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A Study on Cloning and Expression of HIV-1 NEF Protein in HEK 293 Cells by Transient Transfection.

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ABSTRACT

HIV continues to be a major global public health issue, there were approximately 34 million people living with HIV in 2011. However, the development of anti-viral has blunted the AIDS epidemic in the Western world but globally the epidemic has not been curtailed. Nef is one of these accessory genes that are only present within HIV and SIV genome and thought to play a key role in the progression to AIDS. The given its central role in HIV pathogenesis, Nef considered as a potential anti-viral target for preventing or at least delaying pathogenesis. The biologically active 27 kDa myristoylated Nef protein expressed from HEK 293 cells is a protein model to be used for significantly specific antibody production to lower the pathogenicity of HIV infection. To express the this protein model, pQBI-6His a mammalian expression vector constructed with base pair 5504 to express 627 bp HIV-1 Nef under CMV promoter. It shows that targeted 27 kDa HIV-1 Nef was not successfully expressed in HEK 293 cells either in transient transfection when transfected. However, non-targeted HIV-1 Nef was detected in western blot by anti-Nef (anti mouse) manufactured by Thermo scientific. The ability of not expressing the targeted myristoylated 27 kDa nef protein was to various unpreventable factors due to time limitation and lacking of skills in the filed cloning and cell culture.

Keywords: Nef; HIV; Protein; Pathogenesis; Cell

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INTRODUCTION

Human immune deficiency virus (HIV) disease continues to be a serious health issue for parts of the world. Worldwide, there were about 2.5 million new cases of HIV in 2011. About 34.2 million people are living with HIV around the world. In 2010, there were about 1.8 million deaths in persons with acquire immunodeficiency disease syndrome (AIDS), and nearly 30 million people with AIDS have died worldwide since the epidemic began. The major public health threat throughout the world is HIV/AIDS even though it was first recognized 28 years ago[1].

HIV continues to be a major global public health issue, having claimed more than 25 million lives over the past three decades there were approximately 34 million people living with HIV in 2011. HIV infection is usually diagnosed through blood tests detecting the presence or absence of HIV antibodies. Since the initial description of the HIV-1 in 1983 [2] and [3] and HIV-2 in 1986 [4], these two viruses have been identified as the primary cause of AIDS. HVI-1 nef was reported to have negative effect on viral replication hence the name 'negative factor' or nef [5]. Although HIV nef was originally named "negative factor", it has been shown to have positive role in viral replication and pathogenesis. Most of the pathogenicity of HIV-1 can be attributed to the presence of six accessory genes coded within the viral genome; nef is an accessory gene that is only present within HIV and SIV genome and thought to play a key role in the progression to AIDS[6]. Figure 1 shows diagram of HIV virus.

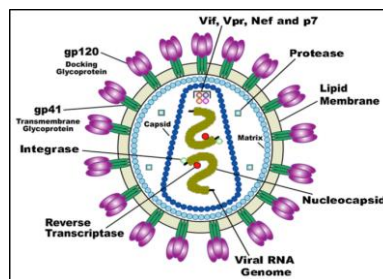


Figure 1: Diagram of the HIV virus (Adapted from, US National Institute of Health (redrawn by en: User: Carl Henderson)

EXPERIMENTAL METHODS

Construction mammalian expression vector

Designing of HIV-1 Nef-specific primers

HIV-1 Nef protein gene specific primers were designed. PCR amplification which was carried out using the set of primer, results in the introduction of *Bam*HI and *Sac*II restriction sites on 5' and 3' of the amplicons by using pQBI-Nef-GFP as a template attached in Figure 2. These restriction enzyme sites have been used for cloning into the cloning/expression vector. Primer sequence of *nef* gene is given in.

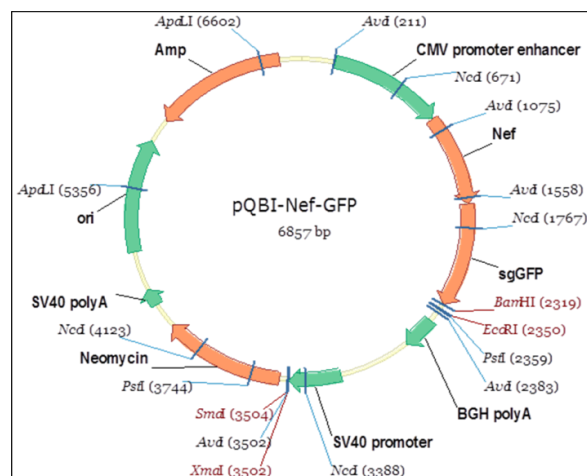


Figure 2: Cloning mapping of pQBI-Nef-GFP (Template)

Preparation Agarose Gel in TAE buffer and PCR amplification of *Nef* gene

A 1% Agarose gel was prepared by adding 0.25 grams of Agarose in 25 ml of 1X Tris Acetic Acid EDTA (TAE) buffer. The solution was heated at Median power in a microwave oven for 2 minutes and the bottle was swirled to mix Agarose until it completely dissolved. The Agarose containing flask was then placed in a 56°C water bath until the temperature was equilibrated. Ethidium bromide was then added into the Agarose to a final concentration of 0.01 mg/ml and the contents were mixed by gentle swirl. The gel was cast by pouring 25 ml of Agarose solution into the gel casting mold followed by the insertion of sample loading comb.

Nef gene 627 base pair long expressing 27 kDa protein was amplified by PCR using pQBI-Nef-GFP as a template shown in Figure 2 by using primer mixture of restriction site of *Bam*HI as reverse and *Sac*II as forward. The primer sequences for *Nef* showed Table 1 mixture was prepared by adding 5 µl of *Sac*II (introducing the restriction site) and 5 µl *Bam*HI with 40 µl of nuclease free water.

Table 1: Primers for Nef Gene

Insert	Primers	Sequences (5'-3')	Size (bp)
Nef	Reverse (BamHI)	gAT <u>ggg ATC CTC</u> CTg CAC CgC AgT TCT Tg	29
	Forward (Sac II)	CCT <u>CCg Cgg</u> gCg CgC ATg ggT ggC AAg Tgg TCA	33

The PCR reaction as given in Table 2 was assembled on ice in order to minimize nonspecific amplifications. The contents of the PCR tubes were mixed by vortex and followed by a brief spun in a micro-centrifuge.

Table 2: Components for PCR Nef amplification

Components	Volume (µl)
Nuclease free water	33.5
Kapa buffer (5x) + Mg ²⁺	10
dNTP	1.5
Primer Mixture (10 µm)	3.0
Template	1
Kapa hifi enzyme	1
Total	40

Optimization PCR Amplification of Vector (pQBI-6His) and Gel Cleanup of vector (pQBI-6His)

Vector pQBI is ≈ 5.5 kb long which will tag with 6his sequence for one step purification. The DNA pQBI-Nef-GFP was used as a template, by inverse PCR with primer sets of *Bam*HI-6His forward and *Nef* as reverse for pQBI-6His amplified. The inverse PCR allowed us to conduct PCR since we only have the information of one internal sequence. Inverse PCR uses standard PCR (polymerase chain reaction), however it has the primers oriented in the reverse direction of the usual orientation. The template for the reverse primers was a restriction fragment that has been ligated upon itself to form a circle [7]. The primers sequences are given in the Table 3.

Table 3: Primers for pQBI-6His

Vector	Primers	Sequences (5'-3')	Size (kb)
pQBI-6His	Forward (BamHI-6His)	ggA ggA TCC CAT CAC CAT CAC CAT CAC TAA ACT AgT AAC ggC CgC Cag Tg	50
	Reverse (Nef)	<u>Cag gCC</u> ATC CAA TCA CAC TAC	21

The PCR reaction was assembled on ice (in order to minimize nonspecific amplifications) as given in Table 4 and Table 5. The PCR amplification Vector (pQBI-6His) with both non-GC buffer and GC rich buffer.

Table 4: Components for PCR non-GC rich buffer

Components	Volume (µl)
Nuclease free water	33.5
5X Kapa buffer + Mg ²⁺	10
Kapa dNTPs (10 mM)	1.5
Primer Mixture (10 µM)	3.0
Template (pQBI-Nef-GFP)	1.0
Kapa hifi DNA polymerase	1.0
Total	50

Table 5: Components for PCR pQBI-6His amplification for GC-rich buffer and gel clean up

Components	Volume (µl) GC-rich buffer	Volume (µl) gel clean up
Nuclease free water	16.25	81.25
Kapa buffer (5x) GC buffer + 2.0 µM Mg ²⁺	5.0	25
dNTP mix (10 nM)	0.75	3.75
Primer Mixture (10 µM)	1.5	7.5
Template (pQBI-Nef-GFP)	1	5
Kapa hifi enzyme	0.5	2.5
Total	25	125

The total volume of the PCR components was scaled up to 125 µl which showed in Table 5. The PCR product of correct size was excised out from the gel and was placed on a UV transilluminator and set the lamp. Quickly, the band was cut using a sterile scalpel blade. The gel slice was transferred to a clean 2 ml microfuge tube and the weight was determined on an analytical balance. Each 100 mg of gel slice was added with 200 µL Buffer NT1. The sample was incubated for 10 minutes at 50°C. The sample was briefly vortexed every 2 minutes until the gel slice was completely dissolved. A NucleoSpin® Gel and PCR Cleanup column was placed in a 2 ml collection tube and the sample was loaded. The collection tube was centrifuged for 30 seconds at 11,000xg. The flow-through was discarded and the column was placed back into the collection tube. 700 µL Buffer NT3 was added to the NucleoSpin® Gel and PCR Cleanup column and centrifuged for 30 seconds at 11,000xg.

PCR Cleanup for Insert (*Nef*)

A single band of the desired size *Nef* was obtained after the PCR amplification. The PCR product was directly used to purify the PCR product free of unused dNTPs, primers and salts. A NucleoSpin® Gel and PCR Cleanup column was placed in a 2 ml collection tube. The sample was loaded into the column and was centrifuged for 30 sec at 11,000xg. The flow-through was discarded and the column was placed back into the collection tube and was added with 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Cleanup column. The collection tube was centrifuged for 30 seconds at 1,000xg. The flow-through was discarded and the column was placed back into the collection tube again and was centrifuged for 1 min at 11,000xg to remove Buffer NT3 completely.

Cloning *Nef* into pQBI-6His

Restriction Enzyme digestion for vector and insert

In order to 'clone' the PCR amplified *nef* gene into the mammalian expression vector pQBI-6His, first both the *vector* (pQBI-6His) and *insert* (PCRed *Nef* gene) was restricted with *Bam*H1 and *Sac*II. The restriction will result in the formation of 'compatible' sticky ends both, in the *vector* pQBI-6His and *insert* which will result in an 'in-frame' ligation. The reaction was set up as given in Table 6.

Table 6: PCR components for restriction enzyme digestion

Components	Volume (in µl)
Vector/Insert (1 µg) <u>in separate tubes</u>	10
Buffer G	2
BamHI	2
Sac II	2
dH2O	4
Total	20

Ligation *Nef* into pQBI-6his vector

The pQBI-6His will be referred to as ‘vector’, whereas the *Nef* gene cassette will be referred to as ‘insert’. The ligation reaction components as showed in Table 7 were assembled on the ice in order to minimize nonspecific amplifications. T4 DNA ligase buffer was thawed on ice after taking out from freezer - 80°C. T4 DNA Ligase buffer (10X) contains ATP which is essential for the activity of T4 DNA Ligase. The DNA (insert and vector) was thawed and incubated at 45°C for 5 minutes. This helps in melting any sticky termini that have re-annealed during fragment preparation. Then the DNA solution was chilled in ice for 2 minutes. Lastly set up the ligation reaction on ice. The contents of the PCR tubes were mixed by vortexes, followed by a brief spun in a micro-centrifuge.

Table 7: Components for ligation reaction

Components	Volume (µl)
Vector	4
Insert	4
T4 Buffer	1
T4 DNA Ligase	1
Total	10

Plasmid extraction

Preparation of Chemically Competent DH5-α *E.Coli*

This Inoue et al., 1990 [8] protocol is less finicky, more reproducible, and therefore more predictable with efficiencies of 1×10^8 to 3×10^8 transformed colonies/mg of plasmid DNA at 18°C rather than the conventional 37°C. The composition or the physical characteristics of bacterial membranes synthesized at 18°C are more favourable for uptake of DNA, or perhaps the phases of the growth cycle that favour efficient transformation are extended. The needed materials are DMSO, Plasmid DNA (recombinant plasmid) LB medium, SOC and SOB medium. Inoue transformation buffer was prepared and chilled to 0°C before use. A single bacterial colony (2-3 mm in diameter) was picked from a plate that was incubated for 16-20 hours at 37°C. The colony then was transferred into 25 ml of LB broth in a 250 ml flask. And then, the culture was incubated for 6-8 hours at 37°C with vigorous shaking (280 RPM). At about 6 o'clock in the evening, this starter culture was used to inoculate three 1liter flasks, each was containing 250 ml of LB broth. The first flask was received 10 ml of starter culture, the second was received 4 ml, and the third received 2 ml. All three flasks were incubated overnight at 18-22°C with moderate shaking (280 RPM).

The following morning, OD₆₀₀ of all three cultures taken and continue to monitor the OD every 45 minutes. When the OD₆₀₀ of one of the cultures reached 0.55, the culture vessel was transferred to an ice-water bath for 10 minutes and the other two was discarded. The cells were harvested by centrifuged at 2500xg for 10 minutes at 4°C. The medium was poured off and the opened centrifuge bottle was stored on a stack of paper towels for 2 minutes. A vacuum aspirator was used to remove any drops of remaining medium adhering to the walls of the centrifuge bottle or was trapped in its neck. The cells were re-suspended gently by swirling

in 80 ml of ice-cold Inoue transformation buffer. The cells were harvested by centrifuging at 2500xg for 10 minutes at 4°C.

Transformation Plasmid DNA (pQBI-Nef-6His) into DH5-α competent cell

The vector (pQBI-6His) contains Ampicillin resistance genes to facilitate the selection in *E.coli* and mammalian cells respectively. *E. coli* DH5α (NEB C2987H) was transfected with vector-insert ligation mix and selected on LB agar plates containing 100 µg ml⁻¹ Ampicillin [9]. Cloning of correct inserts was confirmed by *Bam*H1/*Sac*II restriction analysis as well as sequencing. The prepared competent cells were thawed on wet ice and the tubes were tapped gently. 100 µl of cell suspension was transferred to a pre-chilled falcon 17 x 100 mm tube. The tubes were incubated on ice for 30 minutes with a gentle mixed every 10 minutes. The cells were heat shocked by placing the tubes in a pre-heated 42°C circulating water bath for exactly 90 seconds. The tubes were immediately transferred to ice and incubated for 2 minutes. The cells then added with 900 µl of pre-warmed LB to the each tube.

Colony PCR for pQBI-Nef-6His

This method was made highly specific by using a combination of vector and insert-specific primers in which the forward primer anneals to the plasmid backbone near the 5' of the insert and reverse primer anneals to the 3' region of the insert. Alternatively, forward primer can anneal to the 5' region of the insert and the reverse primer binds to the plasmid backbone near the 3' of the insert. An additional advantage of this strategy is that the PCR amplified fragment can be sequenced and plasmid extracted only from the colonies containing correct sequence without any secondary mutations.

The used materials were PCR reagents, thermal cycler, Agarose, ethidium bromide and TAE buffer (10x), dNTPs (10 mM) 10X PCR buffer Mg²⁺, Taq polymerase (5 U/µl). Based on the number of screened colonies, 200 µl PCR tubes and was labelled and in every tube, 10 µl of PCR water was added. A plate with ~100 well-isolated colonies post 18 hours incubation was selected. For patching, the LB-agar plate with appropriate antibiotic and a grid was drawn with appropriate number of squares (20). By using a sterile toothpick a single colony was picked up and re-suspended in 20 µL of water in the PCR tube by smearing the toothpick or tip along the walls. By using the same toothpick it was marked as 'X' on the correspondingly labeled squares on the selection agar plate and the plate was incubated at 37°C for up to 16 hours.

Restriction Enzyme analysis for vector and insert

Upon to 'clone' the PCR amplified *nef* gene into the mamalian expression vector pQBI-6His, the plasmid DNA pQBI-nef-6His restricted with *Bam*H1 and *Sac*II and the correct sequence of clone in the selected colonies verified. The restriction components prepared as showed in Table 8. The stated components were briefly vortexed and the reaction tubes were briefly centrifuged. The reaction tube was incubated at 37°C for 2 hours. The restricted 'plasmid' was resolved on 1.0% Agarose/TAE gel at 100v for 25 minutes and visualized in Fluochem M FM 0287.

Table 8: Components for restriction enzyme analysis

Components	Volume (in µl)
Plasmid (pQBI-nef-6His)	5
Buffer G	2
BamHI	1
Sac II	2
dH2O	10
Total	20

Endotoxin-Free Plasmid DNA (pQBI-Nef-6His) Purification

The starter culture was prepared by inoculating a 3-5 ml starter culture of LB medium with a single colony was picked from a freshly streaked agar plate. The plate and liquid culture was confirmed that containing ampicillin antibiotic to guarantee plasmid propagation and was shake at 37°C at 300 rpm for 8 hours. After the 8 Hours, large overnight culture was prepared. An overnight culture was inoculated by diluting

the starter culture 1/1000 into the given 100 ml of LB medium also containing the appropriate selective antibiotic. The culture was growing for overnight at 37 °C at 300 rpm for 12–16 hours. Then the bacterial cells were harvested by confirming the cell culture OD and the recommended culture volume was determined according the following formula: Vol (mL) = 400/OD 600. The cell pellet was centrifuged at 6,000xg for 10 minutes at 4°C and the supernatant was discarded completely. Continuously the cell pellet was completely re-suspended in 8 ml Re-suspension Buffer RES-EF + RNase A by pipetting the cells up and down the cells. For an efficient cell lysis it is important that no clumps remain in the suspension. Then 8 ml Lysis Buffer + LYS-EF was added to the suspension and gently mixed by inverting the tube 5 times. The mixture was incubated at room temperature (18-25°C) for 5 minutes. Whenever the white precipitate was visible, the buffer was warmed for several minutes at 30–40°C until precipitate dissolved completely. The buffer was cooled down to room temperature (18–25°C).

Before loading the plasmid DNA, a NucleoBond Xtra Column together with the inserted column filter was equilibrated with 7 ml Equilibration Buffer EQU-EF. (The buffer applied onto the rim of the column filter and it makes sure that it wet the entire filter). The column was allowed to empty by gravity flow and the column did not run dry. Then, 5 ml Neutralization Buffer NEU-EF was added to the suspension and immediately mixed the lysate gently by inverting the tube 10–15 times without vortex. The tube used for this step was not filled more than two thirds to be allowed homogeneous mixing. (It was making sure that it neutralized completely to precipitate all the protein and chromosomal DNA. The lysate was not turn from a slimy, viscous consistency to a low viscosity, homogeneous suspension of an off-white flocculate). The crude lysate was incubated in ice and a homogeneous suspension was precipitated by inverting the tube 3 times directly before applying the lysate to the equilibrated NucleoBond Xtra and Column Filter was to avoid clogging of the filter. The lysate simultaneously was cleared and loaded onto the column. The filter was refilled whenever more lysate was loaded to make sure the filter is able to hold. The column was allowed to empty by gravity flow. The precipitate was removed by centrifugation at 5,000xg for 10 minutes. Whenever the supernatant still contains suspended matter it was transferred to a new tube and the centrifugation repeated, at higher speed, or the lysate applied to the equilibrated NucleoBond Xtra Column Filter. This clarification step is extremely important since residual precipitate may clog the NucleoBond Xtra Column. The cleared lysate was applied to load the column to the equilibrated filter. The column was allowed to empty by gravity flow and now, the column filter and column washed.

Quantification Plasmid DNA (pQBI-Nef-6His) by Qubit Fluorometer

For the accurate quantification of DNA, Qubit® Fluorometer with Qubit™ DNA Assay Kit was used. The kit provides concentrated assay reagent (stored at 4°C), dilution buffer (stored at room temperature), and pre diluted DNA standards (stored at 4°C). The assay was performed at room temperature, and the signal was stable for 3 hours. The need materials were Qubit® Fluorometer (Qubit™ DNA Assay Kit) and 0.5 ml tubes.

Two 0.5 ml tubes were needed for standards and appropriate number of samples and the tube lids were labelled accordingly. The Qubit™ working solution was made by diluting the Qubit™ DNA reagent 1:200 in Qubit™ DNA buffer. 190 µl of Qubit™ working solution was loaded into each of the tubes used for standards and was added with 10 µl of each Qubit™ standard to the appropriate tube. It was mixed by vortexing it 2-3 seconds. The Qubit™ working solution was loaded into individual assay tubes so that the final volume in each tube after adding sample was 200 µl. Each of the samples was added to assay tubes containing the correct volume of Qubit™ working solution. It was mixed by vortexing it 2–3 seconds. All tubes were incubated at room temperature for 2 minutes.

The prepared tube for standard was inserted into the Qubit® Fluorometer for calibration. The sample tubes was inserted and completed with the measurement. The Qubit® Fluorometer gives values for the Qubit™ DNA assay in ug/mL. This value corresponds to the concentration after the sample was diluted into the assay tube. The concentration of the samples calculated by multiplying QF Value with (200/x), where QF value was the value given by the Qubit® Fluorometer and x was the number of microliters of sample was added to the assay tube.

RESULTS AND DISCUSSION

Amplification of Nef insert sequences and pQBI vector tagged 6His

pQBI Vector and Nef insert was amplified from the same template which is pQBI-Nef-GFP as shown in Figure 2 by using different set of primer mix. Figure 3 shows the PCR amplification product of pQBI-6His and Nef by using KAPA DNA polymerase buffer in Agarose gel electrophoresis. Lane M shows ladder 1ul of 1 kb DNA (NEB standard ladder labelling). Lane 1 shows amplification product of vector pQBI-6His ≈5.5 kb by set of primer mixture BamH1-6His as forward and Nef as reverse by using pQBI-Nef-GFP as the template through inverse PCR. The result shows multiple non-specific bands indicate that the targeted product was not clean. Lane 2 shows amplification product Nef insert 627 bp which uses set of primer mixture of SacII as forward and BamH1 as reverse. As shows in the Figure 3 insert Nef successfully was amplified from the template without any multiple band. The product of Nef insert was subjected PCR clean up.

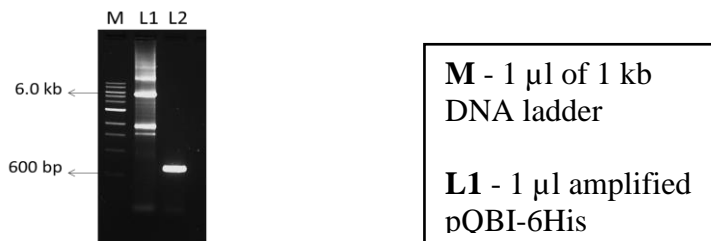


Figure 3: PCR amplification product for pQBI-6His and Nef by using KAPA DNA polymerase buffer

Figure4 shows comparison of PCR amplification performance by GC-rich buffer and Non-GC rich buffer for pQBI-6His vector ≈5.5 kb by set of primer mix BamH1-6His as forward and Nef as reverse from the template pQBI-Nef-GFP as shown in Figure 2. Lane M in both diagram **A** and **B** are 1 kb DNA ladder (NEB). L1 in diagram **B** shows PCR amplification product of pQBI-6His vector with GC rich buffer on the other hand diagram **B** shows non-GC rich buffer. Diagram **A** shows high yield of amplification product of pQBI-6His compared to diagram **B** when resolves on 1% Agarose gel at 100 v for 25 minutes. It clearly shows that GC rich buffer needed for more productive vector amplification since the vector's GC content of this region is by 50%, BamHI (F) has 54% GC percentage and Nef (R) has 52.38 percentage. GC-rich templates have strong secondary structures which resist denaturation and prevent efficient primer annealing. By using GC rich buffer it will enhance denaturation and promote efficient primer annealing.

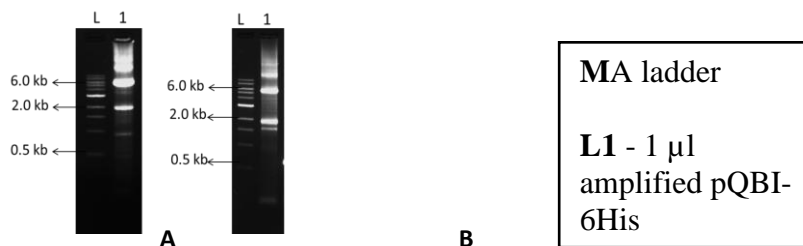


Figure 4: PCR product for pQBI-6his by using KAPA DNA polymerase A: GC buffer and B non GC buffer

PCR cleanup for Nef insert

PCR clean up step is to purify PCR amplification products away from undesirable reaction components such as salts, excess nucleotides, primers, adjuncts and proteins (e.g., Taq polymerase). The removal of these components improves the reliability of downstream analytical methods routinely performed in genomics labs, thereby increasing process flow and decreasing handling costs. Figure 5 shows PCR clean up product for Nef insert. Lane M shows 1 Kb DNA ladder (NEB). Lane 1 shows the band for 627 bp of clean product of Nef insert out of 40ul of elution.

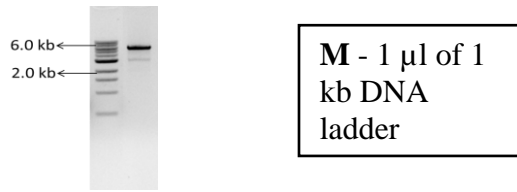


Figure 5: Clean up product of pQBI-6His

Gel clean-up for vector pQBI-6His

Fragments of interest was identified by resolving DNA samples on 1% TAE Agarose gel at 100 v for 25 minutes, the corresponding band was isolated, the DNA from those band was isolated, and desired DNA fragment was excised accompanying salts and stain. Figure 6 shows the desired pQBI-6His vector which is 5.5 kb was excised out from the gel. Lane M shows 1 kb DNA ladder (NEB), Lane 1 and Lane 2 are PCR amplification of pQBI-6His by pQBI BamHI -6His (F) and Nef (R). Figure 7 shows the end product pQBI-6His after gel clean. Lane M shows 1 Kb DNA ladder. Lane 1 is 1 μ l of 5.5 kb pQBI-6His vector which is from 40 μ l eluted DNA product.

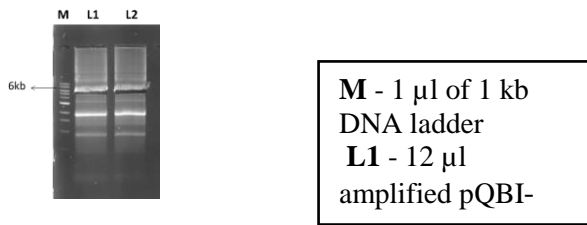


Figure 6: Gel extraction of pQBI-6His

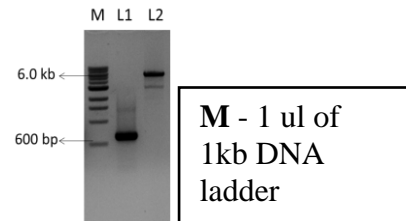


Figure 7: Clean product of pQBI-6His and nef

Cloning of Nef in vector pQBI-6His

Figure 7 shows the clean product used for ligation where the pQBI-6His vector and Nef insert resolved in 1% TAE Agarose gel. Lane M is 1 kb DNA ladder. Lane 1 Nef insert 627 bp and Lane 2 is pQBI-6His vector 5.5 kb. Before ligation, the vector has linearized as shown in Figure 8. Lane M1 was 100bp DNA ladder and M2 was 1.0 Kb DNA ladder. Lane 2 shows pQBI-6His vector restricted by BamHI-6His 5`G GATC C 3`and Nef 5`CCGC GG3` where else Lane 1 shows insert was restricted by SacII 5`CC GC GG3` and BamHI 5`GG ATC3`.

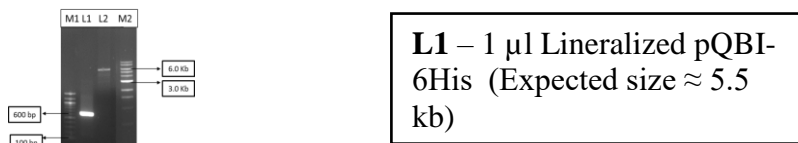


Figure 8: Restricted product of pQBI-6His vector and Nef insert

These restriction enzymes (RE) function by cutting double stranded DNA at specific base pair inverted repeat recognition sequences within the target DNA. The products of DNA cleavage are containing 5' or 3' overhangs as stated below. The sticky ends are universally compatible with other sticky end DNA and possess a 5' phosphate group to promote ligation. In addition to this calf intestine alkaline phosphatase (CIAP) is added to this restricted mixture. CIAP treatment prevents the random joining of two vector molecules thus enhances the overall vector: insert ligation efficiency. The *SacII/BamHI* restriction of PCR nef amplicons 627 bp will be ligated overnight with BamHI-6His/Nef restriction of pQBI-6His 5.534 kb expression vector. The cloning product 6.1 kb pQBI-Nef-6His was amplified.

Transformation

Ligation of pQBI-6His and Nef will produce ≈ 6.1 kb pQBI-nef-6His plasmid DNA refer to cloning map shown in Figure 9. The prepared plasmid has gene of interest (*Nef*), with a CMV promoter sequence in front of the gene, in addition to the origin of replication (*ori*) and the ampicillin resistance gene. The plasmid DNA transformed into chemically prepared competent DH5- α cells, *E.Coli* strain for routine cloning applications to replicate a specific DNA fragment. As part of the transformation procedure, the heat shock treatment (temperature is briefly raised to 42°C) changes the structure of the bacterial membrane encourage the competent cells to bring the extra DNA into the cell. Once the bacteria are transformed, those cells containing the plasmid (pQBI-Nef-6His) was selected for and maintained using selective pressure from the selectable marker ampicillin resistance. In normal condition DH5- α bacteria die in the presence of the antibiotic ampicillin.

Sac II

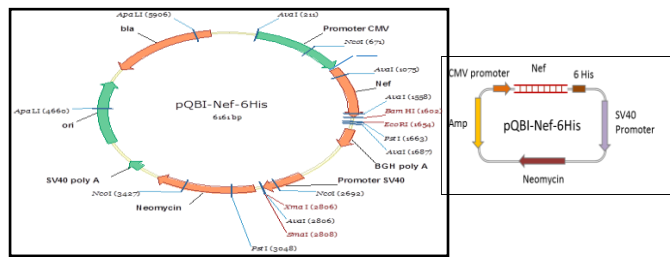


Figure 9: Cloning map of pQBI-Nef-6His Plasmid DNA sequence.

However, the bacteria that have taken up the plasmid (transformed) and express the ampicillin resistance gene product from the plasmid as shown in colonies in Figure 10 was be able to grow on media containing ampicillin while all the other bacteria was did not survive, which called as selection. At this point, the transformed bacteria used as a factory to produce large amounts of the pQBI-Nef-6His when the plate was incubated in 37°C incubator for 18 hours.

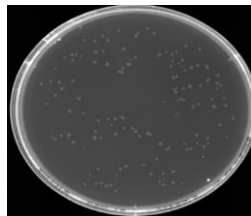


Figure 10: Colonies of transformed DH5- α with pQBI-Nef-6His

Colony PCR for pQBI-Nef-6his

Colony PCR was carried out after a transformation to screen colonies for the desired plasmid. *SacII* (F) and *BamH1*(R) are set primers used which generate a PCR product of 6.1 kb. Randomly 10 colonies were selected from the LB ampicillin plate shown Figure 10 and the selected colonies were subjected to colony PCR. Figure 11 shows product of colony PCR for pQBI-Nef-6His. Thus, the colonies which give rise to an amplification product of the expected size were likely to contain the correct DNA sequence. Lane M was 1kb DNA ladder. Lane 1-10 is 5 μ l of product of colony PCR and Lane 11 was negative control. Expected size plasmid DNA identified in lane 1,3,4,6,7,8,9 and 10. Lane 2 which is colony 2 has multiple bands even though there is very fade band detected on the expected size which makes the colony was not selected. On the other hand lane 5 which was colony 5 was not detected at all. This could be because of plasmid have self-ligated or the plasmid is without an insert transformed into the bacteria. Colony 1, 3 and 4 was subjected to plasmid DNA extraction.

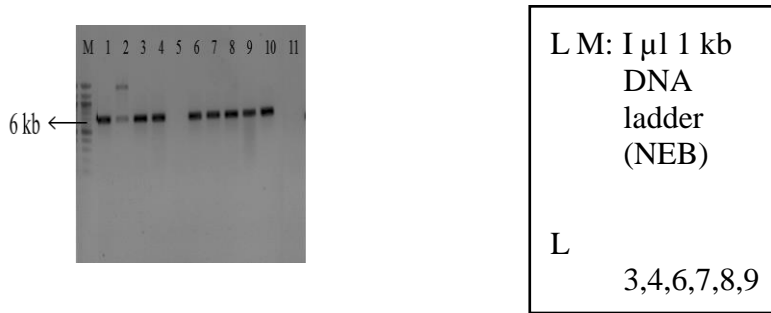


Figure 11: Product of colony PCR for pQBI-Nef-6His

Plasmid DNA pQBI-Nef-6his extraction

The starting *E. coli* culture volume was 15-25 ml of Luria broth (LB) prepared by using selected positive colonies and the expected DNA yield is 100-350 μ g. Selected colonies are 1,3 and 4 which were expected to have high yield which was shown by brighter bands in these colonies. The culture medium for the selected colonies was purified by using endotoxin free plasmid extraction of Nucleobond Xtra midi kit. The total volume of DNA concentrated was 35 μ l. The plasmid DNA was extracted from each colony and resolved in 1% TAE Agarose gel and visualised. Figure 12 shows uncut plasmid DNA was extracted from the DH5- α cell. Lane M was 1kb DNA ladder. Lane 1 was plasmid DNA from colony 1, Lane 2 plasmid DNA from colony 3 and Lane 3 plasmid DNA from colony 4.

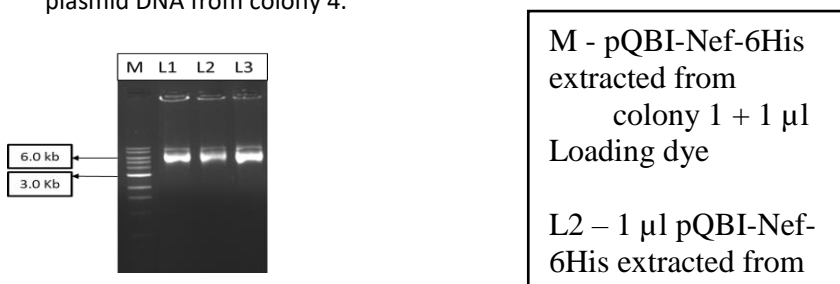


Figure 12: Product of plasmid DNA extraction

Restriction enzyme analysis of pQBI-Nef-6His

Restriction enzyme analysis was used to confirm the size of plasmid DNA produced by DH5- α cells even though it has been verified by colony PCR. It is because colony PCR is basically a "preliminary screening" method. There are few limitations in colony PCR which trace amount of insert may be attached to the agar or the colony and we can accidentally pick them into the PCR mixture, so the band and thought the colony carries our gene. Moreover, colony picking was extraordinarily tricky as well. Another problem was the PCR primer. We also don't know whether the insert is ligated into the vector of our desired, or instead something else. This could happen in cases where the insert was not purified by PCR amplification, but instead held in a cloning vector before digestion and subsequent gel-purification and ligation. Therefore, restriction analysis was processed as shown in Figure 15. Lane M was 1kb DNA ladder. Lane 1 was of pQBI-nef-6his uncut product with an expected size of 6.1 kb which is supercoiled and lane 2 shows pQBI-nef-6his cut with *Bam*HI restriction enzyme with a size of 6.1 kb linearized DNA due to *Bam*HI binding at the recognition sequence 5'-GGATCC-3', and cleaves these sequences just after the 5'-guanine on each strand. This cleavage results in sticky ends. Lane 3 shows vector pQBI-6his and lane 4 shows the band of Nef insert. Lane 3 was the gel extraction clean up, and lane 4 was PCR clean up.

Gene sequencing verification

The sequencing of pQBI-Nef-6His was sent to a gene sequencing facility at IPPT for Gene Expression using Beckman-Coulter Genome GeXP instrumentation. The plasmid DNA was prepared by a midi prep kit method and prepared by ethanol precipitation. The sequencing data was interpreted using BioEdit software. It was shown that the nef sequences were 100% matched with the reference gene.

Quantification of extracted pQBI-Nef-6his.

The extracted pQBI-Nef-6his plasmid DNA from midi prep kit was quantified by using Qubit fluorometer. The reading was taken after calibrated with standard 1 and standard 2. The extracted plasmid was 274.5 µg/ 0.15 ml. The plasmid DNA adjusted the concentration to 1 µg/µl. The concentration DNA was 275 µg in 150 µl. 125 µl allocated for transfection of HEK 293 cells.

Transformation

Ligation of pQBI-6His and Nef will produce ≈ 6.1 kb pQBI-nef-6His plasmid DNA refer to cloning map shown in Figure 19. The prepared plasmid has gene of interest (*Nef*), with a CMV promoter sequence in front of the gene, in addition to the origin of replication (*ori*) and the ampicillin resistance gene. The plasmid DNA transformed into chemically prepared competent DH5-α cells, *E.Coli* strain for routine cloning applications to replicate a specific DNA fragment. As part of the transformation procedure, the heat shock treatment (temperature is briefly raised to 42°C) changes the structure of the bacterial membrane encourage the competent cells to bring the extra DNA into the cell. Once the bacteria are transformed, those cells containing the plasmid (pQBI-Nef-6His) was selected for and maintained using selective pressure from the selectable marker ampicillin resistance. In normal condition DH5-α bacteria die in the presence of the antibiotic ampicillin.

However, the bacteria that have taken up the plasmid (transformed) and express the ampicillin resistance gene product from the plasmid as shown in colonies in Figure 10 was be able to grow on media containing ampicillin while all the other bacteria was did not survive, which called as selection. At this point, the transformed bacteria used as a factory to produce large amounts of the pQBI-Nef-6His when the plate was incubated in 37°C incubator for 18 hours.

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CONCLUSION

Based on this study, the results conclude that targeted myristoylated 27 kDa HIV-1 Nef has not expressed in HEK 293 cells in transient transfection when transfected with pQBI-Nef-6his which was constructed by ligation of 5534 bp pQBI-6His as the vector with 627 bp Nef as the insert.

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