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Expression of a Novel HIV-1 Gag-Pol-Env-Nef-Rev Multi-Epitope Construct in *Escherichia coli*

Elahe Akbari[1, 2], Soheila Ajdary[3], Esmat Mirabzadeh Ardakani[4], Elnaz Agi[5], Vahid Khalaj[2*], Azam Bolhassani[1*]

[1]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran; [2]Department of Medical Biotechnology, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; [3]Department of Immunology, Pasteur Institute of Iran, Tehran, Iran; [4]Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran; [5]Iranian Comprehensive Hemophilia Care Center, Tehran, Iran

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***Correspondence**
Email: A_bolhasani@pasteur.ac.ir; azam.bolhassani@yahoo.com & khalajs@pasteur.ac.ir
Tel: +982166953311 (Ext. 2240)
Fax: +982166465132

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ABSTRACT

Introduction: Recombinant subunit vaccines have been explored against various human pathogens, however, developing an effective therapeutic toward human immunodeficiency virus (HIV) infection has been challenging. So far, several recombinant HIV-1 antigens have been produced and examined for activation of desired immune responses. This study aimed to express an HIV-1 multiepitope protein as an antigen candidate to develop a vaccine. **Methods:** In this study, the codon-optimized encoding sequence of the designed multi-epitope construct (Gag-Pol-Env-Nef-Rev) was synthesized and subcloned into the pET-24a (+) expression vector. Then, expression of the target antigen was evaluated in *E. coli* BL21 (DE3) and Rosetta strains under different conditions (temperature, optical density/ OD_{600}, isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, and time). Finally, the expression of the Gag-Pol-Env-Nef-Rev multi-epitope protein was confirmed using SDS-PAGE and western blot analysis. **Results:** The highly conserved and immunodominant T-cell epitopes of HIV-1 Gag, Pol, Env, Nef, and Rev proteins were used to prepare a novel Gag-Pol-Env-Nef-Rev multi-epitope construct. The *gag-pol-env-nef-rev* gene was successfully sub-cloned in pET-24a (+) vector and subsequently expressed in BL21 (DE3) *E. coli* strain under optimized conditions (1 mM IPTG, 16 h post-induction, OD_{600}= 0.6, and 37ºC). A clear band of ~ 35 kDa was detected by western blotting using an anti-His antibody, indicating the successful expression of our target multi-epitope protein. **Conclusion:** Expression of the recombinant HIV-1 multi-epitope protein was optimized in a bacterial system. The expressed protein will be purified to use as a multi-epitope protein vaccine candidate in the future.

INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) epidemic caused by the human immunodeficiency virus (HIV) represents 70 million infections and 32.7 million deaths according to the United Nations Program on HIV/AIDS [1]. Despite the discovery of HIV nearly 50 years ago, no effective vaccine is still available due to several unique characteristics of the virus, such as heterogeneity, hyper-variability, and high mutation rate [2, 3]. Therefore, designing an effective therapeutic vaccine against HIV infection is still a new promising approach for preventing the disease progression to AIDS by priming cellular immune responses [4]. Indeed, the broadly cross-reactive antiviral T-cell immunity, particularly cytotoxic T lymphocytes (CTLs) responses, is critical for controlling HIV-1 infection [5]. In addition, T-cell-based therapeutic vaccines can induce more efficient and broader immune responses to control virus replication [6].

Among different therapeutic vaccine strategies, multi-epitope-based subunit vaccines have advantages due to overcoming the high HIV-1 mutation rate. This vaccine strategy focuses on favorable and highly immunogenic regions of antigens and can enhance the population coverage of the vaccine candidates [7]. Epitope mapping using bioinformatics methods is a powerful tool to select protective epitopes from main viral antigens [3, 8]. Developing an HIV-1 multi-epitope protein-based

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vaccine has potential benefits such as biosafety, stability, cost-effectiveness, and immune system activation [9].

The RNA genome of HIV contains nine genes named *gag, pol, env, nef, vif, vpu, vpr, tat,* and *rev* genes that encode fifteen proteins such as three structural proteins (Gag, Pol and Env), four accessory proteins (Vpr, Vpu, Vif, and Nef), and two regulatory proteins (Tat and Rev) which play essential roles in replication and virus pathogenesis [7]. Vaccine candidates harboring the conserved epitopes of Gag, Pol, Env, and Nef proteins could significantly activate specific $CD8^+$ and $CD4^+$ T cells in different clinical trials [10, 11]. Furthermore, Rev and Nef proteins are frequent targets of cytotoxic T lymphocytes [12].

A novel multi-epitope construct (*gag-pol-env-nef-rev*) containing the conserved T-cell epitopes derived from HIV-1 Gag, Pol, Env, Nef & Rev proteins have been designed in our previous study [13] using immunoinformatics approach. In the current study, the designed *gag-pol-env-nef-rev* coding sequence was synthesized and subcloned in the pET-24a expression vector. Next, protein expression was investigated in two *E. coli* strains under different conditions of temperature, optical density, IPTG concentration, and post-induction cultivation time. Finally, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were utilized to detect the expressed Gag-Pol-Env-Nef-Rev multi-epitope protein.

MATERIAL AND METHODS

Design of the multi-epitope DNA construct. Based on our previous study [13], designing the artificial multi-epitope immunogen was carried out by *in silico* tools. Briefly, the MHC-I and MHC-II binding epitopes were predicted using NetMHCpan 4.0 and NetMHCIIpan 3.2 servers for selecting highly conserved T-cell epitopes. The MHC-I immunogenicity, antigen processing, population coverage, and conservancy analysis of epitopes were studied by the IEDB database. The AllergenFP v.1.0, ToxinPred, and HemoPI web servers were used for allergenicity, toxicity, and hemotoxicity analyses. The peptide-MHC-I/MHC-II molecular docking was done by GalaxyPepDock. The final selected T-cell epitopes were linked by the AAY proteolytic linker to form a multi-epitope fusion construct. The codon-optimized nucleotide sequence of the selected *gag-pol-env-nef-rev* construct was retrieved by amino acid reverse translation tools, and the appropriate restriction enzyme sites were added to facilitate the cloning procedure. The final DNA fragment was synthesized commercially (Biomatik Corporation, Canada) and provided in a pUC57 cloning vector.

Confirmation of the pUC57-*gag-pol-env-nef-rev* construct. At first, the competent *E. coli* DH5α strain was transformed by pUC57-*gag-pol-env-nef-rev* using the standard heat shock method. Then, ten nanograms of the pUC57-*gag-pol-env-nef-rev* construct were added to 100 µl competent *E. coli* cells. The transformation mixture was incubated for 20 m on ice and subsequently for 90 s in 42°C. After that, the mixture was added to 1 ml of Luria-Bertani (LB) medium (Sigma-Aldrich, Germany) and incubated for 1 h at 37°C. Finally, bacterial suspension was plated on LB agar containing 100 µg/ml ampicillin (Sigma) and incubated overnight at 37°C. The pUC57-*gag-pol-env-nef-rev* transformants were recovered from LB-ampicillin (100 µg/ml) plates, and the vector was purified from an ampicillin-resistant single colony using plasmid extraction Mini-kit (Yekta Tajhiz Azma, Iran). The concentration and purity of DNA construct were determined by NanoDrop spectrophotometer (Thermo Fisher Scientific). The presence of the inserted *gag-pol-env-nef-rev* fragment in pUC57-*gag-pol-env-nef-rev* was confirmed by digestion with *Eco*RI/*Hind*III restriction enzymes (Thermo Fisher Scientific) and detection using 1% agarose gel electrophoresis.

Subcloning of the *gag-pol-env-nef-rev* gene into pET-24a (+) expression vector. The *gag-pol-env-nef-rev* gene fragment (without stop codon) was excised from pUC57-*gag-pol-env-nef-rev* using *Eco*RI/*Hind*III (Thermo Fisher Scientific) enzymes and subcloned into the same enzymatic sites of the pET-24a (+) prokaryotic expression vector using T4 DNA ligase (Thermo Fisher Scientific). The ligation product was transformed into the competent *E. coli* DH5α strain. The recombinant pET-24a (+)-*gag-pol-env-nef-rev* vector was extracted from kanamycin-resistant *E. coli* colonies, and the concentration and purity of DNA were determined as above. The correct cloning procedure was validated by restriction analysis of pET-24a (+)-*gag-pol-env-nef-rev*. The recombinant pET-24a(+)-*gag-pol-env-nef-rev* vector was then transformed into the competent *E. coli* BL21(DE3) and Rosetta strains as expression hosts.

Expression of the recombinant Gag-Pol-Env-Nef-Rev multi-epitope protein. A recombinant kanamycin-resistant clone of *E. coli* BL21 (DE3) and Rosetta harboring pET-24a(+)-*gag-pol-env-nef-rev* construct was cultured in 5 mL LB medium containing 50 µg/ml kanamycin at 37°C overnight in a shaker incubator (150 *rpm*). Then, 100 *µl* of each BL21 (DE3) and Rosetta overnight culture was inoculated in 50 ml fresh 2xYT medium (Peptone 1.6%, Yeast 1%, NaCl 0.5%) in separate tubes to assess the protein expression under different conditions of optical density (OD_{600}), isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, temperature and incubation time after IPTG induction. The cultures were incubated at 37°C for 3 to 4 h with constant shaking until the OD_{600} reached 0.4, 0.6, and 0.8. Then, the protein expression was induced by adding IPTG (Fermentas) at final concentrations of 0.25, 0.5, and 1 mM, followed by incubation for 2, 3, 4, and 16 h at 30 or 37°C. Afterward, the bacterial pellet was harvested by centrifugation (Eppendorf) at 1000 g, and the protein expression pattern was analyzed.

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Expression analysis of the recombinant Gag-Pol-Env-Nef-Rev multi-epitope protein. SDS-PAGE and western blot analyses were performed to detect His-tagged Gag-Pol-Env-Nef-Rev protein. For western blotting, the cell pellet from each sample was mixed with sample loading buffer (0.5 M Tris-HCl (pH 6.8), 5% SDS, 10% glycerol, 0.25% bromphenol blue, 5% mβ-mercaptoethanol), and incubated for 5 min at 100°C for cell disruption. The samples were loaded onto 12.5% SDS-polyacrylamide gel and then were electro-transferred to a nitrocellulose membrane (Whatman, UK). TBS buffer (Tris-buffered saline including 3% bovine serum albumin) was used for blocking the membrane. The membrane was immersed in 1:10000 dilution of peroxidase-conjugated anti-His-tag monoclonal antibody (Abcam, UK) for 2 h at room temperature. Subsequently, the protein band was visualized using 3, 3' Diaminobenzidine (DAB)/H_2O_2 substrate solution (Roche, Germany). The BL21 (DE3) and Rosetta bacterial strains carrying the empty pET-24a (+) vector were used as the negative controls.

RESULTS

Preparation of the multi-epitope *gag-pol-env-nef-rev* DNA construct. In our previous work [13], a multi-epitope *gag-pol-env-nef-rev* cassette was designed and validated using bioinformatics. Totally, 10 cytotoxic T lymphocytes (CTL) and 8 helper T lymphocytes (HTL) epitopes from HIV-1 Gag, Pol, Env, Nef, and Rev proteins were selected to design the multi-epitope construct. The AAY proteasomal cleavage site was used between epitopes to form the fusion construct. **Figure 1** shows the amino-acid sequence of the Gag-Pol-Env-Nef-Rev construct.

Confirmation of the pUC57-*gag-pol-env-nef-rev* construct. The synthetic DNA sequence of the multi-epitope construct was received as an insert cloned in the pUC57 vector. This plasmid was successfully propagated and confirmed by enzymatic digestion using *Eco*RI/*Hind*III restriction enzymes. The *gag-pol-env-nef-rev* insert was detected as an expected band of ~ 894 bp on 1% agarose gel **(Fig. 2).** The confirmed ampicillin-resistant colonies were prepared in 25% glycerol solution and stored at -70º C for future use.

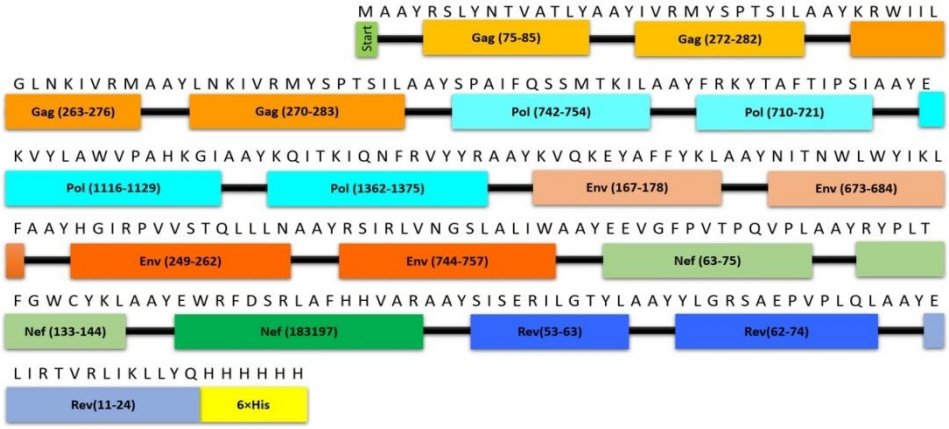

Fig. 1. The designed Gag-Pol-Env-Nef-Rev multi-epitope construct [Ref. 13]

Preparation of the pET24a-*gag-pol-env-nef-rev* construct. A schematic model for the sub-cloning of the *gag-pol-env-nef-rev* gene from pUC57 into pET-24a (+) plasmid is shown in **Figure 3**. For making the expression construct, the target insert was cut from the intermediate plasmid and then subcloned into the pET-24a (+) expression vector. The double digestion of the pET construct using *Eco*RI/*Hind*III enzymes resulted in an apparent fragment of ~894 bp, indicating the successful cloning steps **(Fig. 4).** Transformation of the *E .coli* Rosetta and BL21(DE3) strains with the recombinant pET-24a(+)-*gag-pol-env-nef-rev* vector was successful, and kanamycin-resistant colonies were prepared in 25% glycerol solution and stored at -70 º C for future use.

Expression of the Gag-Pol-Env-Nef-Rev multi-epitope protein. The total cell lysates of transformants were analyzed by SDS-PAGE at 2, 3, 4, and 16 h post-induction. The results confirmed that the Gag-Pol-Env-Nef-Rev construct was expressed in BL21 (DE3) *E. coli* strain **(Figures 5 & 6)**. **Figure 6** shows a clear band of ~35 kDa on 12.5% SDS-polyacrylamide gel at 16-18 h post-induction compared to other incubation times. Moreover, a decrease in the IPTG concentration reduced the expression of the Gag-Pol-Env-Nef-Rev protein. The optimum conditions for protein expression in BL21 (DE3) strain were OD_{600} 0.6, 1mM IPTG, 37ºC, and 16-18 h (overnight) post-induction **(Fig. 7)**. Western blot analysis also confirmed the proper expression of the recombinant Gag-Pol-Env-Nef-Rev protein within the

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crude bacterial lysate with the expected molecular weight of ~ 35 kDa **(Fig. 8).**

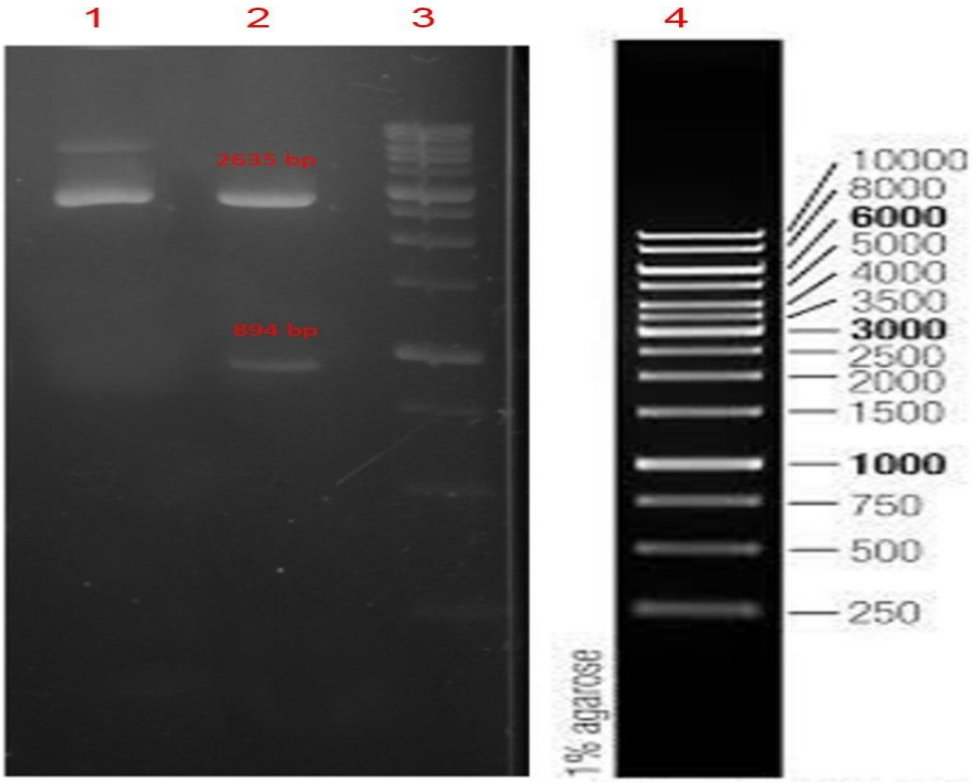

Fig. 2. Confirmation of the pUC57-*gag-pol-env-nef-rev* construct using enzymatic digestion: Lane 1, undigested pUC57-*gag-pol-env-nef-rev* vector; lane 2, double digestion of pUC57-*gag-pol-env-nef-rev* by *Eco*RI/*Hind*III restriction enzymes produced two clear bands of ~ 894 bp for the *gag-pol-env-nef-rev* gene and ~ 2635 bp for pUC57; lanes 3 and 4, DNA ladder (1 kb, Fermentas).

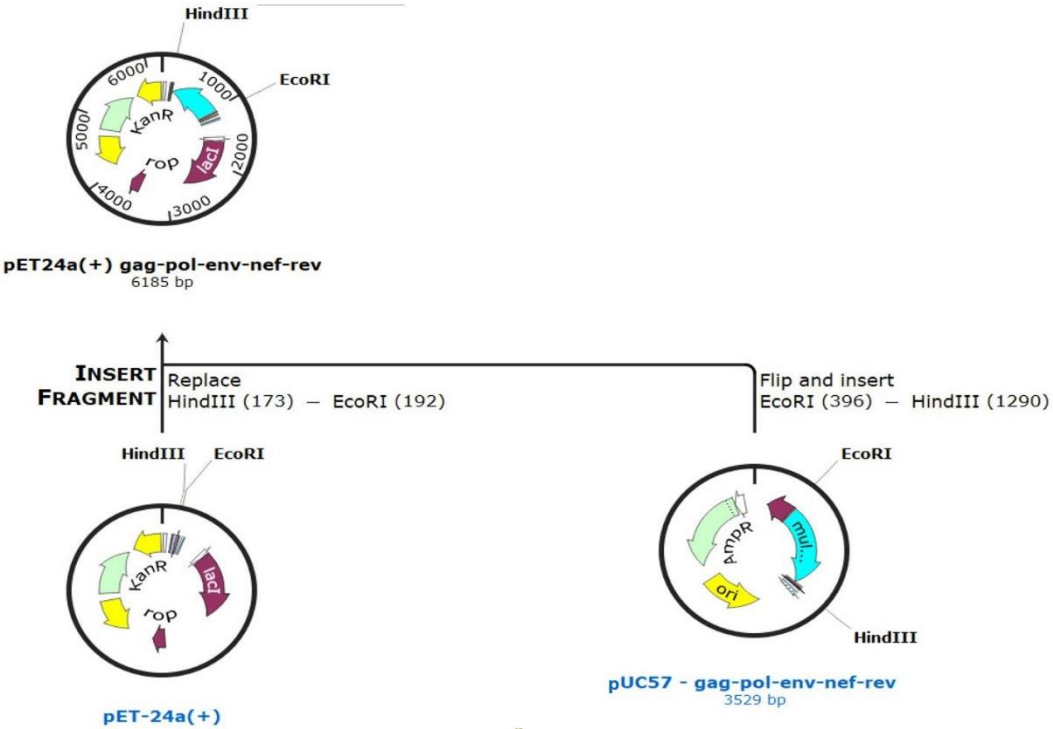

Fig. 3. Schematic diagram of subcloning of the *gag-pol-env-nef-rev* gene from pUC57 vector into pET-24a (+) vector.

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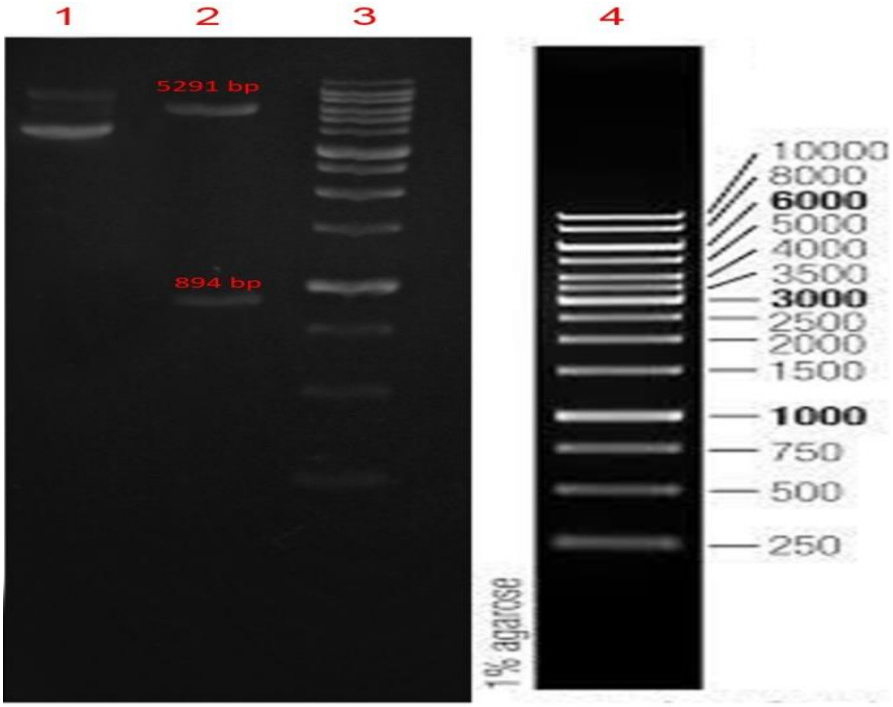

Fig. 4. Confirmation of the *gag-pol-env-nef-rev* gene inserted in pET-24a (+) vector using double digestion: Lane 1, undigested pET-24a (+)-*gag-pol-env-nef-rev* vector; lane 2, double digestion of pET-24a(+)-*gag-pol-env-nef-rev* using *Eco*RI/*Hind*III restriction enzymes produced two clear bands of ~894 bp for the *gag-pol-env-nef-rev* gene, and ~5291 bp for pET-24a(+) vector, lanes 3 and 4, DNA ladder

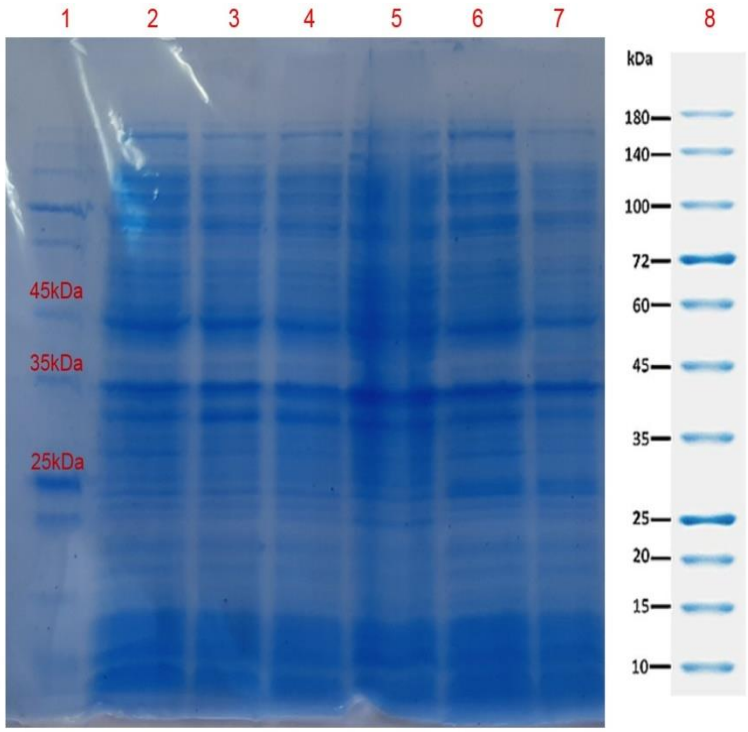

Fig. 5. SDS-PAGE analysis of the Gag-Pol-Env-Nef-Rev protein expression in *Escherichia coli* Rosetta strain: Lane 1, protein MW marker (Fermentas); lane 2, crude bacterial extract before induction; Lane 3, crude bacterial lysate 2 h after induction by 1 mM IPTG; lane 4, crude bacterial lysate 3 h after induction by 1 mM IPTG; lane 5, crude bacterial lysate 4 h after induction by 1 mM IPTG; lane 6: crude bacterial lysate 16 h (overnight) after induction by 1 mM IPTG; lane 7, crude transformed Rosetta by empty pET-24a (+) vector at 16 h after induction by 1 mM IPTG as negative control; lane 8, protein MW marker (10-180 kDa, Fermentas)

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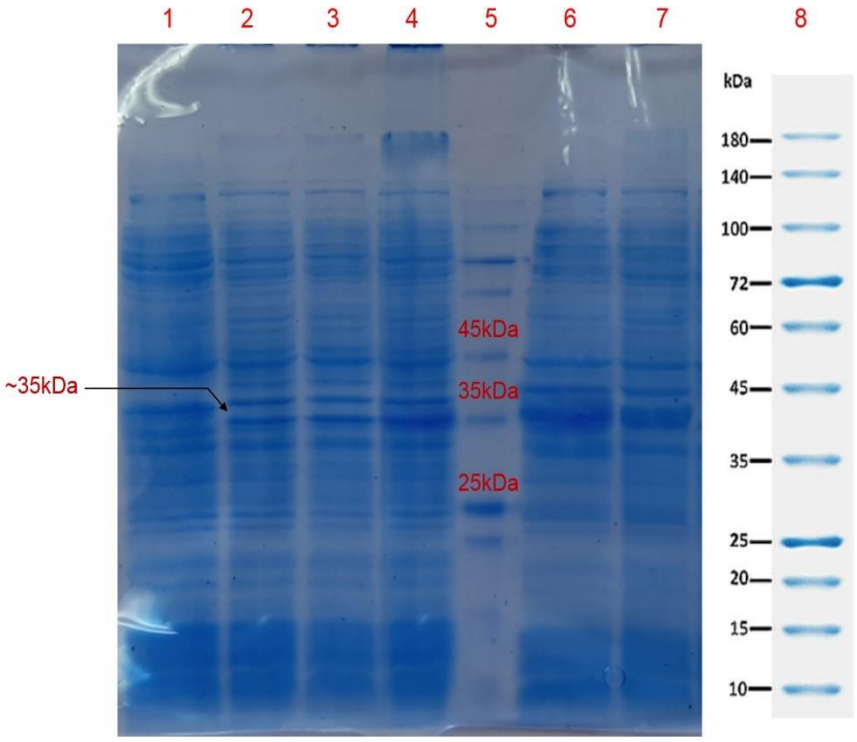

Fig. 6. SDS-PAGE analysis of the Gag-Pol-Env-Nef-Rev protein expression in *Escherichia coli* BL21 (DE3) strain: Lane 1, Protein MW marker (Fermentas); lane 2: crude bacterial extract before induction; lane 3: crude bacterial lysate 2 h after induction by 1 mM IPTG; Lane 4, crude bacterial lysate 3 h after induction by 1 mM IPTG; lane 5: crude bacterial lysate 4 h after induction by 1 mM IPTG; lanes 6 and 7, crude bacterial lysate 16 h after induction by 1 mM IPTG; lane 8, protein MW marker (10-180 kDa, Fermentas)

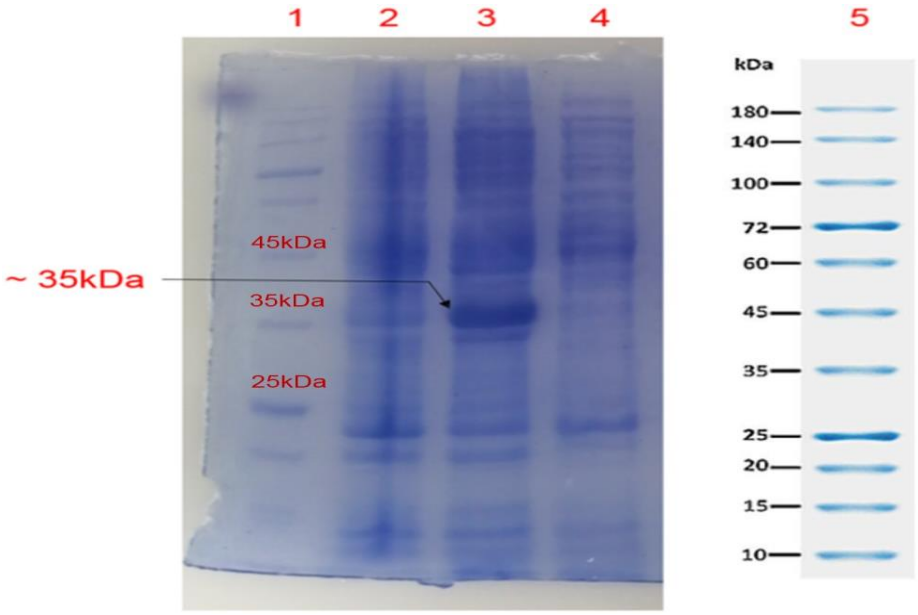

Fig. 7. SDS-PAGE analysis of the expressed Gag-Pol-Env-Nef-Rev protein in *Escherichia coli* BL21 (DE3) strain under the optimized condition: Lane 1, protein MW marker (Fermentas); Lane 2, crude bacterial extract before induction; lane 3, crude bacterial lysate 16 h after induction by 1 mM IPTG; lane 4, crude transformed BL21 (DE3) by empty pET-24a (+) vector at 16 h after induction by 1 mM IPTG as negative control; lane 5, protein MW marker (10-180 kDa, Fermentas)

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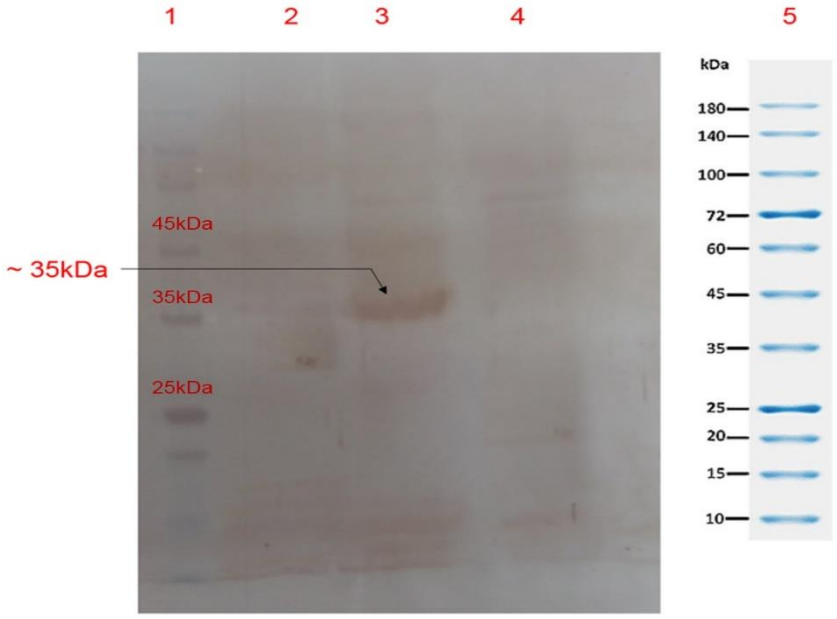

Fig. 8. Western blot analysis of the expressed Gag-Pol-Env-Nef-Rev protein in *Escherichia* BL21 (DE3) strain: Lane 1, protein MW marker; lane 2, crude bacterial extract before induction; Lane 3, crude bacterial lysate 16 h after induction by 1 mM IPTG; lane 4, crude transformed BL21 (DE3) by empty pET-24a (+) vector at 16 h after induction by 1 mM IPTG as negative control; lane 5, Protein MW marker (10-180 kDa, Fermentas)

DISCUSSION

High genetic diversity and antigenic variability in HIV impede the development of a beneficial vaccine using traditional methods [14]. While expression and purification of individual antigens are technically feasible to develop vaccines, combining the protective regions into a single fusion protein is a more efficient, cost-effective, and practical approach. The clinical studies indicated that using a multi-epitope vaccine strategy against HIV infection leads to the production of broader immunity as observed in the T-cell (t) HIV-1consvX therapeutic vaccine [15] or the F4/AS01B HIV-1 vaccine [16] candidates. The tHIVconsvX vaccine construct containing six conserved Gag and Pol proteins regions significantly reduced plasma viral load and increased $CD4^+$ T-cell count in HIV-1-infected individuals [15]. Letourneau *et al*. designed an immunogen harboring 14 most conserved regions of HIV-1 proteome into one chimeric protein (HIVconsv). The HIVconsv construct was able to shift pre-existing immune responses towards vaccine-encoded conserved regions in HIV-infected individuals [17]. Therefore, this study aimed to express an artificial multi-epitope immunogen containing the conserved T-cell epitopes derived from five HIV-1 proteins (Gag, Pol, Env, Nef, and Rev). The construction of mosaic immunogen harboring the potential T-cell epitopes linked by the AAY proteasomal degradation sequence facilitates epitope processing *in vivo*.

As known, *E. coli* was successfully used as a vehicle for the expression of fusion proteins because of its fast growth rate, high plasmid yield, and high transformation efficiency [18]. The BL21 (DE3) *E. coli* is the most common prokaryotic strain used to express the recombinant proteins until now [19, 20]. Also, the Rosetta strain is a BL21 derivative designed to enhance the expression of eukaryotic proteins containing the rare codons used in *E. coli* [21]. In this study, both *E. coli* BL21 (DE3) and Rosetta strains were used to evaluate the multi-epitope protein expression. Our results showed that the BL21 (DE3) strain had higher potency in the expression of the *gag-pol-env-nef-rev* gene than the Rosetta strain. Thus, supplying rare tRNAs in the Rosetta strain was not effective for increasing the expression of Gag-Pol-Env-Nef-Rev protein likely due to the codon optimization of the multi-epitope Gag-Pol-Env-Nef-Rev DNA construct. In our expression studies, the optimal temperature and incubation time were determined as 37° C and 16-18 h post-induction, respectively. The optimum concentration of IPTG was found to be 1 mM and the cell density at OD_{600} value of 0.6 provided the highest expression level in the BL21 (DE3) *E. coli* strain. Host strain, IPTG concentration, and incubation times post-induction are essential parameters that influence total protein production [22].
Arabi et al. showed that the HIV-1 *tat/pol/gag/env* (HIVtop4) multi-epitope construct cloned in pET-23a expression vector was produced in *E. coli* BL21 (DE3) strain using 1 mM IPTG at 3 h post-induction. This construct could induce the secretion of antibody and IL-4 and IFN-γ cytokines in mice [23]. Davoodi et al. indicated that a poly-epitope Nef-Vpr-Gp160-P24 construct was expressed in *E. coli* (Rosetta strain) under

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certain expression conditions [24]. Also, Namazi *et al*. (2019) produced a recombinant Nef-Tat-Gp160-P24 polyepitope immunogen in the Rosetta strain under optimum conditions [25]. Therefore, the pET/ *E. coli* BL21 or Rosetta expression systems have been widely used to generate HIV immunogenic constructs in several studies [24-28]. Similar to the above studies, in this research, the designed *gag-pol-env-nef-rev* fusion gene was successfully expressed in *E.coli* BL21 (DE3) cells under the optimal expression conditions. The recombinant Gag-Pol-Env-Nef-Rev protein will be a potential target for future studies to design a possible therapeutic HIV vaccine candidate.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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