tree, which has a strong adjuvant activity: it induces both antigen-specific lymphoproliferation and CTLs to several antigens.

[0217] Experiments have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and T<sub>H1</sub> type cellular immune responses.

[0218] The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprises 5% squalene, 5% tocopherol, 2% Tween 80 and has an average particle size of 180 nm (see WO 95/17210).

[0219] Experiments performed have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

## Preparation of the Oil/Water Emulsion (2 Fold Concentrate)

[0220] Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5 g of DL alpha tocopherol and 5 ml of squalene are vortexed to mix thoroughly. 90 ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

## Preparation of Oil in Water Formulation.

[0221] Antigens (100 µg gp120, 20 µg Neffat, and 20 µg SIV Nef, alone or in combination) were diluted in 10 fold concentrated PBS pH 6.8 and H<sub>2</sub>O before consecutive addition of the oil in water emulsion, 3D-MPL (50 µg), QS21 (50 µg) and 1 µg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (250 µl for a dose of 500 µl).

[0222] All incubations were carried out at room temperature with agitation.

[0223] CpG oligonucleotide (CpG) is a synthetic unmethylated oligonucleotide containing one or several CpG sequence motifs. CpG is a very potent inducer of T<sub>H1</sub> type immunity compared to the oil in water formulation that induces mainly a mixed T<sub>H1</sub>/T<sub>H2</sub> response. CpG induces lower level of antibodies than the oil in water formulation and a good cell mediated immune response. CpG is expected to induce lower local reactogenicity.

Preparation of CpG Oligonucleotide Solution: CpG Dry Powder is Dissolved in H<sub>2</sub>O to give a Solution of 5 mg/ml CpG.

## Preparation of CpG Formulation.

[0224] The 3 antigens were dialyzed against NaCl 150 mM to eliminate the phosphate ions that inhibit the adsorption of gp120 on aluminium hydroxide.

[0225] The antigens diluted in H<sub>2</sub>O (100 µg gp120, 20 µg Neffat and 20 µg SIV Nef) were incubated with the CpG solution (500 µg CpG) for 30 min before adsorption on Al(OH)<sub>3</sub> to favor a potential interaction between the H is tail of Neftat and Nef antigens and the oligonucleotide (stronger immunostimulatory effect of CpG described when bound to the antigen compared to free CpG). Then were consecutively added at 5 min interval Al(OH)<sub>3</sub> (500 µg), 10 fold concentrated NaCl and 1 µg/ml thiomersal as preservative.

[0226] All incubations were carried out at room temperature with agitation.

## Example 14

## Immunization and SHIV Challenge Experiment in Rhesus Monkeys

## First Study

[0227] Groups of 4 rhesus monkeys were immunized intramuscularly at 0, 1 and 3 months with the following vaccine compositions:

Group 1:	Adjuvant 2	+ gp120		
Group 2:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 3:	Adjuvant 2		+ NefTat*	+ SIV Nef
Group 4	Adjuvant 6	+ gp120	+ NefTat	+ SIV Nef
Group 5	Adjuvant 2		+ NefTat	+ SIV Nef
Group 6	Adjuvant 2			

Adjuvant 2 comprises squalene/tocopherol/Tween 80/3D-MPL/QS21 and Adjuvant 6 comprises alum and CpG.

Tat\* represents mutated Tat, in which Lys41→Ala and in RGD motif Arg78→Lys and Asp80→Glu (Virology 235: 48-64, 1997).

[0228] One month after the last immunization all animals were challenged with a pathogenic SHIV (strain 89.6p). From the week of challenge (wk16) blood samples were taken periodically at the indicated time points to determine the % of CD4-positive cells among peripheral blood mononuclear cells by FACS analysis (FIG. 14) and the concentration of RNA viral genomes in the plasma by bDNA assay (FIG. 15).

## Results

[0229] All animals become infected after challenge with SV89.6p.

[0230] CD4-positive cells decline after challenge in all animals of groups 1, 3, 5 and 6 except one animal in each of groups 1 and 6 (control group). All animals in group 2 exhibit a slight decrease in CD4-positive cells and recover to baseline levels over time. A similar trend is observed in group 4 animals (FIG. 14).

[0231] Virus load data are almost the inverse of CD4 data. Virus load declines below the level of detection in 3/4 group 2 animals (and in the one control animal that maintains its CD4-positive cells), and the fourth animal shows only marginal virus load. Most of the other animals maintain a high or intermediate virus load (FIG. 15).

[0232] Surprisingly, anti-Tat and anti-Nef antibody titres measured by ELISA were 2 to 3-fold higher in Group 3 (with mutated Tat) than in Group 5 (the equivalent Group with non-mutated Tat) throughout the course of the study.

[0233] At week 68 (56 weeks post challenge) all animals from the groups that had received the full antigen combination (groups 2 and 4) were still alive, while most of the animals in the other groupshad to be euthanized due to AIDS-like symptoms. The surviving animals per group were:

Group 1:	2/4
Group 2:	4/4
Group 3:	0/4
Group 4	4/4
Group 5	0/4
Group 6	1/4

## CONCLUSIONS

[0234] The combination of gp120 and Neffat (in the presence of SIV Nef) prevents the loss of CD4-positive cells,

reduces the virus load in animals infected with pathogenic SHIV<sub>89.6p</sub>, and delays or prevents the development of AIDS-like disease symptoms, while gp120 or Neffat/SIV Nef alone do not protect from the pathologic consequences of the SHIV challenge.

[0235] The adjuvant 2 which is an oil in water emulsion comprising squalene, tocopherol and Tween 80, together with 3D-MPL and QS21 seems to have a stronger effect on the study endpoints than the alum/CpG adjuvant.

## Second Study

[0236] A second rhesus monkey SHIV challenge study was conducted to confirm the efficacy of the candidate vaccine gp120/Neffat+adjuvant and to compare different Tat-based antigens. The study was conducted by a different laboratory.

[0237] The design of the study was as follows.

[0238] Groups of 6 rhesus monkeys were immunized at 0, 4 and 12 weeks with injections i.m. and challenged at week 16 with a standard dose of pathogenic SHIV<sub>89.6p</sub>.

[0239] Group 1 is the repeat of Group 2 in the first study.

Group 1:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 2:	Adjuvant 2	+ gp120	+ Tat (oxidised)	
Group 3:	Adjuvant 2	+ gp120	+ Tat (reduced)	
Group 4	Adjuvant 2			

[0240] The follow-up/endpoints were again % CD4-positive cells, virus load by RT-PCR, morbidity and mortality

## Results

[0241] All animals except one in group 2 become infected after challenge with SHIV<sub>89.6p</sub>.

[0242] CD4-positive cells decline significantly after challenge in all animals of control group 4 and group 3, and in all but one animals of group 2. Only one animal in group 1 shows a marked decrease in CD4-positive cells. Unlike the animals from the first study, the monkeys in the second experiment display a stabilisation of CD4-positive cells at different levels one month after virus challenge (FIG. 16). The stabilisation is generally lower than the initial % of CD4-positive cells, but will never lead to a complete loss of the cells. This may be indicative of a lower susceptibility to SHIV-induced disease in the monkey population that was used for the second study. Nonetheless, a beneficial effect of the gp120/Neffat/SIV Nef vaccine and the two gp120/Tat vaccines is demonstrable. The number of animals with a % of CD4-positive cells above 20 is 5 for the vaccinated animals, while none of the control animals from the adjuvant group remains above that level.

[0243] Analysis of RNA plasma virus loads confirms the relatively low susceptibility of the study animals (FIG. 17). Only 2 of the 6 control animals maintain a high virus load, while the virus disappears from the plasma in the other animals. Thus, a vaccine effect is difficult to demonstrate for the virus load parameter.

## Conclusions

[0244] Analysis of CD4-positive cells indicates that the vaccine gp120/Neffat+adjuvant (in the presence of SIV Nef) prevents the drop of CD4-positive cells in most vaccinated animals. This is a confirmation of the result obtained in the first SHIV study. Due to the lack of susceptibility of the study animals, the virus load parameter could not be used to demonstrate a vaccine effect. Taken together, the combination of

gp120 and Tat and Nef HIV antigens provides protection against the pathologic consequences of HIV infection, as evidenced in a SHIV model.

[0245] The Tat alone antigens in combination with gp120 also provide some protection from the decline of CD4-positive cells. The effect is less pronounced than with the gp120/Nef/Tat/SIV Nef antigen combination, but it demonstrates that gp120 and Tat are able to mediate some protective efficacy against SHIV-induced disease manifestations.

[0246] The second SHIV challenge study was performed with rhesus monkeys from a source completely unrelated to the source of animals from the first study. Both parameters, % of CD4-positive cells and plasma virus load, suggest that the animals in the second study were less susceptible to SHIV-induced disease, and that there was considerably greater variability among the animals. Nonetheless, a beneficial effect on the maintenance of CD4-positive cells of the gp120/Nef/Tat/SIV Nef vaccine was seen with the experimental vaccine containing gp120/Nef/Tat and SIV Nef. This indicates that the vaccine effect was not only repeated in a separate study, but furthermore demonstrated in an unrelated monkey population.

#### Example 15

##### Generation of PMID Vectors for gp120 and Nef/Tat

[0247] Expression vectors were constructed as described below. Endotoxin free plasmid preps were prepared and used to transfect subconfluent 293T cell monolayers in 24 well tissue culture plates with 1 µg of DNA using Lipofectamine 2000. Samples were harvested 24 hours post transfection and examined by Western Blot to assess expression levels. (FIGS. 18 and 19)

[0248] Codon optimisation of gp120 resulted in a substantial increase of REV independent expression.

##### Generation of Plasmids

[0249] See also FIGS. 20-23

gp120:

[0250] Both wild type and optimised gp120 were compared.

Wild Type: (pgp120w)

[0251] PCR cloned from pRIT 13968 (see Example 1) using primers:

(Seq ID No: 32)  
g120w-5' GAATTCGGCCGCAATGAAAGTGAAGGAGACCG

(Seq ID No: 33)  
g120w-3' GAATTCTGGATCCTTATCTCTGCACCACTTTC

[0252] The gene was cloned into vector p7313-ie as a NotI-BamHI fragment and sequenced. A single base change (conservative) was found relative to the reference sequence, but this change was also found on sequencing pRIT 13968 (T1170C in gp120).

Codon Optimised (pgp120c)

[0253] The gene sequence was based on the gp120 sequence from pRIT 13968. This has a RSCU value of 0.297. Optimisation was performed using SynGene 2d, resulting in a RSCU value of 0.749. The sequence was split into 40 overlapping oligonucleotide, PCR assembled and recovered using the end primers. The gene was cloned into vector

p7313-ie as a NotI-BamHI fragment and sequenced. Restriction fragments from three initial clones were combined to generate a single correct clone.

Nef/Tat (pNTm and ptrNTm)

[0254] The gene for the Nef/Tat fusion protein was provided in plasmid pRIT15244. The plasmid pRIT 15244 is identical to pRIT 14913 previously described except that the His tail has been deleted. The Tat in this plasmid contains three mutations as previously described. The fusion contains full length Nef which has an immune modulatory function (Collins and Baltimore (1999)) that may be abrogated by N-terminal truncation. Therefore constructs were generated for both full length Nef/mutant Tat(pNTm) and truncated Nef/mutant Tat(ptrNTm), in which the first 65 amino acids of Nef were removed. These sequences were PCR amplified from pRIT15244 using primers:

5'Nef:	(Seq ID Nos: 34, 35 and 36 respectively) GAATTCGGCCGCCATGGTGGCAAGTGGTCAAAAAG
5'trNef:	GAATTCGGCCGCCATGGTGGGTTTCCAGTCACACG
3'Tat:	GAATTCTGGATCCTTATCTCTGCACCACTTTC

[0255] The genes were cloned into vector p7313-ie as NotI-BamHI fragments and sequenced.

##### Dual Expression Vectors: (pRIX1 and pRIX2)

[0256] The Nef/Tat and trNef/Tat expression cassettes were excised as ClaI-XbaI restriction fragments, and ligated into the ClaI and blunted Sse8387 I sites of the vector containing the codon optimised gp120 to provide single plasmids for expression of both proteins (pRIX1 and pRIX2 respectively).

##### Composition of Plasmid p7313-ie

[0257] The plasmid was constructed by replacing the beta-lactamase gene containing Eam11051-PstI fragment of pUC19 (available from Amersham Pharmacia Biotech UK Ltd., Amersham Place, Little Chalfont, Bucks, HP7 9NA) with an EcoRI fragment of pUC4K (Amersham-Pharmacia) containing the Kanamycin resistance gene, following blunt ending of both fragments using T4 DNA polymerase. The human Cytomegalovirus iE1 promoter/enhancer, Intron A (nucleotides-489 to +118 relative to the transcription start), was derived from plasmid JW4303 obtained from Dr Harriet Robinson, University of Massachusetts, and inserted into the SalI site of pUC19 as a XhoI-SalI fragment, incorporating the bovine growth hormone polyadenylation signal. Deletion of the 5' SalI-BanI fragment from the promoter generated the minimal promoter used in the vector (WO00/23592—Powderject Vaccines Inc.). HBV Surface antigen 3'UTR was derived from Hepatitis B Virus, serotype adw, in the vector pAM6 (Moriarty et al., Proc.Natl.Acad.Sci. USA, 78, 2606-10, 1981). pAM6 (pBR322 based vector) was obtained from the American Type Culture Collection, catalogue number ATCC 45020. The 3'UTR was inserted 5' to the polyadenylation signal as a 1.4 kb BamHI fragment, blunt ended for insertion to remove the BamHI sites. In a series of steps (including digestion with Bgl II, Klenow polymerase treatment with mung bean nuclease to remove overhang and further digestion with BstX I), modifications were made to the region between the 3'untranslated enhancer region of the HBV S gene and bGHpA signal to remove all open reading frames of greater than 5 codons between the X gene promoter and the bGHpA signal. This resulted in deletion of sequence encoding the translatable portion of the X protein (9 amino acids)

and the X gene start codon. The bovine growth hormone polyadenylation signal was substituted with the rabbit beta globin polyadenylation signal. The 5'non-coding and coding sequences of the S antigen were excised and replaced with an oligonucleotide linker to provide multiple cloning sites as shown to produce plasmid p7313-PL.

```
(Seq ID No: 37)
Hind---NotI--- EcoRV- -NdeI- -BamHI
AGCTTGCGGCGCTAGCGATATCGGTACCATATGTCGACGGATCC.....
....ACGCCGGCGATCGCTATAGCCATGGTCTACAGCTGGCTAGGCCGG
-NheI- -KpnI- -SalI- ANotI
```

[0258] This polylinker was further extended by insertion of an additional oligonucleotide linker between the KpnI and SalI sites:

```
(Seq ID No: 38)
AspI- -MunI- NaeI- NdeI-- BglII-
GTACCGGTCAATTGGCGCCGGCGCCATATGACGTCAAGATCTG---
----GCCAGTTAACCGCGGCCGCGGTATACTGCAGTCTAGAGAGCT
--AgiI- -NarI-- AatII- SalI
```

[0259] The ColE1 cer sequence was obtained from a subclone from plasmid pDAH212 from David Hodgeson (Warwick University) and amplified by PCR using primers to place EcoRI restriction sites at the ends of the sequence. The cer sequence was then inserted into the EcoRI site of p7313-PL to produce plasmid p7313-PLc. The sequence of the amplified cer was verified against the Genbank entry M11411.

[0260] The HBV 3'UTR sequence between the promoter and polyadenylation signal was removed by PCR amplification of the polyadenylation signal using primers:

[0261] sense: CCATGGATCCGATCTTTCCCTCT-GCC (Seq ID No: 39) antisense: GTTAGGGTGAAAAGCT-TCCGAGTGAGAGACAC (Seq ID No: 40)

[0262] The resulting product was cut with BamHI and XmnI and used to replace the corresponding fragment containing both the polyadenylation signal and the 3'UTR.

[0263] The Intron A sequence was removed from the plasmid by PCR amplification of the CMV promoter/enhancer using primers:

[0264] sense: GCTAGCCTGCAGGCTGACCGC-CCAACGAC (Seq ID No: 41) antisense: GTTCTC-CATCGCGGCCGACTCTGGCACGGGG (Seq ID No: 42)

[0265] The resulting product was cut with Sse8387 I and NotI, and inserted back into the Sse8387 I and NotI sites of the parental vector.

#### Example 16

##### Immunogenicity Studies

[0266] A mouse immunogenicity study may be performed to test the immunogenicity of gp120 and Neftat constructs delivered by particle mediated immunotherapeutic delivery (PMID) to show that the construct can generate an immune response in vivo. The DNA constructs may be used in combination with protein to determine whether there is an advantage of the combined approach compared to immunisation with the individual components. The aim is to detect and quantify cellular and humoral immune responses in mice following priming with protein and boosting with DNA (PMID) or priming with DNA (PMID) and boosting with protein.

[0267] The DNA is precipitated onto gold microparticles, which are used to coat the inner walls of a tefzel cartridge. PMID cartridges are prepared using a DNA loading rate of 2, which gives approximately 0.5 [[□]] µg DNA per cartridge.

[0268] Mice are immunised by PMID on the abdomen by providing two shots containing gp120 or Neftat. Following a PMID boost spleens are removed and the spleen cells are used to determine immunogenicity to individual constructs. Responses are evaluated using the IFN-[□]<sub>n</sub> ELIsport assay. In this assay peptides from gp120 and Nef are used to stimulate the spleen cells to secrete IFN-[□<sub>n</sub>], which is captured by an antibody based detection system. The measurement of humoral responses to gp120 and Nef are measured by ELISA.

##### gp120/Neftat Prime Boost Studies

[0269] The aim of the study is to detect and quantify cellular and humoral immune responses in mice following priming with protein and boosting with DNA (PMID) or priming with DNA (PMID) and boosting with protein.

##### Cartridge Preparation

[0270] Gene gun cartridges are suitably prepared using a DNA loading rate (DLR) of 2, which will give approximately 0.5 µg DNA per cartridge.

##### In vivo Immunogenicity

[0271] An experiment (Experiment I) may suitably be carried out as set out in the table below. The readouts for this experiment are carried out by IFN-[□]<sub>n</sub> ELIsport to Balb/c peptides from gp120 and Nef to measure the cellular responses and detection of antibodies to gp120 and Nef by ELISA for the humoral response.

---

##### Experiment I

Group	Mouse strain	Prime	Boost
A	Balb/c	PMID: gp120, 2 × 0.5 [[□]] µg	PMID: gp120, 2 × 0.5 [[□]] µg
	Female		
B	Balb/c	PMID: Neftat, 2 × 0.5 [[□]] µg	PMID: Neftat, 2 × 0.5 [[□]] µg
	Female		

-continued

---

Experiment I

Group	Mouse strain	Prime	Boost
C	Balb/c Female	PMID: Empty vector, 2 × 0.5 [[□]] µg	PMID: Empty vector, 2 × 0.5 [[□]] µg
D	Balb/c Female	Protein: gp120	Protein: gp120
E	Balb/c Female	Protein: NefTat	Protein: NefTat
H	Balb/c Female	Protein: Irrelevant protein	Protein: Irrelevant protein

---

n = 3

[0272] A further experiment (Experiment 11) may be carried out as follows:

---

Experiment II

Group	Mouse strain	Prime	Boost
A	Balb/c Female	PMID: gp120, 2 × 0.5 [[□]] µg	Protein: gp120
B	Balb/c Female	PMID: gp120, 2 × 0.5 [[□]] µg	PMID: gp120, 2 × 0.5 [[□]] µg
C	Balb/c Female	PMID: NefTat, 2 × 0.5 [[□]] µg	Protein: NefTat
D	Balb/c Female	PMID: NefTat, 2 × 0.5 [[□]] µg	PMID: NefTat, 2 × 0.5 [[□]] µg
E	Balb/c Female	PMID: gp120, 2 × 0.5 [[□]] µg NefTat, 2 × 0.5 [[□]] µg	Protein: gp120 + NefTat
F	Balb/c Female	PMID: gp120, 2 × 0.5 [[□]] µg NefTat, 2 × 0.5 [[□]] µg	PMID: gp120, 2 × 0.5 [[□]] µg NefTat, 2 × 0.5 [[□]] µg
G	Balb/c Female	PMID: Empty vector, 2 × 0.5 [[□]] µg	Protein: Irrelevant protein
H	Balb/c Female	Protein: gp120	PMID: gp120, 2 × 0.5 [[□]] µg
I	Balb/c Female	Protein: gp120	Protein: gp120
J	Balb/c Female	Protein: NefTat	PMID: NefTat, 2 × 0.5 [[□]] µg
K	Balb/c Female	Protein: NefTat	Protein: NefTat
L	Balb/c Female	Protein: gp120 + NefTat	PMID: gp120, 2 × 0.5 [[□]] µg NefTat, 2 × 0.5 [[□]] µg
M	Balb/c Female	Protein: gp120 + NefTat	Protein: gp120 + NefTat
N	Balb/c Female	Protein: Irrelevant protein	PMID: Empty vector, 2 × 0.5 [[□]] µg

---

n = 3

#### Sample Protocol

[0273] Female Balb/c mice are used. All mice are pre-bled (tail vein) before the start of the experiment.

[0274] Groups of 3 mice/group are culled after the primary immunisation and spleens are collected for enumeration of IFN-[[□]]γ-producing CD8 cells by ELIspot assay using gp120 and Nef K<sup>d</sup>-restricted peptides.

[0275] Groups of 3 mice/group are culled at a minimum of two time points after the secondary immunisation and cardiac blood samples are collected. These are analysed for antibodies to gp120 and Nef using ELISA assays. Spleens are also be

collected for enumeration of IFN-[[□]]γ-producing CD8 cells by ELIspot assay using gp120 and Nef K<sup>d</sup>-restricted peptides.

#### Immunological Assays

[0276] ELIspot Assays

[0277] Cellular immune responses are detected using Interferon-[[E]]<sub>y</sub> ELIspot assays. Splenocytes will be isolated from 3 mice per group at each selected time point (one post primary and a minimum of two post-boost), and incubated overnight with peptides to known CD8 epitopes (restricted to a K<sup>d</sup> (Balb/c) background) in plates coated with [[□]]α—Interferon-[[□]]γ. The splenocytes are lysed and plates

developed using a secondary  $[\alpha]^{[32]}-\text{Interferon}-[\gamma]^{[3]}-\text{antibody}$  and biotin-streptavidin amplification system.

#### ELISA Assays

[0278] Humoral responses are detected using standard antibody ELISA assays. 96-well flat-bottomed microtitre plates will be coated with protein and blocked. Serial dilutions of serum collected prior to immunisation and after boost are collected and incubated in the plates. The plates are developed after incubation with an anti-mouse antibody. Responses to gp120, Nef and Tat are analysed.

#### Example 17

##### Immunogenicity Studies

###### Protocol

[0279] Cartridges were prepared for particle mediated delivery using a gene gun using standard methods. A DNA loading rate of 2 which will give approximately 0.5  $\mu\text{g}$  DNA/cartridge was used.

[0280] F1 (C3H $\times$ Balb/c) mice were given a primary immunisation with either gp120 protein and Nef/Tat in adjuvant (administered via the intramuscular route) or with gp120 DNA, codon optimised and cloned into vector p7313-ie as described in Example 15, and a vector expressing a Nef fusion protein (using particle mediated delivery wherein the DNA is coated onto gold beads). The mice were boosted 23 days later either with protein in adjuvant (administered via the intramuscular route) or with DNA (using particle mediated delivery wherein the DNA is coated onto gold beads). Mice were culled 5 days later and spleens were collected. The splenocytes were harvested by teasing out, erythrocytes were lysed and splenocytes were washed and counted. Specialised ELIspot plates (coated with interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37° C./5% CO<sub>2</sub> in the presence of medium control gp120 E7 peptide or various nef peptides. The splenocytes were lysed and the plate developed using standard procedures to demonstrate the number of interferon-gamma secreting cells present.

#### CONCLUSION

[0281] Although the ELIspot assay was carried out using a sub-optimal concentration of gp120 E7 peptide, the results indicated that the most effective schedule was to prime mice

with protein in adjuvant (administered i.m.) and then boost with DNA (administered by particle mediated delivery). See FIG. 24. Similar results were obtained for nef, with the two peptides Nef 19 and 20 only being recognised by mice that had been primed with protein and boosted with DNA. The sequences for these two peptides are Nef 29: HIV-1 Bru (171-190) GMDDPEREVLEWRFDSRLAF (Seq ID No: 43) and Nef 20: HIV-1 Bru (181-200) EWRFDSRLAFHH-VARELHPE (Seq ID No: 44). See FIG. 25.

#### Example 18

##### Immunogenicity Studies

###### Protocol

[0282] For PMID immunisations (DNA) cartridges were prepared using standard methods. A DNA loading rate of 2, which will give approximately 0.5  $\mu\text{g}$  DNA/cartridge was used and each immunisation consisted of two shots. Protein was formulated in adjuvant comprising squalene/tocopherol/Tween 80/3D-MPL/QS21 just before use. Balb/c mice were given a primary immunisation of either gp120 protein in adjuvant (administered via the intramuscular route) or with gp120 codon optimised DNA prepared as described in Example 15 (using PMID). The mice were boosted 21 days later with either protein in adjuvant (administered via the intramuscular route) or with DNA (using PMID). Mice were culled 7 days later and spleens were collected. The splenocytes were harvested by teasing out the spleen cells and erythrocytes were lysed. The splenocytes were washed and counted. Specialised ELIspot plates (coated with interferon-gamma capture antibody and blocked) were used. Splenocytes were transferred to these plates and incubated overnight at 37° C./5% CO<sub>2</sub> in the presence of pools of gp120 15-mer peptides. The splenocytes were lysed and the plate developed using standard procedures to demonstrate the number of interferon-gamma secreting cells present. Results are shown in FIG. 26.

#### CONCLUSION

[0283] Three pools of gp120 15-mer peptides were recognised by mice that had been primed with protein and boosted with DNA. Responses to these three pools of gp120 15-mers were not detected in animals that had been primed with DNA and boosted with protein or immunised twice with either protein or DNA.

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ggagcaatca	caagtagcaa	tacagcagct	accaatgtcg	cttgtgcctg	gctagaagca	180
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ttagagtgg	ggtttgacag	ccgcctagca	tttcatcacg	tggcccgaga	gctgcacccg	600
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Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu  
 50 55 60  
 Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr

65           70           75           80  
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly

Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu

Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr

Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys  
130 135 140

Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu  
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Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro  
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Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His

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	30	

His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly	35	40
	45	

Arg Lys Lys Arg Arg Gln Arg Arg Pro Pro Gln Gly Ser Gln Thr	50	55
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Pro	Gly	Pro	Gly	Val	Arg	Tyr	Pro	Leu	Thr	Phe	Gly	Trp	Cys	Tyr	Lys
130									135						140

Leu	Val	Pro	Val	Glu	Pro	Asp	Lys	Val	Glu	Ala	Asn	Lys	Gly	Glu	
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Asn	Thr	Ser	Leu	Leu	His	Pro	Val	Ser	Leu	His	Gly	Met	Asp	Asp	Pro
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His	Val	Ala	Arg	Glu	Leu	His	Pro	Glu	Tyr	Phe	Lys	Asn	Cys	Thr	Ser
									195						205

Glu	Pro	Val	Asp	Pro	Arg	Leu	Glu	Pro	Trp	Lys	His	Pro	Gly	Ser	Gln
									210						220

Pro	Lys	Thr	Ala	Cys	Thr	Asn	Cys	Tyr	Cys	Lys	Lys	Cys	Cys	Phe	His
225									230						240

Cys	Gln	Val	Cys	Phe	Ile	Thr	Lys	Ala	Leu	Gly	Ile	Ser	Tyr	Gly	Arg
									245						255

Lys	Lys	Arg	Arg	Gln	Arg	Arg	Pro	Pro	Gln	Gly	Ser	Gln	Thr	His	
									260						270

Gln	Val	Ser	Leu	Ser	Lys	Gln	Pro	Thr	Ser	Gln	Ser	Arg	Gly	Asp	Pro
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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275	280	285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His		
290	295	300
<210> SEQ ID NO 14		
<211> LENGTH: 1029		
<212> TYPE: DNA		
<213> ORGANISM: Homo sapiens		
<400> SEQUENCE: 14		
atggatccaa aaaccttagc ccttcttta tttagcagctg gcgtactagc aggttgtagc	60	
agccattcat caaatatggc gaatacccaa atgaaatcag acaaaatcat tattgctac	120	
cgtggtgcta gcggttattt accagagcat acgttagaat cttaaagcact tgctttgca	180	
caacaggctg attattnaga gcaagattt gcaatgacta aggttgtcg tttagtgggt	240	
attcacgatc actttttaga tggcttgact gatgttgcca aaaaattccc acatcgatc	300	
cgtaaagatg gccgttacta tgtcatcgac ttacacctaa aagaattca aagtttagaa	360	
atgacagaaa actttgaaac catgggtggc aagtggtaa aaagttagtgg ggttgtggatgg	420	
cctactgtaa gggaaagaat gagacgagct gagccagcagc cagatggggg gggagcagca	480	
tctcgagacc tggaaaaaca tggagcaatc acaagtagca atacagcagc taccatgt	540	
gttttgtgcgt ggctagaagc acaagaggag gaggagggtgg gttttccagt cacacccatcg	600	
gtacaccttaa gaccaatgac ttacaaggca gctgttagatc tttagccactt tttaaaagaa	660	
aaggggggac tggaaaggcgt aatttactcc caacgaagac aagatacttc tggatctgtgg	720	
atctaccaca cacaaggcta cttccctgat tggcagaact acacaccagg gccagggggtc	780	
agatatccac tgaccttgg atggctgatc aagctgtac cagttgagcc agataaggta	840	
gaagaggccca ataaaggaga gaacaccaggc ttgttacacc ctgtgagccct gcatggaaat	900	
gtatgaccctg agagagaagt gtttagagtgg aggtttgaca gccgcctagc atttcatcac	960	
gtggccccag agctgcattcc ggagttacttc aagaactgca ctagtgccca ccatcaccat	1020	
caccattaa	1029	
<210> SEQ ID NO 15		
<211> LENGTH: 324		
<212> TYPE: PRT		
<213> ORGANISM: Homo sapiens		
<400> SEQUENCE: 15		
Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys Ser Asp		
1	5	10
15		
Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His		
20	25	30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu		
35	40	45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His		
50	55	60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His		
65	70	75
80		
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys		
85	90	95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Glv Glv		

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100	105	110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg		
115	120	125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg		
130	135	140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr		
145	150	155
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Val Gly		
165	170	175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala		
180	185	190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Leu Glu Gly		
195	200	205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr		
210	215	220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro		
225	230	235
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro		
245	250	255
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser		
260	265	270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu		
275	280	285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala		
290	295	300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly His His		
305	310	315
His His His His		

&lt;210&gt; SEQ ID NO 16

&lt;211&gt; LENGTH: 1290

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 16

atggatccaa aaacttttagc cctttcttta ttagcagctg gcgtactagc aggttgttagc	60
agccattcat caaatatggc gaatacccaa atgaaatcat acaaaatcat tattgctcac	120
cgtggtgcta gcggttattt accagagcat acgttagaat ctaaaggact tgcgtttgca	180
caacaggctg attatttaga gcaagattt acaatgacta aggatggtcg ttttagtggtt	240
attcacgatc actttttaga tggcttgact gatgttgcga aaaaattccc acatcgatcat	300
cgtaaagatg gccgttacta tgtcatcgac tttagcttta aagaaattca aagtttagaa	360
atgacagaaaa actttgaaac catgggtggc aagtggtaaa aagtagtgt ggttggatgg	420
cctactgtaa gggaaagaat gagacgagct gagccagcag cagatggggat gggagcagca	480
tctcgagacc tggaaaaaca tggagcaatc acaagtagca atacagcagc taccaatgt	540
gcttggcct ggctagaagc acaagaggag gaggaggtgg gtttccagt cacacccat	600
gtacctttaa gaccaatgac ttacaaggca gctgttagatc tttagccactt tttaaaagaa	660
aaggggggac tggaaaggct aattcactcc caacgaagac aagatatcct tgatctgtgg	720
atctaccaca cacaaggcta cttccctgat tggcagaact acacaccagg gccaggggtc	780

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agatatccac	tgaccttgg	atggtgctac	aagctagtagc	cagttgagcc	agataaggta	840
gaagaggcca	ataaaaggaga	gaacaccagc	ttgttacacc	ctgtgagcct	gcatggaatg	900
gatgaccctg	agagagaagt	gttagagtgg	aggtttgaca	gccgcctagc	atttcatcac	960
gtggcccgag	agctgcatcc	ggagtaactc	aagaactgca	ctagtgagcc	agtagatct	1020
agactagago	cctggaagca	tccaggaagt	cagcctaaaa	ctgcttgtac	caattgctat	1080
tgtaaaaagt	gttgcttca	ttgccaagtt	tgtttcataa	caaaagcctt	aggcatctcc	1140
tatggcagga	agaagcggag	acagcga	agacccctc	aaggcagtca	gactcatcaa	1200
gtttcttat	caaagcaacc	cacccc	tcccggggg	acccgacagg	cccgaaaggaa	1260
actagtggcc	accatcacca	tcaccattaa				1290

<210> SEQ ID NO 17

<211> LENGTH: 411

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Cys	Ser	Ser	His	Ser	Ser	Asn	Met	Ala	Asn	Thr	Gln	Met	Lys	Ser	Asp
1							5			10		15			

Lys	Ile	Ile	Ile	Ala	His	Arg	Gly	Ala	Ser	Gly	Tyr	Leu	Pro	Glu	His
	20					25					30				

Thr	Leu	Glu	Ser	Lys	Ala	Leu	Ala	Phe	Ala	Gln	Gln	Ala	Asp	Tyr	Leu
	35					40					45				

Glu	Gln	Asp	Leu	Ala	Met	Thr	Lys	Asp	Gly	Arg	Leu	Val	Val	Ile	His
	50					55				60					

Asp	His	Phe	Leu	Asp	Gly	Leu	Thr	Asp	Val	Ala	Lys	Lys	Phe	Pro	His
	65					70			75			80			

Arg	His	Arg	Lys	Asp	Gly	Arg	Tyr	Tyr	Val	Ile	Asp	Phe	Thr	Leu	Lys
	85					90				95					

Glu	Ile	Gln	Ser	Leu	Glu	Met	Thr	Glu	Asn	Phe	Glu	Thr	Met	Gly	Gly
	100					105					110				

Lys	Trp	Ser	Lys	Ser	Ser	Val	Val	Gly	Trp	Pro	Thr	Val	Arg	Glu	Arg
	115					120					125				

Met	Arg	Arg	Ala	Glu	Pro	Ala	Ala	Asp	Gly	Val	Gly	Ala	Ala	Ser	Arg
	130					135				140					

Asp	Leu	Glu	Lys	His	Gly	Ala	Ile	Thr	Ser	Ser	Asn	Thr	Ala	Ala	Thr
	145					150				155					160

Asn	Ala	Ala	Cys	Ala	Trp	Leu	Glu	Ala	Gln	Glu	Glu	Glu	Val	Gly	
	165					170					175				

Phe	Pro	Val	Thr	Pro	Gln	Val	Pro	Leu	Arg	Pro	Met	Thr	Tyr	Lys	Ala
	180					185				190					

Ala	Val	Asp	Leu	Ser	His	Phe	Leu	Lys	Glu	Lys	Gly	Leu	Glu	Gly	
	195					200				205					

Leu	Ile	His	Ser	Gln	Arg	Arg	Gln	Asp	Ile	Leu	Asp	Leu	Trp	Ile	Tyr
	210					215				220					

His	Thr	Gln	Gly	Tyr	Phe	Pro	Asp	Trp	Gln	Asn	Tyr	Thr	Pro	Gly	Pro
	225					230				235					240

Gly	Val	Arg	Tyr	Pro	Leu	Thr	Phe	Gly	Trp	Cys	Tyr	Lys	Leu	Val	Pro
	245					250					255				

Val	Glu	Pro	Asp	Lys	Val	Glu	Glu	Ala	Asn	Lys	Gly	Glu	Asn	Thr	Ser
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260	265	270	
Leu Leu His Pro Val Ser	Leu His Gly Met Asp Asp	Pro Glu Arg Glu	
275	280	285	
Val Leu Glu Trp Arg Phe Asp Ser Arg	Leu Ala Phe His His	Val Ala	
290	295	300	
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu Pro Val			
305	310	315	320
Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr			
325	330	335	
Ala Cys Thr Asn Cys Tyr Cys Lys Cys Cys Phe His Cys Gln Val			
340	345	350	
Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg			
355	360	365	
Arg Gln Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser			
370	375	380	
Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr Gly Pro			
385	390	395	400
Lys Glu Thr Ser Gly His His His His His			
405	410		

&lt;210&gt; SEQ ID NO 18

&lt;211&gt; LENGTH: 981

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapien

&lt;400&gt; SEQUENCE: 18

atggatccaa gcagccattc atcaaatacg ggcataccg aaatgaaatc agacaaaatc	60
attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca	120
cttcgttttgc cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatgg	180
cgttttagtgg ttattcacga tcactttta gatggcttgc ctgtatgtgc gaaaaatttc	240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt	300
caaagttagt aaatgacaga aaaccttggaa accatgggtg gcaagtggc aaaaagttagt	360
gtgggtggat ggctactgt aaggaaaga atgagacgag ctgagccagc agcagatgg	420
gtggggcagc catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca	480
gctaccatg ctgcttgctc ctggctagaa gcacaagagg aggaggagg gggtttcca	540
gtcacacctc aggtacctt aagaccaatg acttacaagg cagctgtaga tcttagccac	600
tttttaaaag aaaagggggg actggaaaggg ctaatttactt cccaaacgaa acaagatatc	660
cttgcgttgtt ggtatctacca cacacaaggc tacttccctg attggcagaa ctacacacca	720
gggccagggg tcagatatcc actgacctt ggtatggctc acaagctagt accagtttag	780
ccagataagg tagaagaggc caataaaggc gagaacacca gcttgcata ccctgtgagc	840
ctgcgtggaa tggatgaccc tgagagagaa gtgttagagt ggagggttgc cagccgccta	900
gcatttcatc acgtggcccg agagctgcat ccggagttact tcaagaactg cactagtggc	960
caccatcacc atcaccatta a	981

&lt;210&gt; SEQ ID NO 19

&lt;211&gt; LENGTH: 326

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapien

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<400> SEQUENCE: 19

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Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1           5          10          15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
 20          25          30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
 35          40          45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
 50          55          60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
 65          70          75          80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
 85          90          95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100         105         110

Gly Gly Lys Trp Ser Lys Ser Val Val Gly Trp Pro Thr Val Arg
115         120         125

Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
130         135         140

Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145         150         155         160

Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
165         170         175

Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
180         185         190

Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Leu
195         200         205

Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
210         215         220

Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225         230         235         240

Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
245         250         255

Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
260         265         270

Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
275         280         285

Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
290         295         300

Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly
305         310         315         320

His His His His His
 325

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<210> SEQ ID NO 20

<211> LENGTH: 1242

<212> TYPE: DNA

<213> ORGANISM: Homo sapien

<400> SEQUENCE: 20

atggatccaa gcagccattc atcaaatatg gcgataaccc aaatgaaatc agacaaaatc 60

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attattgctc accgtggtgc tagcggttat ttaccagac atacgtaga atctaaagca	120
cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatgg	180
cgttttagtgg ttattcacga tcactttta gatggcttga ctgatgttc gaaaaattc	240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaaatt	300
caaagttag aaatgacaga aaactttgaa accatgggtg gcaagtggc aaaaagttagt	360
gtgggttggat ggcctactgt aaggggaaaga atgagacgag ctgagccagc agcagatggg	420
gtgggagcag catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca	480
gctaccatg ctgcttgc ctggctagaa gcacaagagg aggaggaggt gggtttcca	540
gtcacacctc aggtacctt aagaccaatg acttacaagg cagctgtaga tcttagccac	600
tttttaaaag aaaagggggg actggaaggg ctaattcact cccaacaa gcaagatatc	660
cttgatctgt gnatctacca cacacaaggc tacttccctg attggcagaa ctacacacca	720
gggcaggggg ttagatatcc actgacctt ggtgggtgc acaagctagt accagtttag	780
ccagataagg tagaaagggc caataaaaggaa gagaacacca gcttgcataa ccctgtgagc	840
ctgcatggaa tggatgaccc tgagagagaa gtgttagagt ggagggttga cagccgccta	900
gcatttcatac acgtggcccg agagctgcat ccggagact tcaagaactg cactagttag	960
ccagtagatc ctagactaga gccctgaaag catccaggaa gtcagcctaa aactgcttg	1020
accaattgct attgtaaaaa gtgttgcctt cattgccaag tttgttcat aacaaaagcc	1080
ttaggcattc cctatggcag gaagaagcgg agacagcgc acgacactcc tcaaggcagt	1140
cagactcatac aagttctct atcaaagcaa cccacccccc aatcccgggg ggacccgaca	1200
ggcccgaaagg aaactagtgg ccaccatcac catcaccatt aa	1242

&lt;210&gt; SEQ ID NO 21

&lt;211&gt; LENGTH: 413

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapien

&lt;400&gt; SEQUENCE: 21

Met	Asp	Pro	Ser	Ser	His	Ser	Ser	Asn	Met	Ala	Asn	Thr	Gln	Met	Lys
1									10					15	

Ser	Asp	Lys	Ile	Ile	Ile	Ala	His	Arg	Gly	Ala	Ser	Gly	Tyr	Leu	Pro
			20				25						30		

Glu	His	Thr	Leu	Glu	Ser	Lys	Ala	Leu	Ala	Phe	Ala	Gln	Gln	Ala	Asp
			35				40						45		

Tyr	Leu	Glu	Gln	Asp	Leu	Ala	Met	Thr	Lys	Asp	Gly	Arg	Leu	Val	Val
							50						55		60

Ile	His	Asp	His	Phe	Leu	Asp	Gly	Leu	Thr	Asp	Val	Ala	Lys	Lys	Phe
								65					70		75

Pro	His	Arg	His	Arg	Lys	Asp	Gly	Arg	Tyr	Tyr	Val	Ile	Asp	Phe	Thr
								85					90		95

Leu	Lys	Glu	Ile	Gln	Ser	Leu	Glu	Met	Thr	Glu	Asn	Phe	Glu	Thr	Met
								100					105		110

Gly	Gly	Lys	Trp	Ser	Lys	Ser	Ser	Val	Val	Gly	Trp	Pro	Thr	Val	Arg
								115					120		125

Glu	Arg	Met	Arg	Arg	Ala	Glu	Pro	Ala	Ala	Asp	Gly	Val	Gly	Ala	Ala
								130					135		140

Ser	Arg	Asp	Leu	Glu	Lys	His	Gly	Ala	Ile	Thr	Ser	Ser	Asn	Thr	Ala
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145	150	155	160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu			
165	170	175	
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr			
180	185	190	
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu			
195	200	205	
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp			
210	215	220	
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro			
225	230	235	240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu			
245	250	255	
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn			
260	265	270	
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu			
275	280	285	
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His			
290	295	300	
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu			
305	310	315	320
Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro			
325	330	335	
Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys			
340	345	350	
Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys			
355	360	365	
Lys Arg Arg Gln Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln			
370	375	380	
Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr			
385	390	395	400
Gly Pro Lys Glu Thr Ser Gly His His His His His His His His			
405	410		

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<210> SEQ ID NO 22
<211> LENGTH: 288
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 22

atggagccag tagatcttag actagagccc tggaaagcata caggaagtca gcctaaaaact      60
gcttgtacca attgctattg taaaaagtgt tgctttcatt gccaagtttg tttcataaca      120
gctgccttag gcatctctta tggcaggaag aagcggagac agcgacgaag acctcctcaa      180
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc caaaggggag      240
ccgacaggcc cgaaggaaac tagtggccac catcaccatc accattaa      288

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<210> SEQ ID NO 23
<211> LENGTH: 95
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 23

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Met	Glu	Pro	Val	Asp	Pro	Arg	Leu	Glu	Pro	Trp	Lys	His	Pro	Gly	Ser
1								10							15
Gln	Pro	Lys	Thr	Ala	Cys	Thr	Asn	Cys	Tyr	Cys	Lys	Lys	Cys	Cys	Phe
								25							30
His	Cys	Gln	Val	Cys	Phe	Ile	Thr	Ala	Ala	Leu	Gly	Ile	Ser	Tyr	Gly
								35							45
Arg	Lys	Lys	Arg	Arg	Gln	Arg	Arg	Arg	Pro	Pro	Gln	Gly	Ser	Gln	Thr
								50							60
His	Gln	Val	Ser	Leu	Ser	Lys	Gln	Pro	Thr	Ser	Gln	Ser	Lys	Gly	Glu
								65							80
Pro	Thr	Gly	Pro	Lys	Glu	Thr	Ser	Gly	His	His	His	His	His	His	
								85							95

&lt;210&gt; SEQ ID NO 24

&lt;211&gt; LENGTH: 909

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Homo sapien

&lt;400&gt; SEQUENCE: 24

atgggtggca	agtggtaaaa	aagtagtgtg	tttggatggc	ctactgttaag	ggaaagaatg	60
agacgagctg	agccagcagc	agatggggtg	ggaggcagcat	ctcgagacct	ggaaaaacat	120
ggagcaatca	caagtagcaa	tacagcagct	accaatgctg	cttgcctg	gctagaagca	180
caagaggagg	aggaggtggg	ttttccagtc	acacctcagg	taccttaag	accaatgact	240
tacaaggcag	ctgttagatct	tagccactt	ttaaaagaaa	aggggggact	ggaagggcta	300
attcactccc	aacgaagaca	agatatcatt	gatctgttga	tctaccacac	acaaggctac	360
ttccctgatt	ggcagaacta	cacaccagg	ccaggggtca	gatatccact	gaccttttga	420
tggtgctaca	agctagtagacc	agttgagcca	gataaggtag	aagaggccaa	taaaggagag	480
aacaccagct	tgttacaccc	tgtgagctg	catggatgg	atgaccctga	gagagaagtg	540
ttagagtgga	ggtttgacag	ccgcctagca	tttcatcag	tggcccgaga	gctgcatecg	600
gagtagttca	agaactgcac	tagtgagcca	gtagatccta	gactagagcc	ctggaaagcat	660
ccaggaagtc	agcctaaaac	tgcttgtaacc	aattgttatt	gtaaaaagt	ttgttttcat	720
tgccaaagt	tttccataac	agctgcctt	ggcatctcct	atggcaggaa	gaagcggaga	780
cagcgacgaa	gacccctca	aggcagtcag	actcatcaag	tttctctatc	aaagcaaccc	840
acctccaaat	ccaaaggaga	gccgacaggc	ccgaaggaaa	ctagtggcca	ccatcaccat	900
caccattaa						909

&lt;210&gt; SEQ ID NO 25

&lt;211&gt; LENGTH: 302

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapien

&lt;400&gt; SEQUENCE: 25

Met	Gly	Gly	Lys	Trp	Ser	Lys	Ser	Ser	Val	Val	Gly	Trp	Pro	Thr	Val
1									10						15
Arg	Glu	Arg	Met	Arg	Arg	Ala	Glu	Pro	Ala	Ala	Asp	Gly	Val	Gly	Ala
								20							30
Ala	Ser	Arg	Asp	Leu	Glu	Lys	His	Gly	Ala	Ile	Thr	Ser	Ser	Asn	Thr
								35							45
Ala	Ala	Thr	Asn	Ala	Ala	Cys	Ala	Trp	Leu	Glu	Ala	Gln	Glu	Glu	

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50	55	60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr		
65	70	75
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly		
85	90	95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu		
100	105	110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr		
115	120	125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys		
130	135	140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu		
145	150	155
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro		
165	170	175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His		
180	185	190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser		
195	200	205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln		
210	215	220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His		
225	230	235
Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly Arg		
245	250	255
Lys Lys Arg Arg Gln Arg Arg Pro Pro Gln Gly Ser Gln Thr His		
260	265	270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu Pro		
275	280	285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His		
290	295	300

<210> SEQ ID NO 26  
<211> LENGTH: 57  
<212> TYPE: DNA  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 26

ttcgaaacca tggccgcggca ctagtggcca ccatcaccat caccattaac ggaattc 57

<210> SEQ ID NO 27  
<211> LENGTH: 9  
<212> TYPE: PRT  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 27

Thr Ser Gly His His His His His His  
1 5

<210> SEQ ID NO 28  
<211> LENGTH: 58  
<212> TYPE: DNA  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 28

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ttcgaaacca tggccgcgga ctagtggcca ccatcacat caccattaac gcgaattc 58

<210> SEQ ID NO 29  
<211> LENGTH: 9  
<212> TYPE: PRT  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 29

Thr	Ser	Gly	His	His	His	His	His	His
1								5

<210> SEQ ID NO 30  
<211> LENGTH: 819  
<212> TYPE: DNA  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 30

atgggtggag	ctatcccatt	gaggcggtcc	aggccgtctg	gagatctgcg	acagagactc	60
ttgcggcgc	gtggggagac	ttatggaga	ctcttagag	aggtgaaaga	tggatactcg	120
caatccccag	gaggattaga	caagggctt	agctcactct	cttgcggg	acagaaatac	180
aatcagggac	agtatatgaa	tactccatgg	agaaacccag	ctgaagagag	agaaaaattta	240
gcatacagaa	aacaaaatata	ggatgatata	gatgaggaag	atgatgactt	ggtaggggta	300
tcagtgggc	caaaaagtcc	cctaagaaca	atgagttaca	aattggcaat	agacatgtct	360
cattttataa	aagaaaaggg	gggactggaa	gggattttatt	acagtgcag	aagacataga	420
atcttagaca	tatacttaga	aaaggaagaa	ggcatcatac	cagattggca	ggattacacc	480
tcaggaccag	gaatttagata	cccaaagaca	tttggctggc	tatggaaatt	agtccctgtta	540
aatgtatcag	atgaggcaca	ggaggatgag	gagcattatt	taatgcattcc	agctcaaact	600
tcccagtgg	atgacccttg	gggagaggtt	ctagcatgga	agtttgatcc	aactctggcc	660
tacacttatg	aggcatatgt	tagataccca	gaagagtttgc	gaagcaagtc	aggectgtca	720
gaggaagagg	ttagaagaag	gctaaccgca	agaggccttc	ttaacatggc	tgacaagaag	780
gaaactcgca	ctagtggcca	ccatcacat	caccattaa			819

<210> SEQ ID NO 31  
<211> LENGTH: 272  
<212> TYPE: PRT  
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 31

Met	Gly	Gly	Ala	Ile	Ser	Met	Arg	Arg	Ser	Arg	Pro	Ser	Gly	Asp	Leu
1															15

Arg	Gln	Arg	Leu	Leu	Arg	Ala	Arg	Gly	Glu	Thr	Tyr	Gly	Arg	Leu	Leu
20															30

Gly	Glu	Val	Glu	Asp	Gly	Tyr	Ser	Gln	Ser	Pro	Gly	Gly	Leu	Asp	Lys
35															45

Gly	Leu	Ser	Ser	Leu	Ser	Cys	Glu	Gly	Gln	Lys	Tyr	Asn	Gln	Gly	Gln
50															60

Tyr	Met	Asn	Thr	Pro	Trp	Arg	Asn	Pro	Ala	Glu	Glu	Arg	Glu	Lys	Leu
65															80

Ala	Tyr	Arg	Lys	Gln	Asn	Met	Asp	Asp	Ile	Asp	Glu	Glu	Asp	Asp	Asp
85															95

Leu Val Gly Val Ser Val Arg Pro Lys Val Pro Leu Arg Thr Met Ser

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100	105	110	
Tyr Lys Leu Ala Ile Asp Met Ser His Phe Ile Lys Glu Lys Gly Gly			
115	120	125	
Leu Glu Gly Ile Tyr Tyr Ser Ala Arg Arg His Arg Ile Leu Asp Ile			
130	135	140	
Tyr Leu Glu Lys Glu Glu Gly Ile Ile Pro Asp Trp Gln Asp Tyr Thr			
145	150	155	160
Ser Gly Pro Gly Ile Arg Tyr Pro Lys Thr Phe Gly Trp Leu Trp Lys			
165	170	175	
Leu Val Pro Val Asn Val Ser Asp Glu Ala Gln Glu Asp Glu Glu His			
180	185	190	
Tyr Leu Met His Pro Ala Gln Thr Ser Gln Trp Asp Asp Pro Trp Gly			
195	200	205	
Glu Val Leu Ala Trp Lys Phe Asp Pro Thr Leu Ala Tyr Thr Tyr Glu			
210	215	220	
Ala Tyr Val Arg Tyr Pro Glu Glu Phe Gly Ser Lys Ser Gly Leu Ser			
225	230	235	240
Glu Glu Glu Val Arg Arg Arg Leu Thr Ala Arg Gly Leu Leu Asn Met			
245	250	255	
Ala Asp Lys Lys Glu Thr Arg Thr Ser Gly His His His His His His			
260	265	270	

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<210> SEQ ID NO 32
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 32

gaattcgcgg ccgcaatgaa agtgaaggag accag 35

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<210> SEQ ID NO 33
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 33

gaattcggat ccttatctct gcaccactct tc 32

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<210> SEQ ID NO 34
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 34

gaattcgcgg ccgccatggg tggcaagtgg tcaaaaag 38

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<210> SEQ ID NO 35
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

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&lt;400&gt; SEQUENCE: 35

gaattcgcgg cggccatggt gggtttcca gtcacacc

38

&lt;210&gt; SEQ ID NO 36

&lt;211&gt; LENGTH: 34

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 36

gaattcggat ccttattcct tcggggctgt cggg

34

&lt;210&gt; SEQ ID NO 37

&lt;211&gt; LENGTH: 45

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 37

agcttgcggc cgcttagcgat atcggtagcca tatgtcgacg gatcc

45

&lt;210&gt; SEQ ID NO 38

&lt;211&gt; LENGTH: 44

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 38

gtaccggta attggcgccg gcgcgcata tgacgtcaga tctg

44

&lt;210&gt; SEQ ID NO 39

&lt;211&gt; LENGTH: 28

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 39

ccatggatcc gatcttttc cctctgcc

28

&lt;210&gt; SEQ ID NO 40

&lt;211&gt; LENGTH: 32

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 40

gttagggta aaagcttccg agtgagagac ac

32

&lt;210&gt; SEQ ID NO 41

&lt;211&gt; LENGTH: 29

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 41

gctagctgc aggctgaccg cccaacgac

29

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<210> SEQ ID NO 42  
<211> LENGTH: 33  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 42

gttctccatc gcggccgcac tcttggcacg ggg 33

<210> SEQ ID NO 43  
<211> LENGTH: 56  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 43

gaattcgcgg ccgcaatgaa ggtcaaggag accagaaaaga actaccagca tctgtg 56

<210> SEQ ID NO 44  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 44

cctggaaatg ctgatgatct gctccgcgc cgagcagctg tgggtcaccg tctactacgg 60

<210> SEQ ID NO 45  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 45

ggccacgacc accctttct gcgcgagcga cgccaaggcc tacgacacgg aagtgcataa 60

<210> SEQ ID NO 46  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 46

ttgcgtgcct acggacccca acccccagga ggtggtgctg ggaaacgtga ccgagtactt 60

<210> SEQ ID NO 47  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 47

catggtgat cagatgcacg aggacatcat ctctctgtgg gaccagtccc tgaagccctg 60

<210> SEQ ID NO 48  
<211> LENGTH: 60  
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 48

ctgcgtgaca ctggactgtg acgacgtcaa caccaccaac agcactacca ccaccagcaa 60

<210> SEQ ID NO 49  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 49

tcggaagggc gagatcaaga actgctcatt caatatcacg acctcgatca gagacaagg 60

<210> SEQ ID NO 50  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 50

gtttataat ctcgatgtgg tccccatgaa cgacgacaat gccaccacca agaacaagac 60

<210> SEQ ID NO 51  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 51

cattcaactgc aacagcagcg tcatgacqca ggcctgcccc aaggtgtcct tcgaaccaat 60

<210> SEQ ID NO 52  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 52

ccctgccgga ttcgcgatcc tcaagtgtaa caacaagacc ttcgacggga agggcctgtg 60

<210> SEQ ID NO 53  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 53

gcagtgcacc catggcatcc gccccgttgt gaggcacccag ctgctgtga acgggtccct 60

<210> SEQ ID NO 54  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 54

gatccggctcg gacaacttca tggacaacac caagacaata atcggtccagg tgaacgagtc 60

<210> SEQ ID NO 55

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 55

ccggcctaacc aacaacaccc gtaaggcat ccacatcggt cctggacggg ctttatgc 60

<210> SEQ ID NO 56

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 56

cgacatccgg caggcccatt gcaacctctc ccgcgcggc tggataaca ccctgaagca 60

<210> SEQ ID NO 57

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 57

agagcacttt ggaaacaaga ccatcaagtt caatcagagt tctggcgag accccgagat 60

<210> SEQ ID NO 58

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 58

ctgcgggggc gagttttct actgcgatac gacacagctc ttcaactcca cctggAACGG 60

<210> SEQ ID NO 59

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 59

agagggaaac tccactatca ccctcccttg ccgcataaag cagatcatca acatgtggca 60

<210> SEQ ID NO 60

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 60

gtatgcccccc cccatcgcccc gccagatccg ctgcctctca aacatcaccc gcctgtgt 60

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<210> SEQ ID NO 61
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 61

cgagggcaac ggcacggaga acgagacgga gatcttcagg cccggcggcg gcgacatgag      60

<210> SEQ ID NO 62
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 62

gctgtacaag tacaagggtgg tgaagggtga gccgctcggc gtggccccc ca cccggggcaa      60

<210> SEQ ID NO 63
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 63

gaattcggat cctcatctc gcacgacgca gcgcttggcc cgggtgggg ccacg      55

<210> SEQ ID NO 64
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 64

accacccgt acttgtacag ctgcgtccgc cagttatccc tcatgtcgcc gccgccggc      60

<210> SEQ ID NO 65
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 65

ttctccgtgc cggtggccctc ggtgcccccg tctctggta gcagcaggcc ggtgtatgg      60

<210> SEQ ID NO 66
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 66

cccccgatgg gggggcata catggcctt cccacccctt gccacatgtt gatgtatgc      60

<210> SEQ ID NO 67
<211> LENGTH: 60
<212> TYPE: DNA
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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 67

gtgatagtggtttccctc tgtgttgttgc cccctcggtgc cgttccagggt ggagttgaag 60

<210> SEQ ID NO 68  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 68

tagaagaact cggccccggca gttgaaggag tgccgcacga tctcggggtc tccgccagaa 60

<210> SEQ ID NO 69  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 69

gtcttggtttc caaaatgtctc tctcagcttg atcacgatct gcttcagggt gttattccac 60

<210> SEQ ID NO 70  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 70

caatgggcct gcccggatgtc gccgatgtac ttgcggggcg catagaaggc ccgtccaggc 60

<210> SEQ ID NO 71  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 71

cgggtgttgt tgtaggcggc ggtacagtta atcgccacag actcggttcag ctggacgtat 60

<210> SEQ ID NO 72  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 72

atgaagttgt ccgaccggat caccacctcc tcctcagcca gggaccgggt cagcagcagc 60

<210> SEQ ID NO 73  
<211> LENGTH: 60  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 73

cggatgccat ggggtgactg caccgtgtcg acgttgggtgc acaggccctt cccgtcgaag 60

<210> SEQ ID NO 74

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 74

aggatgcgca atccggcagg ggcacagtaa tggatggga ttggttcgaa ggacaccttg 60

<210> SEQ ID NO 75

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 75

acgctgctgt tgcaagtgaat gagtctgaaa ttacgctcg tcttggttttt ggtggggca 60

<210> SEQ ID NO 76

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 76

accacatcga gattataaaaa cagcgctat tccttctgca ccttgcgtct gatcgaggtc 60

<210> SEQ ID NO 77

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 77

ttcttgatct cgcccttccg aatctctccg gtccagccgt tgctgggtgg ggttagtgctg 60

<210> SEQ ID NO 78

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 78

tcacagtcga gtgtcacgca gagaggcgctc agcttcacgc agggcttcag ggactggcc 60

<210> SEQ ID NO 79

<211> LENGTH: 60

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: primer

<400> SEQUENCE: 79

tctgtcatct gatccacat gttatttttc cacatgttga agtactcggt cacgtttccc 60

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<210> SEQ ID NO 80
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 80
ttggggtccg taggcacgca agcatgcgtc gcccacacgt tatgcacttc cgtgtcgtag      60

<210> SEQ ID NO 81
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 81
cagaagaggg tggtcgtggc ctccctccac acaggcacgc cgtagtagac ggtgaccac      60

<210> SEQ ID NO 82
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 82
cagatcatca gcattccag gagcatggtg ccccagcgcc acagatgctg gtatcttt      60

<210> SEQ ID NO 83
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 83
cggcgtgcct gtgttggagg agg      24

<210> SEQ ID NO 84
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 84
gacaagggtgc agaaggata cgcg      24

<210> SEQ ID NO 85
<211> LENGTH: 24
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25

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26

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&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

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26

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&lt;211&gt; LENGTH: 25

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

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&lt;210&gt; SEQ ID NO 95

&lt;211&gt; LENGTH: 25

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

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&lt;210&gt; SEQ ID NO 96

&lt;211&gt; LENGTH: 24

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 96

gttattccat tgtgctctac taag

24

&lt;210&gt; SEQ ID NO 97

&lt;211&gt; LENGTH: 24

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 97

ggcacaggcct gtgtcatgac tgag

24

&lt;210&gt; SEQ ID NO 98

&lt;211&gt; LENGTH: 24

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: primer

&lt;400&gt; SEQUENCE: 98

ctacttcttg tgggttgggg tctg

24

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1. A method for eliciting an immune response against human immunodeficiency virus (HIV), comprising:
  - a) administering a priming dose of an HIV gp120 protein and a fusion polypeptide comprising an HIV Nef protein and an HIV Tat protein; and
  - b) administering at least one boosting dose of a nucleic acid comprising an HIV Nef polynucleotide polynucleotide sequence and an HIV Tat polynucleotide sequence.
2. The method of claim 1, further comprising administering at least one boosting dose of a nucleic acid comprising an HIV gp120 polynucleotide sequence.
3. The method of claim 1, wherein at least one of the polynucleotide sequences is codon-optimised DNA.
4. The method of claim 1, wherein the nucleic acid comprising an HIV Nef polynucleotide polynucleotide sequence and an HIV Tat polynucleotide sequence encodes a fusion protein comprising a Nef polypeptide and a Tat polypeptide.
5. The method of claim 1, wherein at the nucleic acid of the at least one boosting dose is coated onto a particle suitable for propulsion into the skin.
6. The method of claim 5, wherein the particles are gold beads.
7. The method of claim 6, wherein the gold beads are 0.4-4.0  $\mu\text{m}$  in diameter.
8. The method of claim 6, wherein the gold beads are 0.6 to 2.0  $\mu\text{m}$  in diameter.
9. The method of claim 1, wherein administering the priming dose comprises administering a vaccine formulation comprising the HIV gp120 protein and the fusion polypeptide comprising an HIV Nef protein and an HIV Tat protein.
10. The method of claim 1, wherein the HIV gp120 and fusion polypeptides are adjuvanted by a combination of a monophosphoryl lipid A and a saponin derivative.
11. The method of claim 10, wherein the adjuvant is a combination of QS21 and 3D-MPL.
12. The method of claim 11, wherein the QS21 is quenched in cholesterol containing liposomes.
13. The method of claim 10, wherein the adjuvant is formulated as an oil in water emulsion.
14. The method of claim 1, further comprising administering a priming dose of at least one additional HIV regulatory or structural protein.
15. The method of claim 14, wherein the additional HIV regulatory or structural protein is selected from Rev, Vif, Vpu, Vpr and proteins encoded by the HIV gag or pol genes.
16. The method of claim 1, further comprising administering at least one boosting dose of a polynucleotide encoding an additional HIV regulatory or structural protein.
17. The method of claim 16, wherein the additional HIV regulatory or structural protein is selected from Rev, Vif, Vpu, Vpr and proteins encoded by the HIV gag or pol genes.
18. A plurality of particles coated with recombinant nucleic acid comprising an HIV Nef polynucleotide sequence and an HIV Tat polynucleotide sequence.
19. The particles of claim 18, wherein the recombinant nucleic acid encodes a fusion protein comprising a Nef polypeptide and a Tat polypeptide.
20. The particles of claim 18, wherein the recombinant nucleic acid further comprises a polynucleotide sequence that encodes an HIV gp120 protein.
21. The particles of claim 18, wherein the recombinant nucleic acid encodes at least one polynucleotide sequence that is optimized for expression in human cells.
22. The particles of claim 18, wherein the recombinant nucleic acid further comprises an HCMV IE1 promoter.
23. The particles of claim 18, wherein the recombinant nucleic acid comprises the vector P7313.
24. A device comprising the plurality of particles of claim 18.

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