Engineering and Validation of a Vector for Concomitant Expression of Rare Transfer RNA (tRNA) and HIV-1 nef Genes in Escherichia coli

Siti Aisyah Mualif1, Sin-Yeang Teow1, Tasyriq Che Omar1, Yik Wei Chew1, Narazah Mohd Yusoff2, Syed A. Ali1*

1 Oncological and Radiological Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia, 2 Regenerative Medicine, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Pulau Pinang, Malaysia

* ali2@amdi.usm.edu.my

Abstract

Relative ease in handling and manipulation of Escherichia coli strains make them primary candidate to express proteins heterologously. Overexpression of heterologous genes that contain codons infrequently used by E. coli is related with difficulties such as mRNA instability, early termination of transcription and/or translation, deletions and/or misincorporation, and cell growth inhibition. These codon bias-associated problems are addressed by co-expressing ColE1-compatible, rare tRNA expressing helper plasmids. However, this approach has inadequacies, which we have addressed by engineering an expression vector that concomitantly expresses the heterologous protein of interest, and rare tRNA genes in E. coli. The expression vector contains three (argU, ileY, leuW) rare tRNA genes and a useful multiple cloning site for easy in-frame cloning. To maintain the overall size of the parental plasmid vector, the rare tRNA genes replaced the non-essential DNA segments in the vector. The cloned gene is expressed under the control of T7 promoter and resulting recombinant protein has a C-terminal 6His tag for IMAC-mediated purification. We have evaluated the usefulness of this expression vector by expressing three HIV-1 genes namely HIV-1 p27 (nef), HIV-1 p24 (ca), and HIV-1 vif in NiCo21(DE3) E.coli and demonstrated the advantages of using expression vector that concomitantly expresses rare tRNA and heterologous genes.

Introduction

Bacterium Escherichia coli (E. coli) remains a predominant host for the expression of heterologous proteins. Like other organisms, E. coli uses 61 available amino acid codons for mRNA production. However, not all 61 mRNA codons are used equally [1, 2]. The so-called ‘major’ codons occur in highly expressed genes, whereas ‘rare’ codons are present in low expressing
The frequency of the codon usage in mRNAs also reflects the abundance of their cognate tRNAs in the cells. When the codon usage of the overexpressed heterologous protein differs considerably from the normal codon usage of the expression host, protein synthesis can be inhibited due to the depletion of rare tRNAs cellular pool [4].

Viral proteins are encoded by genes that contain codons rarely used by *E. coli*. For instance, genes of HIV-1 proteins contain 8.21% (in gene encoding Nef protein) up to 23.17% (in gene encoding Vpu protein) codons that are rarely used by *E. coli* (Table 1). These genes express poorly in *E. coli* and as a result, little and/or poor quality protein is produced [4, 5]. To alleviate codon bias-associated problems, one option is to optimize the gene sequence by changing rare codons into more frequently used codons [6]. Alternatively, specialized *E. coli* strains such as BL21-CodonPlus (Stratagene) and Rosetta2(DE3) (EMD Millipore) can be used. These strains harbor CoE1-compatible, rare tRNA expressing helper plasmids, which are maintained under chloramphenicol selective pressure [7].

High level expression of numerous heterologous proteins has been achieved by using either of the two above mentioned strategies. However, there are some issues associated with these approaches. 1. Codon optimization via gene synthesis can be costly and time-consuming especially for genes longer than 500bp. Moreover, codon changes can affect secondary structure of mRNA with unknown consequences [8, 9]. 2. Maintenance of tRNA-expressing helper plasmids together with expression vectors results in additional metabolic pressure because the bacteria constitutively express two antibiotic resistance genes [10]. 3. It complicates expression strategies where multiple vectors are employed for co-expression of protein subunits [11]. 4. Certain engineered strains such as those containing pLysS (to reduce background expression levels) cannot be transformed with rare tRNA vectors that contain p15A ori and constitutively express chloramphenicol acetyltransferase gene for selection [12].

To address abovementioned limitations, we engineered an expression vector that would express both the heterologous protein of interest, and rare tRNA genes in *E. coli*. We started off with cloning HIV-1 nef gene in an expression vector pSA-HP24-6His, which we have previously used for high level expression of HIV-1 p24 [13]. We expressed HIV-1 Nef because it has gained increased interest as a new therapeutic target for HIV/AIDS treatment in recent years [14, 15, 16, 17, 18, 19] and we are engineering cell internalizing antibodies to target this pathogenic factor. We then modified the backbone of the resulting pSA-HNef-6His vector by replacing a non-essential DNA segment between lacI gene and T7 promoter with rare tRNA genes argU, ileY, and leuW. We call this vector pSA-HNef-6His-RIL. In order to further validate the utility of this rare tRNA vector gene, we replaced nef with HIV-1 p24 and vif genes and the

Table 1. Rarely used codons by *E. coli* in genes of HIV-1.

| HIV-1 proteins | Total number of codons per ORF | Number of codons rarely used by *E. coli* | Percentage of codons rarely used by *E. coli* |
|----------------|-------------------------------|------------------------------------------|---------------------------------------------|
| Gag polyprotein | 501                           | 55                                       | 10.97                                       |
| Pol polyprotein | 1004                          | 101                                      | 10.05                                       |
| Vif             | 193                           | 28                                       | 14.5                                        |
| Vpr             | 97                            | 17                                       | 17.52                                       |
| Tat             | 87                            | 11                                       | 12.64                                       |
| Rev             | 117                           | 19                                       | 16.24                                       |
| Vpu             | 82                            | 19                                       | 23.17                                       |
| Env             | 855                           | 95                                       | 11.11                                       |
| Nef             | 207                           | 17                                       | 8.21                                        |

HIV-1 (NL4-3) genes were subjected to rare codon analysis using online tool 'Rare Codon Calculator (RaCC)' [http://nihserver.mbi.ucla.edu/RACC/](http://nihserver.mbi.ucla.edu/RACC/).
resulting plasmids were designated as pSA-HP24-6His-RIL and pSA-HVif-6His-RIL respectively, which were then used to express P24 and Vif proteins. To facilitate clone manipulation, we substituted the nef gene with a multiple cloning site (MCS) and the resulting plasmids called pSA-C6His-RIL.

### Materials and Methods

#### Bacterial culture conditions

The E. coli strains DH5α (NEB, #C2987H) and NiCo21(DE3) (NEB, #C2529H) were used for cloning and expression experiments, respectively. Bacteria were grown aerobically in LB (Miller) broth, or on LB (Miller) agar at different temperatures, and in the presence or absence of ampicillin (100μg/ml) and/or chloramphenicol (25μg/ml). Bacterial strains were stored in glycerol (50%)-supplemented LB broth at -80°C. In some experiments, NiCo21(DE3) were transformed with rare tRNA expressing pACYC-RIL (Stratagene), pRARE2 (Novagen), and pLysSRARE2 (Novagen) plasmids and the transformants were selected on chloramphenicol-supplemented LB-agar plates.

#### Plasmid construction

PCR amplifications of DNA fragments, intended for cloning purposes, was carried out using Q5 High-Fidelity DNA Polymerase (NEB, #M0491), whereas Taq DNA Polymerase (NEB, #M0273) was used for colony PCR. DNA fragments were reaction cleaned-up or gel-purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co, #740609). Plasmid DNA was purified using Wizard Plus SV Minipreps DNA Purification System (Promega, #A1465). Vector and insert were mixed in 1:3 molar ratios (unless otherwise specified) and ligated in presence of T4 DNA ligase (NEB, #M0202) at 4°C for 18 h. Construction of engineered vectors (Table 2) is described below.

#### Construction of pSA-HNef-6His

The HIV-1 nef gene encoding 206 residues of wild type Nef protein was PCR amplified from pNL4.3 plasmid (NIH AIDS Reagent Program, #114) using Nef-NdeI-F and Nef-SacI-R primers (Table 3). The resulting 635bp amplicon was gel purified and restricted with NdeI (NEB,

### Table 2. Expression vectors engineered in this study.

| Vector name | Features |
|-------------|----------|
| pSA-HNef-6His (6.517kb) | a. A derivative of pSA-Hp24-6His [13]  
  b. Expresses HIV-1 (NL4-3) Nef protein under the control of T7 promoter.  
  c. Contains a 6His tag on C-terminal of HIV-1 Nef. |
| pSA-HNef-6His-RIL (6.455kb) | a. A derivative of pSA-HNef-6His.  
  b. Expresses argU, ileY, and leuW tRNA genes under the control of their own promoters. |
| pSA-HP24-6His-RIL (6.533kb) | a. A derivative of pSA-HNef-6His-RIL.  
  b. Expresses HIV-1 (NL4-3) P24 protein under the control of T7 promoter. |
| pSA-HVif-6His (6.500kb) | a. A derivative of pSA-HNef-6His.  
  b. Expresses HIV-1 (NL4-3) Vif protein under the control of T7 promoter. |
| pSA-HVif-6His-RIL (6.439kb) | a. A derivative of pSA-HNef-6His-RIL.  
  b. Expresses HIV-1 (NL4-3) Vif protein under the control of T7 promoter. |
| pSA-C6His-RIL (5.885kb) | a. A derivative of pSA-HNef-6His-RIL.  
  b. HIV-1 Nef gene is replaced with a multiple cloning site (MCS). |

doi:10.1371/journal.pone.0130446.t002
and 

SacI (NEB, #R0156L) for 8h at 37°C, and purified. The vector was prepared by restricting pSA-HP24-6His [13] with NdeI/Sacl for 4h., separating plasmid backbone on 1% agarose gel, and purified. Insert (635bp nef) and vector (5.914kb pSA-6His) were ligated, transformed into chemically competent DH5α E. coli cells, and selected on ampicillin-containing LB-agar plates after 18h incubation at 30°C. Ten randomly selected bacterial colonies were subjected to colony PCR using vector-specific pMXB10-up101-F and insert-specific Nef-R primers (Table 3). Transformants that contained an amplicon of expected size by PCR were then verified using DNA restriction and sequence analyses. This vector was called pSA-HNef-6His.

**Construction of pSA-HNef-6His-RIL**

The pACYC-RIL vector (5μg) was restricted with SspI (NEB, #R0132) and FspI (NEB, #R0135) for 4h at 37°C, and an 874bp DNA fragment that contained argU, ileY, leuW tRNA genes, was purified from 1.5% agarose gel, and purified. Insert (635bp nef) and vector (5.914kb pSA-6His) were ligated, transformed into chemically competent DH5α E. coli cells, and selected on ampicillin-containing LB-agar plates after 18h incubation at 30°C. Ten randomly selected bacterial colonies were subjected to colony PCR using vector-specific pMXB10-up101-F and insert-specific Nef-R primers (Table 3). Transformants that contained an amplicon of expected size by PCR were then verified using DNA restriction and sequence analyses. This vector was called pSA-HNef-6His.

**Table 3. Oligonucleotides used to construct and verify pSA-HNef-6His, pSA-HNef-6His-RIL, pSA-Hp24-6His-RIL, pSA-HVif-6His-RIL, and pSA-6His-RIL expression vectors.**

| Primer           | Sequence (5’ to 3’)                                      |
|------------------|----------------------------------------------------------|
| Nef-NdeI-F       | GGTGTGCTATATGGGCTGGCAGATGGCTGGCAAAAAG                   |
| Nef-Sacl-F       | GGTGTGCTGTCGTCGTCGTAAGTGGCATTTGCAGATCCCGGG             |
| Up-T7-Smal-F     | TCAGACCCGGGGCCAGAAATTGGGGATCGGG                        |
| Up-lacl-Smal-R   | TCAGACCCGGGGCCAGTGCACATTCTCTCGG                        |
| pMXB10-up101-F   | GATTCGCCGAAATATACG                                      |
| Nef-R            | GCAGTTCTGGAATGACTCCGG                                   |
| pMXB4560-81-Seq-F| CTCTCTGATTAGGAGAAAGCCGAACCC                             |
| RIL-R            | CCCATCGTCAACGCTTTTC                                     |
| P24-NdeI-F       | GGTGTGCTATATGGCAGATGGGGATCCCGGG                        |
| P24-Sacl-R       | GGTGTGAGCTCCAAAATCTCTTTGTTATGGCC                       |
| P24-R            | CAAGAATCCTTGTCTTATAGCC                                  |
| Vif-Nhel-F       | GTGGTGGCTGACGATGGGAAACAGATGGCCGGTTT                   |
| Vif-Sacl-R       | CTTTAAAGGTCTGTCGCCATCATGTTAGTGGCTCC                   |
| Vif-R            | GTGTCCATCTCATTGTATGGCTCC                               |
| pSA-Nhel-R       | CATCATGCTAGCGTATATCTCTCTCTCTCTAAAGTTAAC                |
| pSA-F            | CAAGAATCGCAGCCGACCAA                                    |
| MCS-F            | TATGAGATCTGCTAGCGAATTCTGCGACCGCGGGGTCCGAGCGGCGGCGAGGCT |
| MCS-R            | CTGCAGCGGCGGTAGCGAATCTCAGCAGGATCTCA                   |

The restriction sites are in italics.

doi:10.1371/journal.pone.0130446.t003

RARE tRNA Gene-Containing E. coli Expression Vector

PLOS ONE | DOI:10.1371/journal.pone.0130446 July 6, 2015 4/2
Construction of pSA-HP24-6His-RIL

The HIV-1 p24 gene encoding 232 residues of wild type P24 protein was PCR amplified from pNL4.3 plasmid (NIH AIDS Reagent Program, #114) using P24-NdeI-F and P24-Sacl-R primers (Table 3). The resulting 713bp amplicon was gel purified and restricted with NdeI (NEB, #R0111L) and Sacl (NEB, #R0156L) for 8h at 37°C, and purified. The vector was prepared by restricting pSA-HNef-6His-RIL vector (5 μg) with NdeI (NEB, #R0111L) and Sacl (NEB, #R0156L) for 8h at 37°C, and purified. Insert (702bp p24) and vector (5.831kb pSA-6His-RIL) were ligated, transformed into chemically competent DH5α E. coli cells, and selected on ampicillin-containing LB-agar plates after 18h incubation at 30°C. Ten randomly selected bacterial colonies were subjected to colony PCR using vector-specific pMXB10-up101-F and insert-specific P24-R primers (Table 3). Transformants that contained an amplicon of expected size by PCR were then verified using DNA restriction and sequence analyses.

Construction of pSA-HVif-6His and pSA-HVif-6His-RIL

The HIV-1 vif gene encoding 192 residues of wild type Vif protein was PCR amplified from pNL4.3 plasmid (NIH AIDS Reagent Program, #114) using Vif-NheI-F and Vif-Sacl-R primers (Table 3). The resulting 600bp amplicon was gel purified and restricted with NheI (NEB, #R0131L) and Sacl (NEB, #R0156L) for 8h at 37°C, and purified. Two vector backbones were prepared by whole plasmid PCR of 100 ng pSA-HNef-6His and pSA-HNef-6His-RIL using pSA-NheI-R and pSA-F primers (Table 3) and 25 cycles. The resulting 5.914kb (pSA-6His) and 5.853kb (pSA-6His-RIL) PCR-amplified vector backbones were reaction cleaned-up and restricted with NheI (NEB, # R0131L) and Sacl (NEB, #R0156L) for 8h at 37°C. Ten units of DpnI (NEB, #R0176) and 1 unit of Shrimp Alkaline Phosphatase (NEB, #M0371) was added to the same reaction and incubated for 60min at 37°C. The vector backbone was then purified from 1% agarose gel. In two separate reactions, the insert (586bp vif) and vectors (5.914kb pSA-6His or 5.853kb pSA-6His-RIL) were ligated, transformed into chemically competent DH5α E. coli cells, and selected on ampicillin-containing LB-agar plates after 18h incubation at 30°C. Ten randomly selected bacterial colonies were subjected to colony PCR using vector-specific pMXB10-up101-F and insert-specific Vif-R primers (Table 3). Transformants that contained the amplicons of expected size by PCR were then verified using DNA restriction and sequence analyses.

Construction of pSA-C6His-RIL

Vector pSA-C6His-RIL was constructed by replacing nef gene with a multiple cloning site (MCS). The MCS introducing BglII, Nhel, EcoRI, NruI, SacII, SalI, and NotI restriction sites was constructed by annealing two oligonucleotides, MCS-F and MCS-R (Table 3). The oligonucleotides anneal to create NdeI and Sacl cohesive ends for ligation into a pSA-6His-RIL vector restricted with 5’ NdeI and 3’ Sacl sites, maintaining the correct frame for the C-terminal 6His tag and stop codon. The MCS-F and MCS-R oligonucleotides were resuspended in annealing buffer (10mM Tris, pH 7.5–8.0, 50mM NaCl, 1 mM EDTA) and mixed in equimolar concentrations (200μM each) in 50μl reaction. The annealing reaction was incubated for 5 min in a hot block at 90°C. The block was removed from the heat source and the reaction was left to gradually cool to room temperature for 45 min and stored at -20°C. The pSA-HNef-6His-RIL vector (5μg) was restricted with NdeI/ Sacl at 37°C for 4h and 5.837kb vector backbone (pSA-6His-RIL) was purified from 1% agarose gel. Annealed oligonucleotides were diluted 1:10 with nuclease-free water and ligated with NdeI/ Sacl-restricted vector in a 4:3 molar ratio. Ligation reaction was transformed into DH5α/ E. coli cells, and selected on ampicillin-containing...
LB-agar plates after 18h incubation at 30°C. Randomly selected bacterial colonies were used to prepare plasmid minipreps, which were subjected to DNA restriction and sequence analyses.

Expression of Nef/P24/Vif in pSA-HNef/P24/Vif-6His or pSA-HNef/P24/Vif-6His-RIL-transformed NiCo21(DE3) E. coli

Sequencing-confirmed pSA-HNef/P24/Vif-6His or pSA-HNef/P24/Vif-6His-RIL was transformed into chemically competent NiCo21(DE3) E. coli and transformants selected on ampicillin-containing LB-Agar plates. For expression experiments, a single colony from a freshly streaked (18–22h) plate was inoculated into 10 ml of ampicillin-supplemented LB broth. The starter culture was grown at 30°C while shaking at 250rpm until OD₆₀₀ reached to ~1.0. The cultures were centrifuged at 3000xg for 10 min, re-suspend into fresh ampicillin-containing LB broth, and used to inoculate the main culture at a 1:20 dilution (~0.5 OD₆₀₀) in a baffled flask. The cultures were grown at 30°C while shaking at 250 rpm until OD₆₀₀ reached to 0.5–0.6. The cultures were then equilibrated to induction temperature and expression was induced with various concentrations of Isopropylthio-β-galactoside (IPTG). Induced cultures were grown for different time lengths (6h at 30°C; 12h at 22°C; 16h at 18°C) before pelleting bacterial cells by centrifugation at 5000xg for 10 min in pre-weighed centrifuge tubes/bottles.

Expression of proteins in NiCo21(DE3) transformed with pACYC-RIL, pRARE2, and pLysSRARE2

NiCo21(DE3) bacteria were individually transformed with pACYC-RIL, pRARE2, and pLysSRARE2 vectors and the transformants selected on chloramphenicol-containing LB agar plates. Transformants were grown and competent cells were prepared according to the method of Inoue [20]. The pACYC-RIL, pRARE2, and pLysSRARE2-containing NiCo21(DE3) were then transformed with pSA-HNef/P24/Vif-6His vectors and the transformants selected on LB agar plates containing both ampicillin and chloramphenicol. Cultures were expanded and the expression protocol given above was essentially followed.

Bacterial cell lysis and protein extraction

To every gram of bacterial cell pellet, 4ml of B-PER extraction reagent (Thermo Scientific, #78248) supplemented with DNase I (Thermo Scientific, #90083) and protease cocktail (Thermo Scientific, #87785) was added. The suspension was incubated at 22°C for 60min with gentle shaking. Soluble and insoluble proteins were partitioned by centrifuging bacterial cell lysate at 15,000xg for 10 min at 4°C. Clear supernatant containing soluble proteins was passed through a 0.45μm membrane (Millipore, #HPWP04700) and used for immobilized-metal affinity chromatography (IMAC). Insoluble lysed bacterial biomass was resuspended to its original volume with Tris-buffered saline (TBS). Both, soluble and insoluble fractions were stored in small aliquots at -80°C for further analysis.

Protein assays, SDS-PAGE, and immunoblotting

Total protein was quantified using the Qubit Protein Assay Kit (Life Technologies – Invitrogen, # Q33211) in a Qubit fluorometer (Life Technologies – Invitrogen). For SDS-PAGE separation, protein fractions were mixed with 5x reducing sample buffer (Thermo Scientific, #39000), resolved on 12% (w/v) polyacrylamide gels, and visualized by staining with Coomassie blue G250 [21]. For immunoblot analysis, proteins were electrobotted onto Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences, #RPN2020D). The membrane was washed in Tris-buffered saline (TBS) for 5 min, blocked with 5% nonfat milk in TTBS (TBS with 0.1% Tween 20) for
1h by shaking at room temperature, and probed with either primary anti-Nef MAb (Thermo Scientific, #MA1-71507) or anti-His MAb (Thermo Scientific, #MA1-213157) with shaking at 4°C overnight. After washing with TTBS, membrane was incubated with secondary HRP-conjugated IgG (H+L) antibody (Thermo Scientific, #32430) and protein bands were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, #34080). Images were captured using FluorChem M Imager (Protein Simple).

Purification of recombinant proteins

Recombinant HIV-1 Nef protein was purified in two steps. First, the soluble protein fraction was pre-adsorbed onto chitin resin (NEB, #S6651) to capture bacterial Histidine-rich proteins. An appropriate amount of Chitin beads was added into Econo-Pac Chromatography Column (BioRad, #732–1010) and equilibrated in two resin-bed volumes of Equilibration/Wash Buffer (50mM sodium phosphate, 500mM sodium chloride and 10mM imidazole, pH 7.4). Soluble protein fraction was mixed with an equal volume of Equilibration/Wash Buffer and added to the equilibrated chitin beads (1 ml of chitin resin for each volume of lysate corresponding to 1 gram of NiCo21(DE3) cell pellet). The column was then placed on an end-over-end rotator and revolved for 30min at 4°C. Void volume containing target protein was eluted by gravity flow and used for IMAC purification step.

For IMAC, HisPur Cobalt resin (Thermo Scientific, #89965) was used following the manufacturer’s protocol. Briefly, an appropriate amount of cobalt resin was added in a 15ml centrifuge tube and washed with two resin-bed volumes of Equilibration/Wash Buffer. The chitin bead pre-adsorbed soluble protein fraction was combined with equilibrated cobalt resin and mixed on an end-over-end rotator for 60min at 4°C. The resin was then washed with Equilibration/Wash Buffer until the absorbance at 280nm reached to the baseline. Bound protein was eluted using one resin-bed volume of Elution Buffer (50mM sodium phosphate, 500mM sodium chloride, 150mM imidazole, pH 7.4). This step was repeated 2–3 times while saving individual fractions. Fractions were analyzed by SDS-PAGE/Western blot, dialyzed against phosphate buffered saline (PBS) using Slide-A-Lyzer Dialysis Cassettes, 7K MWCO (Thermo Scientific, #66710) and stored at -80°C in small aliquots.

Results

Construction of a plasmid expressing HIV-1 Nef

pSA-HNef-6His vector (Fig 1) used to produce HIV-1 Nef protein was constructed by modifying the pSA-Hp24-6His vector [13]. The HIV-1 p24 coding sequence was removed by restricting the plasmid with NdeI and SacI, and replaced with HIV-1 nef coding sequence at the same sites. The resulting 6.517kb vector was verified by DNA restriction and sequence analyses (data not shown). The mRNA sequence encoding the nef gene is transcribed from an inducible T7 promoter and the resulting protein has a C-terminal 6His tag for IMAC-mediated purification.

Expression of Nef in pSA-HNef-6His-transformed NiCo21(DE3) E. coli

E. coli NiCo21(DE3) was used to express 6His-tagged HIV-1 Nef using shaker flask culture conditions for expression. Plasmid-borne retroviral sequences are unstable in E. coli when grown at 37°C [22]. Therefore, the cultures of pSA-HNef-6His-transformed NiCo21(DE3) were grown at 30°C until the OD600 reached 0.5–0.6. Cultures were then induced with 0.4mM IPTG and incubated for another 6h at 30°C. When subjected to SDS-PAGE analysis, the expressed Nef protein appeared as an approximately 27kDa band in the IPTG-induced fractions (Fig 2A). Overexpressed Nef protein was mainly present in the soluble fraction. However,
immunoblot analysis with the anti-Nef antibody revealed that some Nef was also present in the insoluble fraction (Fig 2B).

Fig 1. Schematic representation of expression vector pSA-HNef-6His. The nef gene was PCR amplified from pNL4.3 plasmid, restricted with NdeI/SacI, and ligated into pSA-6His vector at the same sites.

doi:10.1371/journal.pone.0130446.g001

Fig 2. SDS-PAGE and immunoblot analysis of recombinant Nef. NiCo21(DE3) E. coli were transformed with pSA-HNef-6His vector and grown overnight from a single colony at 30°C in LB broth supplemented with 100 μg/ml ampicillin. The cultures were diluted 100-fold in the same medium and grown to mid-log phase (OD600 ~0.5–0.6), at which point IPTG was added to a final concentration of 0.4 mM. The induced cells were grown for another 6 h at 30°C and stored on ice. Nine microliters of samples were mixed with 4 x loading dye, electrophoretically resolved on a 12% SDS-PAGE gel and analyzed by (A) Coomassie staining and (B) immuno-blotting. Lanes: M, PageRuler Prestained Protein Ladder Plus; WCL, whole cell lysate; IS, insoluble fraction; S, soluble fraction; M1, MagicMark XP Western Protein Standard. Recombinant Nef was produced in the induced cells but not in the un-induced controls, and mainly present in the soluble fraction.

doi:10.1371/journal.pone.0130446.g002
Optimization of IPTG concentration and induction temperature

In order to optimize Nef expression, we tested ranges of IPTG concentrations (0–0.4mM) and incubation temperatures (30, 22, and 18°C). Expression of HIV-1 Nef was optimal at the lowest tested concentration of IPTG i.e. 0.05mM as determined by the western blot analysis (Fig 3A). HIV-1 Nef production was similar in 0.05mM IPTG-induced cultures when grown at 30°C and 22°C, but lower in 18°C-grown cultures (Fig 3B). However, neither IPTG concentration nor various incubation temperatures enhanced overall Nef production in E. coli NiCo21(DE3) cells.

**Fig 3. Optimization of IPTG concentration and induction temperature.**

(A) For optimization of IPTG concentration, the overnight cultures of pSA-HNef-6His-transformed NiCo21(DE3) were diluted 1:100 in LB +Amp (100 μg/ml) and grown to mid-log phase (OD₆₀₀ ~0.5–0.6). The cultures were then induced with varying concentrations (0, 0.05, 0.1, 0.2, 0.3, and 0.4 mM) of IPTG and grown for another 6 h at 30°C. Nine microliters of samples were mixed with 4x loading dye, electrophoretically resolved on a 12% SDS-PAGE gel and analyzed by immuno-blotting. A concentration of 0.05 mM IPTG (red arrow) was sufficient to induce high level Nef expression. (B) For optimal induction temperature, the overnight cultures of pSA-HNef-6His-transformed NiCo21(DE3) were diluted 1:100 in LB+Amp (100 μg/ml) and grown to mid-log phase (OD₆₀₀ ~0.5–0.6). The cultures were then induced with 0.05 mM of IPTG and grown at 30, 22, and 18°C for 6, 12, and 16 h respectively. Nine microliters of samples were mixed with 4 x loading dye, electrophoretically resolved on a 12% SDS-PAGE gel and analyzed by Coomassie staining. Nef expression was similar at 30 and 22°C but lower at 18°C. Lanes: M, BenchMark Pre-stained protein ladder; M1, PageRuler Prestained Protein Ladder Plus; 1, un-induced NiCo21 at 30°C (soluble); 2, IPTG-induced NiCo21 at 30°C (insoluble); 3, IPTG-induced NiCo21 at 30°C (soluble); 4, un-induced NiCo21 at 22°C (soluble); 5, IPTG-induced NiCo21 at 22°C (insoluble); 6, IPTG-induced NiCo21 at 22°C (soluble); 7, un-induced NiCo21 at 18°C (soluble); 8, IPTG-induced NiCo21 at 18°C (insoluble); 9, IPTG-induced NiCo21 at 18°C (soluble).

doi:10.1371/journal.pone.0130446.g003
### A

| Amino acid | Rare codon(s) | Amino acid position in Nef ORF |
|------------|---------------|--------------------------------|
| *Arginine (Arg)* | AGG | 17,184 |
| | AGA | 19, 21, 77, 105, 106, 134, 178 |
| | CGA | 22, 35, 196 |
| **Leucine (Leu)** | CTA | 37, 58, 100, 145, 189 |
| **Isoleucine (Ile)** | ATA | - |
| **Proline (Pro)** | CCC | - |

*Tandem rare Arg codon double repeats = 2 (22, 106)*

### B

| pSA-HNef-6His | pSA-HNef-6His | pSA-HNef-6His | pSA-HNef-6His |
|---------------|---------------|---------------|---------------|
| + pACYC-RIL   | + pRARE2      | + pRARE2-lysS|
| M             | WCL           | IS            | S             |
| S             | WCL           | IS            | S             |
| S             | WCL           | IS            | S             |

**Fig 4. Effect of rare tRNA supplementation on Nef expression in NiCo21(DE3) E. coli.** (A) ORF coding for HIV-1 nef gene was subjected to rare codon analysis using an online tool 'Rare Codon Calculator (RaCC)' [http://nihserver.mbi.ucla.edu/RACC/](http://nihserver.mbi.ucla.edu/RACC/). Three rare codons encoding arginine and one rare codon encoding leucine are present at 17 amino acid positions in Nef ORF. (B) NiCo21(DE3) E. coli were individually transformed with rare tRNA-expressing helper plasmids (pACYC-RIL, pRARE2, pRARE2-lysS) and selected on LB+Cam. These bacteria were then made competent, transformed with pSA-HNef-6His, and selected on LB+Cam+Amp plates. Cultures were grown overnight from a single colony at 30°C in LB+Cam+Amp. The cultures were then diluted 100-fold in the same medium and grown to mid-log phase (OD$_{600}$ ~0.5–0.6), at which point IPTG was added to a final concentration of 0.05 mM. The induced cells were grown for another 12 h at 22°C and stored on ice. Nine microliters of samples were mixed with 4 x loading dye, electrophoretically resolved on a 12% SDS-PAGE gel analyzed by Coomassie staining. Expression of rare codon tRNA genes resulted in high level expression of Nef. Lanes: M, PageRuler Prestained Protein Ladder Plus; WCL, whole cell lysate; IS, insoluble fraction; S, soluble fraction.

**Effect of rare tRNA supplementation on Nef expression in NiCo21(DE3) E. coli**

Over-expression of heterologous proteins in *E. coli* may be considerably circumscribed by the presence of "rare" codons in the foreign mRNA that are seldom used by *E. coli* [23, 24]. When subjected to 'rare' codon analysis, the 618bp coding sequence for HIV-1 Nef was found to contain (a) three rare codons (AGG, AGA, CGA) for arginine at positions 17, 19, 21, 22, 35, 77, 105, 106, 134, 178, 184, 194, and (b) one rare codon (CTA) for leucine at positions 37, 58, 100, 145, and 189 (Fig 4A). To investigate whether Nef expression could be improved by expressing nef together with rare tRNA genes, NiCo21(DE3) were transformed with rare tRNA expressing pACYC-RIL, pRARE2, and pLysSRARE2 helper plasmids. The pACYC-RIL plasmid supplies tRNA for four rare codons (AUA, AGG, AGA, CUU), whereas pRARE2 supplies those for
seven rare codons (AUA, AGG, AGA, CUA, CCC, CGG, and GGA). In addition to seven rare codons, transformation with pLysSRARE2 also results in lower background expression due to the expression of T7 lysozyme. When expressed in the presence of rare tRNA supplying helper plasmids, Nef expression strikingly improved as shown in Fig 4B. However, this also led some Nef protein to end up in the insoluble fractions, suggesting that bacterial protein folding machinery was saturated by the enhanced expression. There was no significant change in Nef expression between the NiCo21(DE3) containing pACYC-RIL and pRARE2/pLysSRARE2, suggesting that supplementation with four rare codons was sufficient to optimize Nef expression. In a side-by-side comparison, NiCo21(DE3)/pACYC-RIL showed relatively higher production of soluble Nef (Fig 4B).

In attempts to shift the relative soluble/insoluble protein production, protein expression was tested at 18°C, but it did not alter the distribution of recombinant protein between soluble and insoluble fractions, nor did the production of soluble Nef improve further (data not shown). Another modification was tested by the addition of 2–3% ethanol to the culture medium [25, 26] and incubation at 42°C [27]. These conditions lead to overexpression of bacterial chaperones, which may result in improved folding and enhanced solubility of overexpressed recombinant proteins. However these approaches failed to generate more soluble Nef with our production system (data not shown). Expression of Nef protein in the presence or absence of rare tRNA genes had minimal effect on the bacterial growth as determined by the total biomass yield (~12 g/L and 12.5 g/L for NiCo21(DE3) transformed with pSA-HNef-6His, and pSA-HNef-6His + pACYC-RIL respectively, when grown in LB broth.

Construction of pSA-HNef-6His-RIL for concomitant expression of Nef and rare tRNA genes

In order to alleviate issues associated with coexistence of tRNA helper plasmids with expression plasmids, we modified pSA-HNef-6His to express rare tRNA genes in concomitant with nef gene. The modified pSA-HNef-6His-RIL plasmid was constructed by exchanging 936bp nonessential DNA fragment between the lacI gene and T7 promoter with 874bp DNA fragment containing argU, ileY, and leuW rare tRNA genes. Blunt-end ligation of rare tRNA gene fragment into the vector resulted in a plasmid with tRNA gene array arranged in either clockwise (CW) or counter-clockwise (CCW) orientation relative to the T7 promoter (Fig 5A and 5B).

Expression of Nef in NiCo21(DE3) transformed with pSA-HNef-6His-RIL

We expressed Nef in E. coli transformed with pSA-HNef-6His-RIL(CW)/(CCW) and compared Nef production with E. coli transformed with pSA-HNef-6His (served as negative control), and pSA-HNef-6His together with pACYC-RIL (served as positive control). Expression experiments were carried out using shaker flask culture conditions for expression as described above. When subjected to SDS-PAGE analysis, Nef expression in bacteria containing pSA-HNef-6His-RIL was comparable with those that contained both pSA-HNef-6His and pACYC-RIL. Little Nef produced in bacteria that harbored pSA-HNef-6His vector (Fig 6A). There was noticeable background expression in un-induced cultures from pSA-HNef-6His-RIL (CW) compared to pSA-HNef-6His-RIL (CCW) (green arrow, Fig 6A and 6B). This suggests that in addition to T7 promoter, Nef was also expressed under the control of some promoter upstream of T7 promoter, most likely of ileY (Fig 5B). As a result, the pSA-HNef-6His-RIL (CCW) was not used in subsequent expression experiments. Samples from pSA-HNef-6His+ pACYC-RIL showed a prominent 25kDa band present in both un-induced, and IPTG-induced cultures (red arrows, Fig 6A). This band most probably represents chloramphenicol acetyltransferase monomer expressed from pACYC-RIL vector. Proteins were also subjected to
immunoblot analysis (Fig 6B) using anti-Nef MAb as primary antibody and relative band intensities were determined and plotted as shown in Fig 6C. Together, these experiments show that recombinant protein can be produced from a single vector concomitantly expressing rare tRNA and a heterologous gene, and in amounts comparable (or better) to traditional two-vector system.

Construction of pSA-HP24-6His-RIL for concomitant expression of P24/CA and rare tRNA genes

In order to further confirm the hypothesis that recombinant protein(s) can be produced in similar or better amounts from a single vector concomitantly expressing rare tRNA and a heterologous gene compared to traditional two-vector system, we expressed HIV-1 p24 gene. HIV-1 p24 gene contains (a) three rare codons (AGG, AGA, CGA) for arginine at positions 44, 59, 62, 94, 105, 116, 124, 129, 135, 191; (b) one rare codon (CTA) for leucine at positions 18, 134, 173; (c) one rare codon (ATA) for isoleucine at positions 66, 77, 96, 103, 115; and (d) one rare codon (CCC) for proline at positions 122, 186. We have previously shown that pACY-C-RIL plasmid significantly helped with improving the overall yields of P24/CA [13]. Here we
replaced the nef gene in pSA-HNef-6His-RIL with p24 and compared the expression profile of P24/CA from pSA-HP24-6His, pSA-HP24-6His together with pACYC-RIL, and pSA-HP24-6His-RIL (Fig 7). The helper plasmids pRARE2/pRARE2 lysS were omitted because in the previous work they were found not to offer any advantage over pACYC-RIL helper plasmid [13]. As anticipated, almost 2 fold more P24/CA was expressed in cultures containing pSA-HP24-6His+pACYC-RIL and pSA-HP24-6His-RIL. The SDS-PAGE (Fig 8A), Western Blot (Fig 8B), and densitometric (Fig 8C) analyses showed that relatively more p24/CA was produced from the single plasmid (pSA-HP24-6His+pACYC-RIL) compared to traditional two plasmid (pSA-HP24-6His+pACYC-RIL) system.
Construction of pSA-HVif-6His-RIL for concomitant expression of Vif and rare tRNA genes

To further demonstrate the utility of the single plasmid concurrently expressing gene of interest and rare tRNA genes, we exchanged nef gene with HIV-1 vif gene in pSA-HNef-6His and pSA-HNef-6His-RIL vectors. The vif gene contains (a) two rare codons (AGG, AGA) for arginine at positions 6, 17, 19, 25, 35, 43, 79, 92, 95, 123, 134, 169, 175, 186; (b) one rare codon (CTA) for leucine at positions 61, 104, 108, 147, 152; (c) one rare codon (ATA) for isoleucine at positions 53, 68, 89, 122, 126, 130, 156, 161; and (d) one rare codon (CCC) for proline at positions 179. Expression of Vif was compared between the cultures that contained pSA-HVif-6His, pSA-HVif-6His together with pACYC-RIL, and pSA-HVif-6His-RIL (Fig 7). As shown in Fig 9A, 9B and 9C, around two fold more Vif was produced in cultures containing pSA-HVif-6His+pACYC-RIL and pSA-HVif-6His-RIL compared to pSA-HVif-6His. Consistent with previous observations for Nef and P24, relatively more Vif was produced from the vector that expresses both the heterologous gene and rare tRNA genes. However, unlike Nef and P24, Vif expressed as insoluble protein and optimizing the IPTG concentration and temperature did not yield any soluble Vif (data not shown).

Growth profile, expression, and purification of HIV-1 Nef and P24 in NiCo21(DE3) transformed with pSA-HNef-6His-RIL and pSA-HVif-6His-RIL

By expressing three rare codon –contain HIV-1 genes i.e. nef, p24, and vif, we showed the suitability of our proposed vector that concomitantly expresses the gene of interest and rare tRNA genes. Here we compared the growth profile, expression, and purification HIV-1 Nef and P24.
between traditional two-plasmid system with that of single expression plasmid system. Because HIV-1 Vif failed to express as soluble protein, we have omitted it from this set of experiments.

Bacterial cultures were grown using optimized conditions i.e. super broth (SB) as culture medium, 0.05mM IPTG as inducer, and cultivation at 22°C for 12h. Cells were harvested and optical densities at 600nm and total biomass yield were determined. Lysed bacterial pellets were used to purify Nef and P24/CA as described in Materials and Methods. The cultures were grown overnight at 30°C in LB broth containing Amp (for bacteria harboring pSA-HP24-6His or pSA-HP24-6His-RIL) or Amp+Cam (for bacteria harboring pSA-HP24-6His + pACYC-RIL). The cultures were then diluted 100-fold in the same medium and grown to mid-log phase (A600 ~0.5–0.6), at which point IPTG was added to a final concentration of 0.05mM. The induced cells were grown for another 12 hours at 22°C and stored on ice. Three microliters of samples were mixed with 4X loading dye, electrophoretically resolved on a 12% SDS-PAGE gel and analyzed by Coomassie staining (A), immuno-blotting (B), and band densitometry (C). The P24 expression in NiCo21 transformed with pSA-HP24-6His-RIL was comparable with NiCo21 co-transformed with pSA-HP24-6His and pACYC-RIL.

doi:10.1371/journal.pone.0130446.g008
SB, and induced with 0.05mM IPTG for 12h at 22°C. There appeared no difference in the final yields and purity of Nef or P24 produced by single (pSA-HNef/P24-6His-RIL) and traditional double-vector (pSA-HNef/P24-6His + pACYC-RIL) system.

Discussion

We have engineered an expression vector that concomitantly expresses the heterologous protein of interest, and rare tRNA genes in E. coli. We started off with cloning HIV-1 nef gene in an expression vector pSA-HP24-6His, which has previously been used for high level expression of HIV-1 P24 [13]. Nef is an accessory protein of HIV that is responsible for a number of viral pathogenic effects. Progression to AIDS is delayed and in some well-documented cases abolished on infection with naturally occurring HIV strains that contain truncated or mutated nef sequences in their genomes [28, 29]. It has been proposed that specific Nef inhibitors could potentially abrogate or slow down gradual deterioration of immune system in chronically infected patients in absence of functional cure [14, 15, 16, 17, 18, 19]. We are engineering
therapeutic antibodies against HIV-1 Nef, and therefore need large amounts of highly purified recombinant protein.
Little Nef was produced when expressed in NiCo21(DE3) E. coli, and optimization of expression conditions had no effect on the final yield. We then analyzed nef gene for the presence of codons rarely used in E. coli, and found that nef contained 8.21% of such codons. We therefore anticipated that co-expression of rare tRNA genes from a helper plasmid could help with high level Nef expression. Indeed, almost 8-fold increase in expression was achieved when Nef was produced in the presence of a rare tRNA-expressing helper plasmid (pACYC-RIL).

Nef expression is toxic towards expression hosts such as bacteria and yeast [30, 31], though the exact mechanism of this toxicity is not known. We observed that Nef expression was toxic towards E. coli but only when it was expressed in the absence of rare tRNA genes. We anticipate that this could be due to the misincorporation or omission of one or several rare tRNA-coded arginine and/or lysines, which results in the production of mutant Nef protein(s) that is toxic towards the expression host. It is also possible that rare tRNAs get sequestered by ribosomes engaged in the translation of rare codon–containing heterologous gene that may affect the cell’s ability to produce essential endogenous protein needed for optimal cell growth.

In order to construct a vector that could concomitantly express the nef and the rare tRNA genes, we modified the backbone of the resulting pSA-HNef-6His vector by replacing a non-essential DNA segment between lacI gene and T7 promoter with argU, ileY, and leuW tRNA genes. The resultant vector was called pSA-HNef-6His-RIL, which was then used to produce high levels of recombinant HIV-1 Nef protein. In addition to nef gene, we also cloned HIV-1 p24 and vif, two genes that contain 10.77 and 14.5% rare codons respectively. In side-by-side experiments we showed that the p24 and vif genes expressed from single pSA-P24/Vif-6His-RIL vector produced similar or better levels of recombinant proteins compared to traditional two vector system in which rare tRNA genes are expressed from a ColE1–compatible helper plasmid such as pACYC-RIL.

It would be interesting to note that protein expression in presence of rare tRNA genes yielded two fold more P24 and Vif compared to eight fold more Nef, whereas the p27 (nef) gene contains fewer rare codons (8.25% of total codons) compared to p24 (10.77% of total codons) and vif (14.5%) genes. Moreover, HIV-1 p27(nef) gene contains rare codons for two amino acids arginine and leucine, whereas p24 and vif genes contain rare codons for four amino acids arginine, leucine, isoleucine, and proline. These observations suggest that neither total number of rare codons nor all the rare codons affect the heterologous protein expression in E. coli. Upon close examination we noticed four rare codons (the last two as tandem repeat) arranged in a cluster in the 5’ of p27 (nef) gene encoding arginine (highlighted region in S1A Fig). There are another two rare codons for arginine also arranged as tandem repeat in the middle of the nef gene (highlighted region in S1A Fig). We did not notice similar clustering of arginine–encoding rare codons in either p24 or vif genes (S1B and S1C Fig). These observations are in agreement with previous findings that not all rare codons affect the heterologous protein expression and it is the priority among those rare codons (for instance those encode for arginine), their position in the gene, and tandem arrangement that affects the protein expression in E. coli [32].

To facilitate clone manipulation, we substituted nef gene with a multiple cloning site (MCS) clustered with eight unique restriction enzyme sites (Fig 11). Any two of the restriction enzyme sites can be chosen to clone gene of interest into the pSA-C6His-RIL vector. However it will be desirable to clone the gene of interest between Ndel and SacI, in order to avoid the addition of extra amino acids at N- and/or C-terminals of the resultant recombinant protein. Another possibility is to introduce desired restriction enzyme sites to the plasmid using whole-plasmid PCR. Several high fidelity polymerases suitable for whole-plasmid PCR are now available at reasonable cost. Whenever possible, we amplify the entire plasmid backbone with a set of primers containing desired restriction enzyme sites using Phusion or Q5 High-Fidelity DNA.
Polymerases. By using 50–100 ng plasmid vector as template, and using 18–25 PCR cycles, it is possible to obtain sufficient amount of PCR amplified plasmid, which can be used to clone the gene of interest with compatible restriction enzymes sites. To reduce the background, we add 10U of DpnI restriction enzymes and 1U of alkaline phosphatase in the reaction. This treatment effectively eliminates the bacterially-produced methylated-plasmid template, and dephosphorylates the ends of the restricted PCR amplified plasmid vector.

Eschenfeldt and co-workers have recently described the construction of LIC expression vectors containing rare tRNA genes [33]. These vectors contain tRNA genes covering rare codons for arginine (AGG/AGA) and isoleucine (AUA), and accept heterologous genes via LIC (ligation independent cloning). While these vectors are useful, they are limited in their utility due to the presence of only two rare tRNA genes. Furthermore, albeit of its advantages over restriction enzyme–based cloning, LIC is not a widely used method in various labs. The expression vector described in the present study contains three rare tRNA genes and therefore suitable for the expression of an assortment of heterologous genes in E. coli. We understand that cloning and expression of heterologous genes in the described rare tRNA-containing expression vector will save both time, and cost, and prove a useful addition to the existing tools/reagents available to scientific community.

**Supporting Information**

S1 Fig. Rarely used codons in HIV-1 nef, p24, and vif genes. occurrence of rarely used codons in HIV-1 (NL4.3) nef (A), p24 (B) and vif (C) genes.

(TIFF)
Author Contributions
Conceived and designed the experiments: SAA NMY. Performed the experiments: SAM SYT TCO YWC SAA. Analyzed the data: SAM SYT SAA. Contributed reagents/materials/analysis tools: SAA NMY. Wrote the paper: SAA.

References
1. Del Tito BJ Jr, Ward JM, Hodgson J, Gershater CJ, Edwards H, Wysocki LA, et al. Effects of a minor isoleucyl tRNA on heterologous protein translation in Escherichia coli. J Bacteriol. 1995; 177: 7086–7091. PMID: 8522513
2. Kane JF. Effects of rare codon clusters on high-level expression of heterologous proteins in Escherichia coli. Curr Opin Biotechnol. 1995; 6: 494–500. PMID: 7579660
3. Kim S, Lee SB. Rare codon clusters at 5’-end influence heterologous expression of archaeal gene in Escherichia coli. Protein Expr Purif. 2006; 50: 27: 365–374. PMID: 12597898
4. McNulty DE, Claffee BA, Huddleston MJ, Porter ML, Cavnar KM, Kane JF. Mistranslational errors associated with the rare arginine codon CGG in Escherichia coli. Protein Expr Purif. 2003; 27: 625–632. doi: 10.1002/pro.2046 PMID: 12597898
5. Huang Y, O’Mara B, Conover M, Ludwig R, Fu J, Tao L, et al. Glycine to glutamic acid misincorporation observed in a recombinant protein expressed by Escherichia coli cells. Protein Sci. 2012; 21: 625–632. doi: 10.1002/pro.2046 PMID: 22362707
6. Puigbó P, Guzmán E, Romeu A, Garcia-Vallvé S. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res. 2007; 35: W126–131. PMID: 17439967
7. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol. 2006; 72: 211–222. PMID: 16791589
8. Sorensen MA, Kurland CG, Pedersen S. Codon usage determines translation rate in Escherichia coli. J Mol Biol. 1989; 207: 365–377. PMID: 2474074
9. Kolmsee T, Henge R. Rare codons play a positive role in the expression of the stationary phase sigma factor RpoS (σ(S)) in Escherichia coli. RNA Biol. 2011; 8: 913–921. doi: 10.4161/ma.8.5.16265 PMID: 21788735
10. Bentley WE, Mirjalili N, Andersen DC, Davis RH, Kompala DS. Plasmid-encoded protein: the principal factor in the “metabolic burden” associated with recombinant bacteria. Biotechnol Bioeng. 1990; 35 (7):668–681. PMID: 18929563
11. Perrakis A, Romier C. Assembly of protein complexes by coexpression in prokaryotic and eukaryotic hosts: an overview. Methods Mol Biol. 2008; 426:247–256. doi: 10.1007/978-1-60327-058-8_15 PMID: 18542868
12. Samuelson JC. Recent developments in difficult protein expression: a guide to E. coli strains, promoters, and relevant host mutations. Methods Mol Biol. 2011; 705:195–209. doi: 10.1007/978-1-61737-967-3_11 PMID: 21125397
13. Teow SY, Mualif SA, Omar TC, Wei CY, Yusoff NM, Ali SA. Production and purification of polymerization-competent HIV-1 capsid protein p24 (CA) in NCo21(DE3) Escherichia coli. BMC Biotechnol. 2013; 13:107. doi: 10.1186/1472-6750-13-107 PMID: 24304876
14. Lülf S, Matz J, Rouvey MC, Järviilouma A, Saksela K, Benichou S, et al. Structural basis for the inhibition of HIV-1 Nef by a high-affinity binding single-domain antibody. Retrovirology. 2014; 11: 24. doi: 10.1186/1742-4690-11-24 PMID: 24620746
15. Iyer PC, Zhao J, Emernt-Sedlak LA, Moore KK, Smithgall TE, Day BW. Synthesis and structure-activity analysis of diphenylpyrazolodiazene inhibitors of the HIV-1 Nef virulence factor. Bioorg Med Chem Lett. 2014; 24: 1702–1706. doi: 10.1016/j.bmcl.2014.02.045 PMID: 24650642
16. Smithgall TE, Thomas G. Small molecule inhibitors of the HIV-1 virulence factor, Nef. Drug Discov Today Technol. 2013; 10: e623–e629. doi: 10.1016/j.ddtec.2013.07.002 PMID: 24451644
17. Emernt-Sedlak LA, Narute P, Shu ST, Poe JA, Shi H, Yanamala N, et al. Effector kinase coupling enables high-throughput screens for direct HIV-1 Nef antagonists with antiretroviral activity. Chem Biol. 2013; 20: 82–91. doi: 10.1016/j.chembiol.2012.11.005 PMID: 23352142
18. Witkowski W, Verhasselt B. Contributions of HIV-1 Nef to immune dysregulation in HIV-infected patients: a therapeutic target? Expert Opin Ther Targets. 2013; 17:1345–1356. doi: 10.1517/14728223.2013.830712 PMID: 23967871
19. Bouchet J, Basmaciogullari SE, Chrobak P, Stolp B, Bouchard N, Fackler OT, et al. Inhibition of the Nef regulatory protein of HIV-1 by a single-domain antibody. Blood. 2011; 117: 3559–3568. doi: 10.1182/blood-2010-07-296749 PMID: 21292773

20. Sambrook J, Russel D. Molecular cloning: a laboratory manual. 4th Edition, Cold Spring Harbor, New York; 2013.

21. Lawrence AM, Besir HU. Staining of proteins in gels with Coomassie G-250 without organic solvent and acetic acid. J Vis Exp. 2009; 30: 1350. doi: 10.3791/1350 PMID: 19684570

22. Trinh T, Jeeen J, Bloom F, Hirsch V. STBL2: an Escherichia coli strain for the stable propagation of retroviral clones and direct repeat sequences. Focus. 1994; 16: 78–80.

23. Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol. 2005; 115: 113–128. PMID: 15607230

24. Angov E, Hillier CJ, Kincaid RL, Lyon JA. Heterologous protein expression is enhanced by harmonizing the codon usage frequencies of the target gene with those of the expression host. PLoS One. 2008; 3: e2189. doi: 10.1371/journal.pone.0002189 PMID: 18478103

25. Thomas JG, Baneyx F. Divergent effects of chaperone overexpression and ethanol supplementation on inclusion body formation in recombinant Escherichia coli. Protein Expr Purif. 1997; 11: 289–296. PMID: 9425634

26. Winter J, Neubauer P, Glockshuber R, Rudolph R. Increased production of human proinsulin in the periplasmic space of Escherichia coli by fusion to DsbA. J Biotechnol. 2001; 84: 175–185. PMID: 11090689

27. Chen J, Acton TB, Basu SK, Montelione GT, Inouye M. Enhancement of the solubility of proteins overexpressed in Escherichia coli by heat shock. J Mol Microbiol Biotechnol. 2002; 4: 519–524. PMID: 12432951

28. Zaunders J, Dyer WB, Churchill M. The Sydney Blood Bank Cohort: implications for viral fitness as a cause of elite control. Curr Opin HIV AIDS. 2011; 6: 151–156. doi: 10.1097/COH.0b013e3283454d5b PMID: 21378562

29. Gorry PR, Churchill M, Learmont J, Cherry C, Dyer WB, Wesselingh SL, et al. Replication-dependent pathogenicity of attenuated nef-deleted HIV-1 in vivo. J Acquir Immune Defic Syndr. 2007; 46: 390–394. PMID: 17993857

30. Macreadie I, Sankovich S, Failla P, Lowe M, Curtain C, Castelli L, et al. HIV-1 Nef protein causes death in stressed yeast cells due to determinants near the N-terminus and elsewhere in Nef. Biochem Mol Biol Int. 1998; 46: 277–286. PMID: 9801796

31. Macreadie IG, Lowe MG, Curtain CC, Hewish D, Azad AA. Cytotoxicity resulting from addition of HIV-1 Nef N-terminal peptides to yeast and bacterial cells. Biochem Biophys Res Commun. 1997; 232: 707–711. PMID: 9126340

32. Thangadurai C, Suthakaran P, Barfal P, Anandaraj B, Pradhan SN, Ramalingam S, et al. Rare codon priority and its position specificity at the 5’ of the gene modulates heterologous protein expression in Escherichia coli. Biochem Biophys Res Commun. 2008; 376(4):647–652. doi: 10.1016/j.bbrc.2008.09.024 PMID: 18801340

33. Eschenfeldt WH, Makowska-Grzyńska M, Stols L, Donnelly MJ, Jedrzejczak R, Joachimiak A. New LIC vectors for production of proteins from genes containing rare codons. J Struct Funct Genomics. 2013; 14: 135–144. doi: 10.1007/s10960-013-9163-9 PMID: 24057978