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(54) Titre : GENES ENV DE SYNTHÈSE VIH-1 ET VIH-2 POUR L'OPTIMISATION DE L'EXPRESSION DES BACTÉRIES DESTINÉES AUX IMMUNO-ESSAIS DES ANTICORPS ANTI-VIH

(54) Title: SYNTHETIC HIV-1 AND HIV-2 ENVELOPE GENES THAT LEAD TO OPTIMIZED EXPRESSION IN BACTERIA FOR USE IN HIV ANTIBODY IMMUNOASSAYS

(57) **Abrégé/Abstract:**

A novel synthetic gene production method, encoding unique HIV-1 and HIV-2 envelope proteins and their fragments using an E. Coli favorite codon, resulting in high-level expression of these proteins in E. coli either as individual proteins or in fusion with other proteins. The HIV-1 and HIV-2 envelope protein thus expressed in E. coli can be used to detect HIV antibody in in vitro diagnostic devices.



ABSTRACT

A novel synthetic gene production method, encoding unique HIV-1 and HIV-2 envelope proteins and their fragments using an E. Coli favorite codon, resulting in high-level expression of these proteins in E. coli either as individual proteins or in fusion with other proteins. The HIV-1 and HIV-2 envelope protein thus expressed in E. coli can be used to detect HIV antibody in *in vitro* diagnostic devices.

DESCRIPTION

1. Background of the Invention

The present invention relates to recombinant HIV (Human Immunodeficiency Virus) antigens. Recombinant antigens derived from the molecular cloning and expression in a heterologous expression system of the synthetic DNA sequences of the various HIV antigens can be used as reagents for the detection of antibodies in body fluids from individuals exposed to various HIV isolates.

The nucleotide sequence of the proviral genome has been determined for several HIV isolates, including HIV-1 strains HTLV-III (HIV BH10) (Ratner et al., *Nature* (1985) 313:277); ARV-2 (Sanchez-Pescador et al., *Science* (1985) 227:484); LAV (Wain-Hobson et al., *Cell* (1985) 40:9); and CDC-451 (Desai et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:8380). The nucleotide sequence of the HIV-2 ROD isolate was reported by Guyader et al. (*Nature* (1987) 326:662).

HIV antigens have previously been obtained from the virus grown in tissue culture, or from a molecularly cloned genomic fragment expressed in heterologous hosts such as *Escherichia coli*. The tissue culture derived virus involves challenging, laborious, and bio-hazardous procedures of growing virus- infected cells and purifying proteins in highly sterile conditions under proper bio-containment facilities. The expression of molecularly cloned HIV genomic fragments overcomes the biohazard problem. Generally, an HIV genomic fragment from a single HIV isolate with mammalian codons is expressed in a heterologous system, such as bacteria or yeast, and is limited to the use of available restriction sites present in the viral genome for cloning and expression.

It has been difficult to obtain expression in heterologous systems of some of the HIV proteins, such as the HIV-1 envelope antigen gp41. Several researchers have tried deleting the hydrophobic regions of the HIV-1 gp41 to increase expression levels. UK Patent Application GB 2188639 discloses an HTLV-III gag/env gene protein wherein the env fragment of the DNA sequence deleted codons corresponds to the first hydrophobic region of the gp41 protein. U.S. Pat. No. 4,753,873 discloses a peptide fragment that is encoded by a nucleotide sequence wherein the nucleotides coding for a first and second hydrophobic region of HTLV-III gp41 are deleted.

Poor expression can be the result of many factors, including but not limited to the specific nucleic acid sequence of the gene to be expressed, the fact that the mammalian codons of the gene sequence to be expressed may not be efficiently transcribed and translated in a particular heterologous system, and the secondary structure of the transcribed messenger RNA. The use of synthetic DNA fragments can increase expression in heterologous systems.

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2. Summary of the Invention

Recombinant antigens which are derived from the molecular cloning and expression of synthetic DNA sequences in heterologous hosts are provided. Synthetic DNA sequences coding for the recombinant antigens of the invention are further provided. The synthetic DNA sequences selected for expression of various HIV antigens are based on the amino acid sequence of either a single isolate or several isolates, optimized for expression in *Escherichia coli* by specific codon selection. The synthetic DNA sequence gives higher expression of the particular antigen encoded. These antigens can be substituted for viral antigens derived from tissue culture or other recombinant technology procedures for use as diagnostic and therapeutic reagents.

The present invention can be utilized to synthesize HIV transmembrane envelope genes using bacterial favorite codons. Another aspect of the invention involves the linkage of sequences comprising different HIV epitopes to express with high efficiency.

3. Description of Tables and Drawings

FIG. 1 illustrates the alignment of the BLHIV-21 gene amino acid sequence with the sequences of the four HIV different isolates of HTLV-III (HIV BH10), LAV-1, ARV-2/SF2 and CDC-451.

FIG. 2 illustrates the alignment of the BLHIV-21 gene DNA sequence with the sequences of the HIV isolate HTLV-III (HIV BH10).

FIG. 3 illustrates the assembly of 4 oligonucleotides to form the synthetic HIV-1 gp41 part 1, and its cloning into pBLHIV-11, designated pBLHIV-12.

FIG. 4 illustrates the assembly of 2 oligonucleotides to form the synthetic HIV-1 gp41 part 2, and its cloning into pBLHIV-13, designated pBLHIV-16.

FIG. 5 is a schematic diagram of the cloning of HIV-1 envelope gene from pBLHIV-12 and pBLHIV-13 into vectors pBLHIV-11 to generate pBLHIV-16.

FIG. 6 is a schematic diagram of the cloning of HIV-1 envelope gene from pBLHIV-16 into vectors pBL-1 to generate pBLHIV-21.

FIG. 7 is a schematic diagram of the plasmid of pBLHIV-21, indicating restriction enzymes used to assemble the gene, pRpL promoter and amp marker.

FIG. 8 illustrates the DNA and amino acid sequence of the full length synthetic BLHIV-21 gene, indicating restriction enzymes used to assemble the gene.

FIG. 9 illustrates the alignment of the BLHIV-18 gene amino acid sequence with the sequences of the HIV isolates HIV-2 ROD.

FIG. 10 illustrates the alignment of the BLHIV-18 gene DNA sequence with the sequences of the HIV isolates HIV-2 ROD.

FIG. 11 illustrates the assembly of 4 oligonucleotides to form the synthetic HIV-2 envelope gene, and its cloning into pET41a, designated pBLHIV-3.

FIG. 12 is a schematic diagram of the cloning of HIV-2 envelope gene from pBLHIV-3 into expression vectors pMAL c2E to generate pBLHIV-18.

FIG. 13 is a schematic diagram of the plasmid of pBLHIV-18, indicating restriction enzymes used to assemble the gene.

FIG. 14 illustrates the DNA and amino acid sequence of fusion protein of MBP and the full length synthetic BLHIV-18 gene, indicating restriction enzymes used to assemble the gene.

Table 1: List of BioLytical HIV synthetic primers and oligonucleotides

Table 2: BLHIV-21 protein EIA result

Table 3: BLHIV-18 protein EIA result

Table 4: BLHIV-21 protein rapid assay result

Table 5: BLHIV-18 protein rapid assay result

4. Detailed Description of the Invention

Synthetic DNA fragments of the HIV genome can be synthesized based on their corresponding amino acid sequences. By comparing the particular region of interest between different isolates, a sequence can be selected which is different from any sequence that exists in nature, because the sequence is a compilation of the sequences from various isolates. For example, the synthetic HIV-1 envelope protein described in Example 1, is based on the amino acid sequence of four different HIV 1 isolates, namely, HTLV-III (HIV BH10), LAV-1, ARV-2 and CDC-451.

Other properties can be built into the sequence. For example, codons can be switched for optimal expression in bacteria or yeast, specific restriction sites can be introduced, and other restriction sites can be removed. In addition, the sequence should have specific restriction sites at both 5' and 3' ends of the fragment to facilitate cloning in a particular vector. Synthetic DNA fragments can be synthesized as follows: (1) select a unique protein sequence, (2) reverse translate to determine complementary DNA sequence, (3) optimize codons for bacterial or yeast expression, and (4) introduce and/or remove specific restriction sites, (5) combine different antigenic epitopes.

Sixty-one distinct nucleotide codons code for 20 amino acids giving rise to the degeneracy in the genetic code. Researchers have reported the frequencies of codons used in nucleic acids for both eukaryotic and prokaryotic organisms. (Grantham et al., Nucleic Acids Res. [1980] 9:r43; Gouy et al., Nucleic Acids Res. [1982] 10:7055; Sharp et al., Nucleic Acids Res. [1986] 14:7737.) Sequences from the entire E. coli genome, with examples of sequences from the chromosome, transposons, and plasmids, have been analyzed. These sequences code for structural proteins, enzymes and regulatory proteins. Correlation has been shown between the degree of codon bias within a particular gene and the level of gene expression.

It is preferred that the same codon triplet for each particular amino acid of the synthetic DNA sequence be used. However, alternative codon(s) can be used to add or delete a particular restriction site. The sequence should include unique restriction sites which can be used to delete a specific fragment or sequence, or substitute a particular sequence in case of an error in the original synthesis.

Vector systems which can be used include plant, bacterial, yeast, insect, and mammalian expression systems. It is preferred that the codons are optimized for expression in the system used. The proteins and polypeptides provided by the invention, which are cloned and expressed in heterologous systems, as described above, can be used for diagnostic and therapeutic purposes.

A preferred expression system pBL-1 utilizes the lambda pRpL vector system for HIV-1 envelope protein. This expression system is controlled by the temperature-sensitive lambda repressor c1857 and expressed at high levels. HIV-2 envelope gene is inserted into a pMAL vector down-stream from the male gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein. The vector uses the strong Ptac promoter to express large amount of the fusion protein. The fusion protein is then purified by one-step affinity purification specific for MBP.

Other proteins from any source, including bacterial, yeast, insect, plant or mammalian, can be used with the synthetic DNA fragments of the invention to produce fusion proteins. Those which are expressed efficiently in their respective expression systems are especially preferred because they can enhance the expression of the synthetic fragment of the fusion protein.

The synthetic DNA sequences of the present invention, derived from several HIV isolates and optimized for expression in *E. coli* provides continuous availability and uniformity of HIV antigen preparations which will recognize test sera from individuals exposed to genetically distinguishable variant HIV isolates. The recombinant antigen expression is very stable since *E. coli* codons have been used for its synthesis. Moreover, the insertion of specific restriction sites allows addition, deletion, or substitution in important antigenic epitopes in the coding sequences, a property difficult to achieve when naturally occurring HIV sequences are utilized for expression. Construction of synthetic genes also allows the addition of protein sequences at either amino- or carboxyl-terminus of the gene thereby allowing the development of novel reagents. For example, a fusion gene can be produced comprising a fusion between HIV epitopes and other protein. More specifically the HIV-2 envelope synthetic gene comprises the HIV-2 gp36 sequence and MBP sequence, which can be expressed at high levels in heterologous host systems such as *E. coli*.

The following examples further describe the invention. The examples are not intended to limit the invention in any manner.

4.1. General Methods

All restriction enzyme digestions were performed according to suppliers' instructions. At least 5 units of enzyme were used per microgram of DNA, and sufficient incubation was allowed to complete digestions of DNA. Plasmid isolations from *E. coli* strains used QIAprep Spin Miniprep Kit (Qiagen). Gel extraction of DNA fragments used MinElute Gel Extraction Kit (Qiagen) and UltraClean 15 DNA purification kit (Mo Bio). Standard buffers were used for T4 DNA ligase and Sequenase Version 2.0 T7 DNA Polymerase. Standard procedures were used for in vitro amplification of DNA by the Polymerase Chain Reaction, Gel electrophoresis of DNA, SDS-PAGE, and Preparation and transformation of competent *E. Coli* using calcium chloride (Maniatis et al., Molecular Cloning. A Laboratory Manual third edition [New York: Cold Spring Harbor, 2001]). Standard procedure was used for gene synthesis (Maniatis et al., Short Protocols in molecular biology Molecular third edition [John Wiley & Sons, Inc., 1995]).

4.2. Reagents and Enzymes

Media such as LB broth, IPTG (isopropyl-.beta.-D-thiogalactoside), glycerol, Dithiothreitol, SigmaMarker wide molecular weight range, Lysozyme, Anti-human IgG peroxidase conjugate and protein molecular weight standards were purchased from Sigma; Some restriction enzymes,

T4 DNA ligase were purchased from Invitrogen. Pefect DNA Markers, 0.05-10 kbp were purchased from Novagen; Molecular biology agerose, 30% Acrylamide/Bis Solution, N,N,N',N',-Tetramethylethylenediamine (TEMED) and sodium dodecylsulfate (SDS), Bio-Safe Coomassie Sitain were purchased from BioRad Laboratories. PfuUltra high-fidelity DNA polymerase was purchased from Stratagene. dNTP set, Sequenase Version 2.0 T7 DNA Polymerase and Klenow, Exonuclease-Free were purchased from GE Healthcare. SureBlue TMB microwell peroxidase substrate, Wash solution concentrate (20X) were purchased from KPL. Some restriction enzymes, Amylose resin were purchased from New Enhland BioLabs.

4.3. Host Cell Cultures. DNA Sources and Vectors

Subcloning efficiency DH5 α competent cells was purchased from Invitrogen. pET Blue -2 plasmid, pET expression system 29, pET expression system 41, E. coli K12 TB1 cells, pMAL-c2E vectors were purchased from New Enhland BioLabs. pBluescript II KS + purchased from Stratagene. pBL-1 vector was obtained from Chinese Center for Disease Control and Prevention.

5. Example 1

5.1. Construction of HIV-1 Clones

5.1.1. Synthesis and Cloning of HIV-1 gp41 Part 1

5.1.1.1. Synthesis of Subfragment-1

The subfragment-1 located downstream from pBLHIV-21, designated pBLSP-15 through pBLSP-16, were synthesized along with sequences containing a BamHI restriction site at the 5' end and a AlwN1 restriction site at the 3' end to facilitate molecular cloning. The subfragment encoding the N-terminal gp 41 amino acid sequence comprised two overlapping oligonucleotides with 15 bp complementary ends. When annealed, the end served as primers for the extension of the complementary strands. 1 ug of each of the two oligonucleotides was annealed. 10 U Sequenase was added and incubated 30 min at 30 .degree. C., then heated at 70 .degree. C. for 10 min to inactive the DNA polymerase. The reaction was subsequently digested with the BamHI and AlwN1 and checked by

electrophoresis on a 2% agarose gel. The subfragment 1 was extracted and purified by UltraClean 15 DNA purification kit (Mo Bio) for further cloning use.

5.1.1.2. Synthesis of Subfragment-2

The subfragment-2 located downstream from pBLHIV-21, designated BLSP-17 through BLSP-18, were synthesized along with sequences containing a AlwNI restriction site at the 5' end and a HindIII restriction site at the 3' end to facilitate molecular cloning of the individual subfragments. The subfragment encoding the gp 41 amino acid sequence comprised two overlapping oligonucleotides with 15 bp complementary ends. When annealed, the end served as primers for the extension of the complementary strands. 1 ug of each of the two oligonucleotides was annealed. 10 U Sequenase was added and incubated 30 min at 30 .degree. C., then heated at 70 .degree. C. for 10 min to inactivate the DNA polymerase. The reaction was subsequently digested with the HindIII and AlwNI and checked by electrophoresis on a 2% agarose gel. The subfragment-2 was extracted and purified by UltraClean 15 DNA purification kit (Mo Bio) for further cloning use.

5.1.1.3. Cloning of Subfragment-1 and Subfragment-2

The subfragment-1 with a BamHI restriction site at the 5' end and a AlwNI restriction site at the 3' end and the subfragment-2 with AlwNI restriction site at the 5' end and a HindIII restriction site at the 3' end were ligated into the vector pBLHIV-11 that had been digested with BamHI and HindIII and gel-isolated. The ligation product was used to transform DH5α competent cells (clone pBLHIV-12). The desired clone was identified by digestion with BamHI and HindIII and check on a 1.5% agarose gel. Miniprep DNA was prepared from an overnight culture of clone pBLHIV-12 and sequenced with the oligonucleotide primers T7 Promoter forward and T3 Promoter reverse by Cortec DNA Service Laboratories, Inc.

5.1.2. Synthesis and Cloning of HIV gp41 Part 2

5.1.2.1. Synthesis of Subfragment

The subfragment located downstream from pBLHIV-21, designated BLSP-22 through BLSP-23, were synthesized along with sequences containing a EcoRI restriction site at

the 5' end and a XhoI restriction site at the 3' end to facilitate molecular cloning of the individual subfragments. The subfragment encoding the gp 41 amino acid sequence comprised two overlapping oligonucleotides with 15 bp complementary ends. When annealed, the end served as primers for the extension of the complementary strands. 1 ug of each of the two oligonucleotides was annealed. 10 U Sequenase was added and incubated 30 min at 30 .degree. C., then heated at 70 .degree. C. for 10 min to inactive the DNA polymerase. The reaction was subsequently digested with the EcoRI and XhoI and checked by electrophoresis on a 2% agarose gel. The subfragment 1 was extracted and purified by UltraClean 15 DNA purification kit (Mo Bio) for further cloning use.

5.1.2.2. Cloning of Subfragment

The subfragment with a EcoRI restriction site at the 5' end and a XhoI restriction site at the 3' was ligated into the vector pBLHIV-1 that had been digested with EcoRI and XhoI and gel-isolated. The ligation product was used to transform DH5 α competent cells (clone pBLHIV-13). The desired clone was identified by digestion with EcoRI and XhoI and check on a 2% agarose gel. Miniprep DNA was prepared from an overnight culture of clone pBLHIV-13 and sequenced with the oligonucleotide primers T7 Promoter forward and T3 Promoter reverse by Cortec DNA Service Laboratories, Inc.

5.2. Construction of pBLHIV-16

pBLHIV-12 that had been digested with BamHI and EcoRI and pBLHIV-13 that had been digested with EcoRI and XhoI were ligated into the vector pBLHIV-11 that had been digested with BamHI and XhoI and gel-isolated. The ligation product was used to transform DH5 α competent cells (clone pBLHIV-16). The desired clone was identified by digestion with BamHI and XhoI and check on a 2% agarose gel. Miniprep DNA was prepared from an overnight culture of clone BHIV-16 and sequenced with the oligonucleotide primers T7 Promoter forward and T7 Terminator reverse by Cortec DNA Service Laboratories, Inc. pBLHIV-16 encoded the recombinant HIV-1 envelope protein that was consisted of S tag, 38 amino acids of env gp120 (HIV-1 group M), and 192 amino acids of env gp41 (HIV-1 group M), which HIV-1 env gp41 amino acids 6-30 were substituted with a single Serine, amino acids 169-193 were deleted.

5.3. Construction of pBLHIV-21

pBLHIV-21 encoded the recombinant HIV-1 envelope protein that was consisted of 38 amino acids of gp120 (HIV-1 group M), and 192 amino acids of gp41 (HIV-1 group M), which HIV-1 gp41 amino acids 6-30 were substituted with a single Serine, amino acids 169-193 were deleted. The construction of pBLHIV-21 was accomplished as follows.

5.3.1. PCR

A PCR reaction (50 ul) was set up with Stratagene PfuUltra high-fidelity DNA polymerase (2.5 U) and 1X buffer along with 10 mM each dNTP, 100 ng primer BLSP-26, 100 ng primer BLSP-27, and 1 ng pBLHIV-16 miniprep DNA. The reaction was incubated at 95 .degree. C. for 120 seconds then amplified with 30 cycles of 94 .degree. C. for 30 seconds; 50 .degree. C. for 45 seconds; 72 .degree. C. for 120 seconds. Then the BLSP-26/BLSP-27 PCR product was gel isolated.

5.3.2. Blunt Ends

A blunt ends reaction (20 ul) was set up with GE Healthcare Klenow, Exonuclease-Free (5 U) and 1X klenow buffer along with 25 mM each dNTP and 4 ug of each of the vector pBL-1, which was digested with EcoRI and gel-isolated and the BLSP-26/BLSP-27 PCR product, which was gel isolated. The reaction was incubated at room temperature for 15 minutes. Stop the reaction by heating to 75 .degree. C. for 10 minutes.

5.3.3. Cloning

The blunt ends BLSP-26/BLSP-27 PCR product was digested with Pst I and ligated into the blunt ends pBL-1 (EcoRI cut) had been digested with Pst I. The ligation product was used to transform DH5 α competent cells (clone pBLHIV-21). The desired clone was identified by digestion with EcoRI and Pst I, check on a 1 % agarose gel. Miniprep DNA was prepared from an overnight culture of clone pBLHIV-21 and sequenced with the oligonucleotide primers BLSP-35 forward and BLSP-36 reverse by Cortec DNA Service Laboratories, Inc.

6. Example 2

6.1. Procedure for Producing and Purifying BLHIV-21 Protein (HIV-1 Envelope Protein)

In this and the following examples, all chemicals were obtained from Sigma Chemical Corporation Canada unless otherwise noted.

6.1.1. Preparation of Required Solutions

An LB solution was prepared by dissolving 20 g LB Broth in 1 liter of MilliQ water, adjusting the pH to 7.2, and sterilizing by autoclaving. MilliQ water was obtained by filtration of water through a MilliQ water system (Millipore Corp.). The solution was allowed to cool and Ampicillin was added to bring the final concentration to 0.1 mg/ml.

6.1.1.1. Inclusion Body Wash Solution

PH 8.0 50 mM Tris-HCl buffer including 50 mM NaCl, 5 mM EDTA, 0.5 % Triton X100, 0.02 % NaN₃

6.1.1.2. Solubilization Buffer

PH 9.6 Carbonate buffer including 50 mM NaCl, 5 mM EDTA, 8 M Urea, 1mM DTT and 0.02 % NaN₃

6.1.2. Growth of and induction of pBLHIV-21 DH5 α

LB Broth (50 ml) was inoculated with 0.02 ml of a pBLHIV-21 DH5 α glycerol stock and then grown overnight in an incubator shaker at 37.degree. C. and 180 RPM. The 10 ml overnight culture was added to 200 ml LB solution containing 0.1 mg/ml Ampicillin in a 500 ml Flask and the mixture was grown until the optical density was 0.7 to 1.0 at 600 nm (3 hours). The temperature of the culture aliquot was quickly raised to 42.degree. C. The cultures were incubated at 42.degree. C. and 180 RPM for 4 hours. The cells were harvested by centrifugation in a Sorvall RC-5B centrifuge at 5000 RPM for 20 minutes. If the cells were not required for immediate purification, they were stored at -20.degree. C.

6.1.3. Extraction and Purification of Inclusion Bodies

The cell pellet was thawed and resuspended in a total of 20 ml of Novagen BugBuster protein extraction reagent (100 ml/1 L culture ratio) with 20 ul of 100 mM PMSF Stock, 40 ul of 1 M DTT and 10 ul of Benzonase (Novagen). Incubate the cell suspension on a sharking platform for 1 hour at room temperature. The solution was centrifuged in a Sorvall RC-5B at 9000 RPM in a GS-3 rotor for 50 min. Then the pellet was resuspended in a total

of 20 ml of Inclusion Body Wash Solution with 40 μ l of 1 M DTT, 30 μ l of 10 mM PMSF Stock and 560 μ l of 10 mg/ml Lysozyme and incubated at 37.degree. C. water bath for 1 hour.

In order to enable efficient resuspension, the solution was sonicated for 6 X 15 seconds (60 Sonic Dismembrator Fisher Scientific). The solution was then centrifuged in a Sorvall RC-5B at 9000 RPM in a GS-3 rotor for 50 min. The supernatant was collected and stored at 4.degree. C. until an aliquot (10 μ l) had been analyzed by 12% Polyacrylimide Gel Electrophoresis (PAGE). The pellet was resuspend in 10 ml of room temperature Solubilization Buffer. The solubilized recombinant antigens were clarified by centrifugation in a Sorvall RC-5B at 9000 RPM in a GS-3 rotor for 50 min. The crude HIV-1 envelope protein was stored at 4.degree. C until further process.

6.1.4. Purification of BLHIV-21 Protein by Electroelution

6.1.4.1.Preparation of required solutions

8 X Sample Buffer (SDS- reducing buffer): Add 1.0 ml 0.5 M Tris-HCL, PH 6.8, 3.2 ml Glyderol, 1.6 ml 10% SDS, 0.4 ml 0.5% Bromophenol Blue in 1.4 ml Deionized water.

Preparative SDS Polyacrylamide Gels (37 mm ID) were prepared as described in the BIO-RAD Model 491 Prep Cell Instruction Manual. 100 ml (10-11 cm) of a 12% Separating Gel solution and 20 ml (2.5 cm) of Stacking Gel solution were used to pour the gels. The Model 491 Prep Cell (BIO-RAD) was assembled and operated according to the Instruction Manual. Prior to loading, 1 ml of 8 X Sample Buffer and 400 μ l of 1 M DTT were added to 7 ml of crude HIV-1 envelope protein obtained in the previous step. The loading sample was heated to 90.degree. C. for 10 minutes (this was done by transferring small aliquots of the sample into Eppendorf tubes and placing them into a heat block). A total of 8 ml of sample was loaded onto each preparative gel. After loading, the Model 491 Prep Cells was operated under a constant power of 12 W, for 20 h, with an elution buffer flow rate of 24 ml/h (0.4 ml/min), and fractions were collected for 10 min.

Aliquots of sample were collected after the elution of the Bromophenol Blue. These aliquots were run on a 12% SDS polyacrylamide gel in order to determine which of them contained the 27.3 kd HIV-1 envelope protein. The fractions containing HIV-1 envelope protein were

then pooled and further concentrated. The protein concentration of this solution was determined and then 5 ug/lane of sample were run on a 12 % SDS-PAGE to check the purity. Applying HIV-1 envelope protein in the EIA assay and rapid assay would be discussed in Sample 5.

7. Example 3

7.1. Construction of HIV-2 Clones

7.1.1. Synthesis and Cloning of HIV-2 gp36

7.1.1.1. Synthesis of Subfragment-1

The subfragment-1, designated BLSP-9 through BLSP-10, were synthesized along with additional sequences containing a BamHI restriction site at the 5' end and a Nco I restriction site at the 3' end to facilitate molecular cloning. The subfragment encoding the N-terminal gp36 amino acid sequence comprised two overlapping oligonucleotides with 16 bp complementary ends. When annealed, the end served as primers for the extension of the complementary strands. 1 ug of each of the two oligonucleotides was annealed. 10 U Sequenase was added and incubated 30 min at 30 .degree. C., then heated the reaction at 70 .degree. C. for 10 min to inactive the DNA polymerase. The reaction was subsequently digested with the BamHI and Nco I and checked by electrophoresis on a 2% agarose gel. The subfragment 1 was further extracted and purified by UltraClean 15 DNA purification kit (Mo Bio) for cloning use.

7.1.1.2. Synthesis of Subfragment-2

The subfragment-2, designated BLSP-11 through BLSP-12, were synthesized along with additional sequences containing a Nco I restriction site at the 5' end and a EcoR I restriction site at the 3' end to facilitate molecular cloning of the individual subfragments. The subfragment encoding the gp36 amino acid sequence comprised two overlapping oligonucleotides with 15 bp complementary ends. When annealed, the end served as primers for the extension of the complementary strands. 1 ug of each of the two oligonucleotides was annealed. 10 U Sequenase was added and incubated 30 min at

30 .degree. C., then heated the reaction at 70 .degree. C. for 10 min to inactive the DNA polymerase. The reaction was subsequently digested with the Nco I and EcoR I and checked by electrophoresis on a 2 % agerose gel. The subframent-2 was further extracted and purified by UltraClean 15 DNA purification kit (Mo Bio) for cloning use.

7.1.1.3. Cloning of Subfragment-1 and Subfragment-2

The subfragment-1 with a BamHI restriction site at the 5' end and a Nco I restriction site at the 3' end and the subfragment-2 with Nco I restriction site at the 5' end and a EcoR I restriction site at the 3' end were ligated into the vector pET41a (Novagen) that had been digested with BamHI and EcoR I and gel-isolated. The ligation product was used to transform DH5 α competent cells (clone pBLHIV-3). The desired clone was identified by digestion with BamHI and EcoR I and check on a 1 % agerose gel. Miniprep DNA was prepared from an overnight culture of clone pBLHIV-3 and sequenced with the oligonucleotide primers T7 Terminator reverse by Cortec DNA Service Laboratories, Inc.

7.2. Construction of pBLHIV-18

The pBHIV-18 encodes the recombinant HIV-2 envelope protein that was consisted of fusion protein of MBP and 106 amino acids of HIV-2 gp36. The construction of pBLHIV-18 was accomplishes as follows.

7.2.1. PCR

A PCR reaction (50 ul) was set up with Stratagene PfuUltra high-fidelity DNA polymerase (2.5 U) and 1X buffer along with 10 mM each dNTP, 100 ng primer BLSP-24, 100 ng primer BLSP-28, and 1 ng pBLHIV-16 miniprep DNA. The reaction was incubated at 95 .degree. C. for 120 seconds then amplified with 30 cycles of 94 .degree. C. for 30 seconds; 54 .degree. C. for 45 seconds; 72 .degree. C. for 60 seconds. Then the BLSP-x/BLSP-XX PCR product was gel isolated.

7.2.2. Cloning

The BLSP-24/BLSP-28 PCR product was digested with Kpn I and BamH I and ligated into pMAL c2E (BioLabs), which had been digested with Kpn I and BamH I. The ligation product was used to transform TB1 K12 competent cells (clone pBLHIV-18). The desired clone was identified by digestion with Kpn I and BamH I and check on a 1 % agerose gel.

Miniprep DNA was prepared from an overnight culture of clone BLHIV-18 and sequenced with the oligonucleotide primers M13 forward by Cortec DNA Service Laboratories, Inc.

8. Example 4

8.1. Procedure for Producing and Purifying BLHIV-18 Protein (HIV-2 Envelope Protein)

8.1.1. Preparation of Required Solutions

Amylose column buffer was prepared by dissolving 1.2 grams of trizma base, 11.7 grams of NaCl, and 372 milligrams of EDTA in 900 ml of Milli Q water. Once the chemicals were completely dissolved 700 ul of 1 mM DTT was added and pH was adjusted to 7.4 (within 0.1) with 10N HCl. This solution should not be used if stored for longer than 30 days.

Amylose elute buffer was prepared by dissolving 1.2 g of Trizma base, 11.7 g of NaCl, 372 mg of EDTA, and 3.42 g of Maltose in 900 ml of MilliQ water. Once the chemicals were completely dissolved 700 ul of 1 mM DTT was added and the pH of the solution was adjusted to 7.4 (within 0.1) with 10N HCl. This solution should not be used if stored for longer than 30 days.

LB Broth was prepared by dissolving 20 grams of LB broth and 2 grams of glucose in 1 liter of MilliQ water, adjusting the pH to 7.2, and sterilizing by autoclaving. The solution was allowed to cool and Ampicillin was added to a final concentration of 0.1 mg/ml.

8.1.2. Growth of and Induction of pBLHIV-18 TB1 K12

A 250 ml Flask containing 50 ml LB Broth (100 mg/ml Ampicillin) was inoculated with 0.02 ml of pBLHIV-18 TB1 K12 glycerol stock and grown overnight in the incubator shaker at 37.degree. C. and 180 RPM. Each of 500 ml flasks containing 200 ml of LB Broth was inoculated with 10 ml of culture grown in the previous step. Cultures were grown in the incubator shaker at 37.degree. C. and 180 RPM until the O.D. at 600 nm was between 0.7 and 1.0 (3 hours). 1 ml of culture was removed for SDS-PAGE analysis of induction (UNINDUCED).

The cultures were induced by adding 1 ml of 200 mM IPTG to each flask. The flasks were covered and incubated for 4 hours at 37.degree. C. and 180 RPM. An aliquot of 1 ml of culture was removed for SDS-PAGE analysis of induction (INDUCED). The cells were harvested by centrifugation in the Sorvall RC-5B at 5000 RPM for 20 minutes. If cells were not required for immediate purification, they were stored at -20.degree. C.

Induction was analyzed on a 12% SDS-PAGE gel under reducing conditions.

8.1.3. Extraction and Isolating of BLHIV-18 Protein

The cell pellet was thawed and resuspended in a total of 20 ml of Novagen BugBuster protein extraction reagent (100 ml/1 L culture ratio) with 20 ul of 100 mM PMSF Stock, 40 ul of 1 M DTT and 10 ul of Benzonase (Novagen). Incubate the cell suspension on a sharking platform for 1 hour at room temperature. The extract should not be viscous at the end of the incubation. The solution was centrifuged in a Sorvall RC-5B at 9000 RPM in a GS-3 rotor for 50 min. Then the pellet was resuspended in a total of 30 ml of Inclusion Body Wash Solution with 40 ul of 1 M DTT, 30 ul of 10 mM PMSF Stock and 560 ul of 10 mg/ml Lysozyme. Mix well and incubate at 37 .degree. C. water bath for 1 hour.

In order to enable efficient resuspension, the solution was sonicated for 6 X 15 seconds (60 Sonic Dismembrator Fisher Scientific). The solution was then centrifuged in a Sorvall RC-5B at 9000 RPM in a GS-3 rotor for 50 min. The crude HIV-2 envelope protein was stored at 4.degree. C until further process.

8.1.4. Purification of Crude BLHIV-18 Protein on Amylose Column:

The crude HIV-2 envelope protein was diluted 1:5 with Amylose column buffer. 15 ml of diluted crude extract was loaded at a flow rate of 0.2 ml/min to 3 ml of Amylose resin column, which was equilibrated with 8 column volumes of Amylose Column Buffer. The column was washed with 8 -12 column volumes of Amylose Column Buffer. 10 column volumes of the Amylose Elute Buffer were run through the column. The fractions were collected and stored at 4.degree. C..

The purity of the peak fractions was determined by running 10 ul aliquots of each fraction on a 12% SDS-PAGE gel under reducing conditions. The peak fractions were pooled and

further concentrated. The protein concentration of this solution was determined and then 5 ug/lane of sample were run on a 12% SDS-PAGE to check the purity. Applying HIV-1 envelope protein in the EIA assay and rapid assay would be discussed in Sample 5.

9. Example 5

9.1. Diagnostic Utility of Recombinant BLHIV-21 Protein and BLHIV-18 Protein

9.1.1. EIA Assay with of Recombinant BLHIV-21 Protein and BLHIV-18 Protein

The present example demonstrates the utility of the invention for providing an EIA for the detection of anti-HIV antibodies employing the recombinant HIV-1 envelope protein and HIV-2 envelope protein. The recombinant antigens were prepared as described in Example 4. The EIA assay may follow any variety of testing formats known to those of skill in the art, given the information of the present disclosure.

The recombinant BLHIV-21 protein and BLHIV-18 protein were used to coat NUNC MaxiSorp microtiter wells at 500 ng/well. Following 1 hour of coating in 37 .degree. C., the wells will be "blocked" with 1 % BSA, and rinsed 5 times with buffer. 5 ul of anti-HIV antibody will be added to individual wells containing 100 ul of reaction buffer (PBS containing about 1 % BSA). The following steps will be typical EIA format involving incubation for about 20 min at 37 .degree. C., 5 rinses with about PBS buffer, and incubation with goat anti-human IgG-HRP conjugated (SIGMA) for about 20 min at 37 .degree. C.. The wells then were rinsed 5 times and the SureBlue TMB substrate (KPL) was added. Following addition of stop buffer, the plate will be read on an ELISA plate reader at the optical density at 450 nm. Controls will include wells with HIV positive and negative sera. The cut-off value for a positive result was set at 0.200 Absorbance Units above the average absorbance obtained from the negative control.

The results in Table 2 show the reactivity of the recombinant BLHIV-21 protein of the invention with HIV-1 positive and negative samples.

The results in Table 3 show the reactivity of the recombinant BLHIV-18 protein of the invention with HIV-2 positive and negative samples.

9.1.2. Rapid Assay with Recombinant BLHIV-21 Protein and BLHIV-18 Protein

A rapid assay for the presence of antibodies to HIV in test serum, plasma and whole blood was performed as follows: The rapid HIV antibody test consists of a synthetic filtration membrane positioned a top, an absorbent material within a plastic cartridge. The membrane was blotted 1 ul containing 2 ug each of the recombinant BLHIV-21 protein and BLHIV-18 protein of the invention, which reacted with HIV-1 antibodies in the specimen to produce a distinct visual signal on the membrane. The membrane also included a human IgG-capture control which consisted of a protein-A treated spot capable of binding IgG antibodies normally presented in blood and blood components. If the control spot did not appear, the test was considered invalid.

The test was performed by adding 50 ul of the blood, serum, or plasma specimen to the vial of Sample Diluent which lysed the red blood cells. This specimen diluent solution was then poured onto the well of the Membrane Unit. HIV antibodies, if present in the specimen, are captured by the HIV-1 and HIV-2 antigens on the filtration membrane. Color Developer was then added to the Membrane Unit. The Color Developer reacted with the captured antibodies to generate a distinct blue dot at the location of the control spot and, in the case that HIV antibodies were present in the specimen, a blue dot also appeared at the location of the test spot on the membrane. In the final step, the Clarifying Solution was then added to the membrane to decrease background color in order to make the control and test spots more distinct.

The results in Table 4 showed the reactivity of the BLHIV-21 protein of the invention with HIV-1 positive and negative samples in the rapid assay.

The results in Table 5 showed the reactivity of the BLHIV-18 protein of the invention with HIV-2 positive and negative samples in the rapid assay.

CLAIMS

1. A HIV-1 synthetic gene comprising sequence SEQ ID NO: 1.
2. A HIV-2 synthetic gene comprising sequence SEQ ID NO: 2.
3. The synthetic gene of claim 1 encoding for a polypeptide having the amino acid sequence SEQ ID NO: 3.
4. The synthetic gene of claim 2 encoding for a polypeptide having the amino acid sequence SEQ ID NO: 4.
5. An expression vector containing the synthetic gene of claim 1, where said gene encodes for a polypeptide having the amino acid sequence SEQ ID NO: 3.
6. An expression vector containing the synthetic gene of claim 2, where said gene encodes for a polypeptide having the amino acid sequence SEQ ID NO: 4.
7. The expression vector of claim 5 wherein heterologous gene expression is regulated by the lambda pRpL Promoter.
8. The expression vector of claim 6 wherein heterologous gene expression is regulated by the Ptac Promoter.
9. A host transformed with the expression vector of claim 5.
10. A host transformed with the expression vector of claim 6.
11. The transformed host of claim 9 wherein said host is E. coli.
12. The transformed host of claim 10 wherein said host is E. coli.
13. The host of claim 11 wherein said host is E. coli Stain DH5 α .
14. The host of claim 11 wherein said host is E. coli Stain K12 TB1.
15. An immunoassay for the detection of antibodies to Human immunodeficiency virus (HIV) in a subject, comprising the steps of: -contacting a biological test sample with a polypeptide or protein composition according to any one of Claims 1 to 4; and -detecting an immunological complex formed between antibodies to HIV in said biological test sample and said peptide or protein composition, characterized in that the presence of said complex being indicative of the presence of antibodies to HIV in said biological sample.
16. The immunoassay according to Claim 15, characterized in that said polypeptide or protein composition is immobilized to a solid support.
17. The immunoassay according to Claim 16, characterized in that said solid support is a nitrocellulose membrane.

18. The immunoassay according any one of Claims 15 to 17, characterized in that the presence of said complex is determined by the addition of an indicator reagent.
19. The immunoassay according to Claim 18, characterized in that said indicator reagent is a signal-generating component attached to a specific binding molecule capable of binding to a human anti-HIV antibody.
20. The immunoassay according to Claim 19, characterized in that said signal- generating component is Indigo Blue.
21. The immunoassay according to Claim 16, characterized in that said solid support is an assay plate comprising a multiplicity of microtiter wells.
22. The immunoassay according any one of Claims 15, 16, 21, wherein a step comprises binding of labeled antibodies to human Ig and the specific binding of said labeled antibodies are measured.
23. The immunoassay according to Claim 22 wherein said labeled antibodies are identified by an enzyme label.
24. The immunoassay according to Claim 23 wherein said enzyme label is Anti-Human IgG HRP conjugate.