RJC

RASĀYAN *J. Chem.*
Vol. 10 | No. 1 |176-189 | January - March | 2017
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
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STABLE TRANSFECTION STUDY FOR CLONING AND EXPRESSION OF HIV-1 NEF PROTEIN IN HEK 293 CELLS

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ABSTRACT

Human immunodeficiency virus continues to be a major global populace 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 in the progression to AIDS. The given its central purpose 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 is a protein model to be used for significantly specific antibody production to lower the pathogenicity of HIV infection. Cultivated HEK 293 was transfected by 4ug/cubic centimetre of successful clone of pQBI-Nef-6His by stable transfection selected in 0.7 mg/cc of G418 antibiotic. It display that targeted 27 kDa HIV-1 Nef was not successfully expressed in HEK 293 cells either in stable transfection when transfected. However, non-targeted HIV-1 Nef was detected in western blot by anti-Nef (anti manufactured by Thermo scientific. It suggesting that, the Nef protein that was detected not identically synthesis through post-translation modification though it was expressed in the cytoplasm of HEK 293 cells. 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

INTRODUCTION

kDa N-terminally myristoylated protein involved in post integration taint. Nef is found in the viral particles is one of protein that is expressed in plenty during the early phase of HIV infection together with Tat and and Nef also induces high virus load titers both in culture and in Its mRNA is estimated to represent three quarters of the early viral load of the cell[1,2]. Using the nef+ HIV-1 was found to productively infect 5 to more than equal amounts of nef-defective HIV-1 [Nef showed in Figure 2 has been indicated to induce down regulation of CD4 and HLA class I[3,4] from the surface of HIV-1-infected, which may represent an important escape process for the virus to evade an attack mediated by cytotoxic and to avoid recognition by CD4 T nef may also interfere with T cell activation by binding to various proteins that are involved in intracellular signal transduction pathways[5]. A study demonstrated that nef as accessory protein has the ability efficiently down regulate chemokine receptor CXCR4 from the surface of HIV infected primary CD4 T cells and highlight this of nef is important to maximize

interference with superinfection by additional vision at the entry step[6] were generated by transformation of embryonic kidney cell cultures (hence HEK) with sheared adenovirus DNA, and were first described in[7]. The HEK cell has been extensively used for recombinant proteins since it was generated over 25 years ago. Although of epithelial origin, its biochemical machinery is capable of carrying out most of the post-translational folding and processing required generating functional, mature protein from a wide spectrum of both mammalian and non-mammalian nucleic acids. Though popular as a transient l system, this cell type has also seen wide use in stably transfected forms (i.e. transformed to focus playing field of cell-biological[8]. It also showed that, the homo blood cell line HEK 293 has been used extensively for transient gene expression as a result of its and ability to grow in high cell density in blood serum free media[9]. However, HEK 293 do not normally large numbers of exosomes. In contrast to that, once the immunodeficiency Human virus -nef gene is expressed in these they produce large number of exosomes[10]. Moreover, was observed using soluble extracellular Nef expressed from HEK 293 cells transfected with HIV-1, HIV-2 and SIV nef gene.

The first enzyme immunoassay to detect Human immunodeficiency virus antibodies was introduced in 1985. These detected IgG antibodies to HIV-1 using viral lysate as the antigen and were unable to detect antibody response to different HIV-1 clades. They became positive approximately 6-8 weeks following infection. Lack the sensitivity and specificity of current widely-used increased specificity by using recombinant peptides or protein to produce antigen for viral. They were able to detect infection approximately 1 week earlier than the to begin with assays of coevals. The emerging of the third-generation assays represented significant advancement since they could not only detect both HIV-1/2 and IgG but also detect them as soon as 3 weeks after infection[11].

The aim of this study is to express HIV-1 Nef protein in HEK 293 cells transfected by pQBI-Nef-6His. Construction of a mammalian expression vector that will express HIV-1 Nef under CMV promoter and express HIV-1 Nef tagged with 6His for one-step purification and transient and stable (G418 selection and genome integration) transfection of HEK293 with pQBI-Nef-6HIS. Analysis of Nef protein expression by HEK293 by SDS-PAGE/Western blot also has been studied.

EXPERIMENTAL

Transfection HEK 293 with pQBI-6His

Cultivation HEK 293 cells

Maintaining the cells

HEK 293 cells sourced from the American Type Culture Collection (ATCC: Manassas, USA) under the designation d293T with accession code CRL-1573. The base medium for this cell line is ATCC-formulated DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10% fetal bovine serum (FBS) to a final concentration of 10%, 200 mM L-Glutamine, Sodium carbonate (7.5%), Sodium pyruvate (100 mM). Generally, cells can be continuously cultured 5% CO_2, 95% air humidified incubator, will deteriorate as the passage number increases. As a general rule, a new stock of cells acquired from ATCC should initially be grown until they are ~70% confluent, then passaged on two consecutive occasions and then stored frozen under liquid nitrogen in quantities which are sufficient for a number of years. This stock of HEK is regarded as laboratory passage one. Cells defrosted and plated from this stock are generally reliable in terms of their native phenotype, transfection efficiency for 20–30 passages before a new stock needs to be thawed and regenerated. Cells at this stage can also be seeded at 30–50% density if the transfection procedure involves a DNA precipitation reaction as used in the calcium phosphate method.

Trypsinization of HEK 293 cell line

The HEK 293 cells were microscopically observed and the flask was gently shaken on the platform to assess adherence. Whenever too many cells are floating, it is an indication of low viability. The medium was discarded into a waste beaker (spray beaker with ethanol before introducing into hood). The cells were washed with PBS 2ml. (Note: Phosphate Buffer Solution (PBS) does not contain Calcium/Magnesium to minimize cell sticking.) PBS was discarded in waste flask. 500 µl of trypsin/

Ethylenediaminetetraacetic acid solution was added so that trypsin covers the entire surface on which cells are adherent. It was allowed 2-3 minutes to trypsin to work. Whenever the detachment was low, flask was placed in the incubator and was microscopically inspected every 5 minutes. To facilitate detachment, the flask was tapped on a hard surface. Whenever 50% of cells was floating, reaction was terminated with 3 mL DMEM containing 10% FBS. FBS contains alpha-1-antitrypsin which inhibits trypsin. The cells was aspirated with a 10 ml pipette and was transferred into 15 ml BD falcon tube and it was make sure that the tube was airtight before removing it from the hood. The tube was centrifuged at 400 g for 5 minutes at 20^0C and the supernatant was carefully discarded into the waste beaker in the hood. The pellet was re-suspended using DMEM 10% FBS and disintegration of pellet was done via repetitive pipetting.

Calculating the viability of the cells

The HEK 293 cells were trypsinized. Under sterile conditions 50 µl of cell suspension was removed and transferred to 96 well plates. 50 µl of Trypan Blue (dilution factor =2) was added to the transferred cell suspension and was re-suspend the mixture by gentle pipetting. The haemocytometer was cleaned and the coverslip moisten with water. The coverslip slid over the chamber back and forth using slight pressure until Newton's refraction rings appear (Newton's refraction rings are seen as rainbow-like rings under the coverslip). Both sides of the chamber were filled with 10 µl cell suspension and viewed under an inverted phase contrast microscope using x10 then with x20 magnification. Number of viable (seen as bright cells) and non-viable cells (stained blue) were counted. Ideally >100 cells was counted in order to increase the accuracy of the cell count as given below).

Preparing Subculture

Subcultures were prepared by trypsinization. The cells were diluted to a concentration 1-2 X 10^5 cells/ml into fresh DMEM 10% FBS in a new flasks. The culture was kept below approximate density of 10^6 cells/ml.

Cryopreservation of Cell Lines

The DMEM 10% FBS was aspirated from the confluent Tissue Culture plate and the cells are trypnized 400 ul DMSO was added in a drop wise fashion, DMSO, so that the final volume is 10% (v/v) DMSO. 4 ml of medium was added and very gently the tube was inverted few times to mix in the DMSO. Into each pre-labelled (date, HEK 293, 10% FBS and number of passage) cryo vial, and 1mL of the cellular suspension was added. It was make sure all the caps were tightly refastened. The tubes were transferred into frozen container which contains ethanol and was incubated in -80^0C for overnight. The next day the tubes were checked for any leakage and immediately was stored in nitrogen thank -196^0C

Transfection by High efficiency Calcium phosphate (BBS) method

Calcium phosphate and DEAE-dextran reagents were incorporated into the first chemical methods that successfully was transferred nucleic acid directly to cultured mammalian cells in a process was referred to as transfection. All solutions were prepared with deionized, nuclease-free water and tissue culture-grade reagents. Solutions were sterilized by filtration through a 0.2 µm filter.

Kill curve for HEK 293 cell line transfected with pQBI-Nef-GFP

Before the selection, G418 antibiotic was prepared by dissolving G418 antibiotic in water with the concentration of 100 mg/ml and was stored in sterile tube at 100 µl aliquots at -20^0C. DMEM (10% FBS) was prepared at six different concentration (0, 100, 300, 500, 700 and 1000 µg/ml) of G418 contains DMEM 10% FBS was prepared. Two 6 well plates were used to plate the cells from 1 T25 flask in 20 ml medium. The cells in the medium was counted and each well will be receiving ≈ 2X 10^6 cells per well. The two 6 well plates were filled with cell line cultured which was incubated 6-8 hours to allow cells to attach before adding the inhibitor. The medium was removed and replaced with medium containing

antibiotic according to the concentration stated above. The density of the cells in each well was observed after added with antibiotic every day for two weeks. The cells were fed with antibiotic containing medium every three days.

Selection of stably transfected clones of HEK 293 with G418

After transfection, cells were allowed to grow and express the protein under non-selective conditions for at least 24 hours. The G418 resistance selection started after 48 hours. For the selection of stably protein expressing cells, the cells were placed on 90 mm culture plates cells and were cultivated in DMEM (10 % FBS) with 0.7 µg/ml of G418, pre-tested for HEK 293 cells on pQBI-Nef-GFP transfected by the kill curve method. The cells were fed with DMEM 10% FBS with 0.7 µg/ml of G418 two weeks to provide sufficient occurrence of clonally event.

Analysis of expression of HIV-1 Nef protein

Preparation of SDS-PAGE gel

The solution of monomer was prepared by covariate all reagents as shown above and the mixture degased for 15 minutes. For 10 ml monomer solution, immediately prior to pouring the gel, 50 µl 10% APS and 5 µl TEMED was added. Immediately the monomer solution overlaid with water. The gel was allowed to polymerize for 45 minutes to 1 hour. Water was removed after the gel has polymerized. Stacking gel monomer solution was prepared and degased for 15 minutes. 50 µl 10% APS and 10 µl TEMED was added to the degassed stacking gel monomer solution. The stacking gel was poured between the glass plates until the top of the short plate was reached. The desired comb was inserted between the spacers starting at the top of the spacer plate. The comb was seated in the gel cassette by aligning the comb ridge to the top of the short plate. It was allowed to be stacked gel to polymerize for 30-45 minutes. Gently the comb was removed and rinsed the wells thoroughly with distilled water or running buffer. This gel was stored in wet tissue at 4°C until further used.

Extraction of expressing protein

M-PER Mammalian Protein Extraction Reagent extracts cytoplasmic and nuclear protein from cultured mammalian cells using a proprietary detergent in 25 mM bicine buffer (pH 7.6). The simple composition of this reagent was compatible with immunoassays such as Western blot and protein purification .M-PER Reagent enables rapid, mild and efficient lysis. The reagent was dialyzable and the cell lysate was compatible with protein assays such as Coomassie blue staining. 1 ml of PBS was added to the adherent cells to remove all the remaining dead cells and mixed gently. After removing the trypsin, 10 µl of 10 % FBS DMEM was added to re-suspend the culture medium. The cell suspension was centrifuged at 400xg for 5 minutes at 20^0C. The suspended pellet was weighed. M-PER Reagent does not contain protease inhibitors.

SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is a technique that is used to separate proteins from each (according to their size and overall charge) on a polyacrylamide gel.

Western blot

After separation of protein samples, the stacking gel was removed one corner to orientate the resolving gel by using a clean, sharp scalpel. The gel soaked in the protein transferred to 1x Toubin buffer for at least 10–20 minutes. The nitrocellulose membrane was transferred to toubine buffer for 15 minutes. The electro-blotting cassette was assembled and placed between the electrode plates in the blotting unit. The protein was transferred to the membrane for 1 hour at 25v. Following transfer the membrane removed from the blotting cassette and the orientation of the gel was marked on the membrane and it also was rinsed briefly.

For immune detection, the membrane which was electro blotted was incubated in blocking reagent for 1 hour by rolling at room temperature. The blocking reagent was prepared by adding 1.0 g of 5% skim milk

into 20 ml TBS/T20 for one membrane. After 1 hour, the membrane was briefly rinsed with 40ml TBS/T20 twice. The membrane was incubated 40 ml TBS/T20 for 15 minutes on rolling at room temperature and was continued by incubation 40 ml TBS/T20 for 5 minutes on rolling at room temperature for two times. Followed by the washing, 2 ml of primary antibody was added which was anti-Nef (1:2000) and was incubated for 1 hour on rolling at room temperature. After 1 hour, the washing step was proceeding as stated above. Then 2 ml secondary antibody was added which was anti- mouse IgG (1: 20000) and incubated for 1 hour on rolling at room temperature. Lastly the membrane was washed as stated above.

Purification of Nef protein by HisPur cobalt column

Approximately 10^7 HEK 293 cells were transfected with pQBI-Nef-6His and was harvested by centrifugation. The Thermo Scientific HisPur Cobalt Purification Kit contains HisPur Cobalt Spin Columns, pre-formulated Equilibration/Wash and Elution Buffers for the efficient purification of recombinant polyhistidine-tagged proteins from bacterial, mammalian and baculovirus-infected cells. His-tagged proteins were purified from total soluble protein extract using a cobalt-charged tetradentate chelator immobilized onto 6% cross linked agarose. Many immobilized metal affinity chromatography (IMAC) resins contain nickel (Ni^{2+}) for purifying His-tagged proteins. Although $Ni2^{+}$chelate resins achieve high protein yields, purity is often suboptimal, requiring additional clean-up steps. Cobalt achieves both high yield and purity with minimal optimization.

The column was equilibrated to room temperature and bottom tab was removed from the HisPur Cobalt Spin Column while the column was placed into a centrifuge tube and top cap was loosening up. The column was inverted to mix. For maximal binding, the column was incubated for 30 minutes at 4°C on an end-over-end rocking platform. The column centrifuged at 700×*g* for 2 minutes and the flow-through was collected in a new centrifuge tube. The resin was washed with two resin-bed volumes of Equilibration/Wash Buffer. It was centrifuged at 700x*g* for 2 minutes and fraction in a centrifuge tube was collected.

The tagged protein was eluted from the resin by adding one resin-bed volume of Elution Buffer and was centrifuged at 700×g for 2 minutes. After each use and before storing, the procedure as described below was performed to remove residual imidazole and any non-specifically adsorbed protein. To prevent cross contamination of samples, designate was given a column to one specific fusion protein. Firstly, the column washed with 10 column volumes of Regeneration MES Buffer. Secondly, the column washed with 10 column volumes of ultrapure water. For storage, 20% ethanol applied to the column. Lastly, before reuse, the column was re-equilibrated with Equilibration/Binding Buffer until the pH was returned to the buffer value. Expression of HIV-1 Nef protein by cell lysate was assayed by SDS-PAGE/Western blot.[12]

RESULTS AND DISCUSSION

Transfection of HEK 293 cultivated in DMEM 10 % FBS with pQBI-Nef-6His

HEK 293 cells was cultivated in DMEM 10% FBS until it reach 80-90 confluence in 37^0C/5% CO_2 incubator in T75 flask. Before one day of transfection number of cells was counted and plated in tissue culture-treated dishes. The tissue cultures-treated dishes were prepared for the transfection process. The dishes were prepared for transient transfection and stable transfection. 1 ml of medium was transferred to each dish where it contains 1 X 10^6 cells. After 24 hours, each dish was transfected with a 4 µg/ml for optimization DNA concentration of extracted plasmid DNA with high efficiency calcium phosphate co-precipitation method (BBS). The microscopic view of transfected HEK 293 cells given in Figure-1 was 24 hours of post transfection, Figure-2 control and Figure-3 for 48 hours post transfection.

The highlighted region shows moderate level of precipitation due to calcium phosphate accumulation which was not seen in control dish given in Figure-1. Figure-5 shows the observation of 48 hours post transient amount of precipitation in 48 post transfection significantly increased compare to 24 post-transfection. As analysed by Baldi *et al.,* 2005[13], the cells transfectability by the calcium phosphate method in HEK 293 cells did not observed any adverse effect on "aging". However efficiency of the transfection not able to measured due to absence of fluorescent protein.

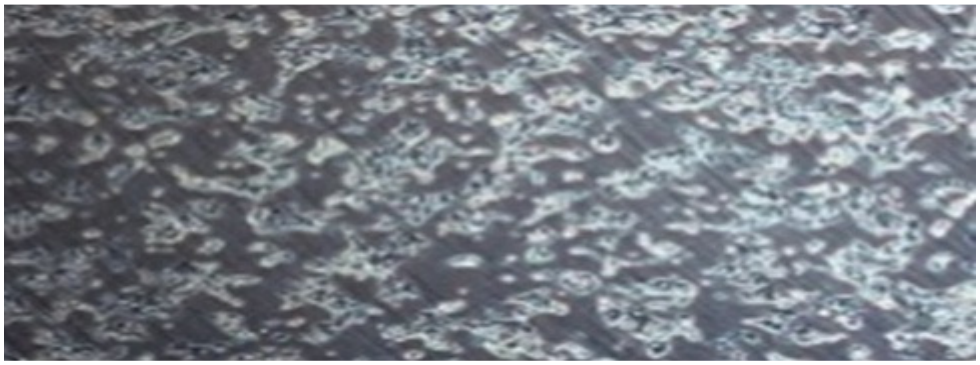


Fig.-1: 24 hours post transfection of HEK 293 cells with pQBI-Nef-6his (X 20)

Fig.-2: Control dishes 24 hours post transfection of HEK 293 cells with deionized water. (X 20)

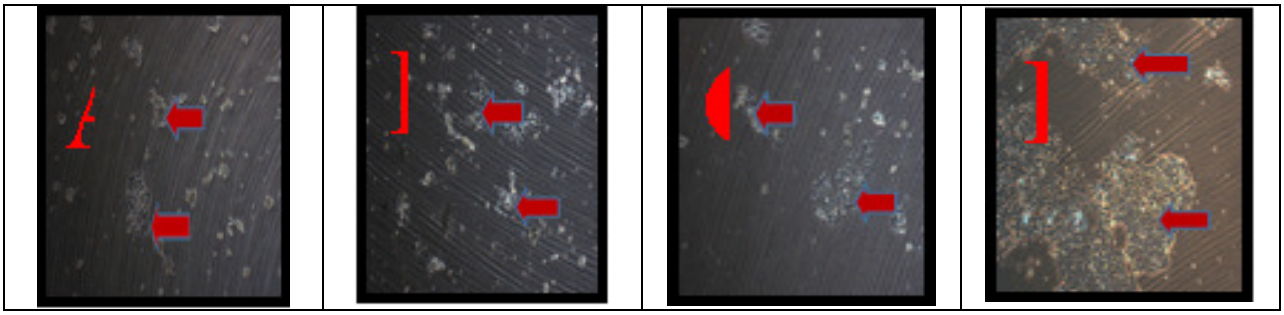


Fig.-3: 48 hours post transfection of HEK 293 cells with pQBI-Nef-6His. (X 20)

Figure-4 shows microscopic observation of HEK 293 cells after 24 hours post-transfection with 4 µg/ml pQBI-Nef-6his under the magnification of X20.

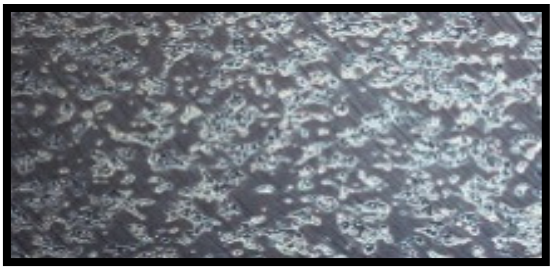


Fig.-4: 24 hours post transfection of HEK 293 cells with pQBI-Nef-6his (X 20)

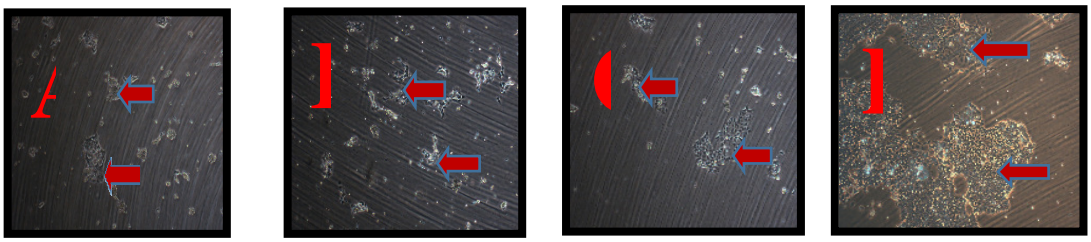
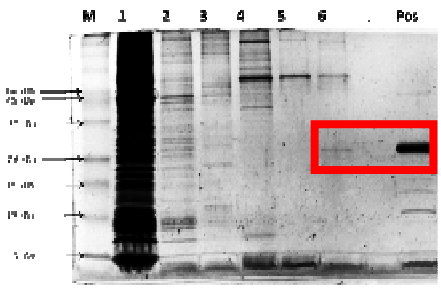


Fig.-5: 48 hours post transfection of HEK 293 cells with pQBI-Nef-6His. (X 20)

Transiently transfected cells express the Nef gene but do not integrate it into their genome. Thus the *nef* gene was not being replicated. These cells express the transiently transfected gene for a finite period of time, several days, after which the *Nef* gene is lost through cell division or other factors[14]. Compared to expression in lower-order organisms, recombinant protein expression in mammalian cells offers significant advantages in protein quality and post-translational modification[15]. Moreover the protein of interest 27 kDa nef is N-terminally myristoylated at the second glycine of the highly conserved sequences MGG[16]. Though transient transfection of mammalian cells has been employed for low recombinant protein expression since the invention of transfection reagents, the ability to express milligram-to-gram amounts of recombinant protein has relied mainly on the creation of stable cell lines[17].
Referring to various studies, it was showed that recombinant protein cannot expressed in high volume by having only transient transfection. In order to that, post-transfection of 48 hours two of the petri dishes contains transfected HEK 293 cells was subjected to cell lysis and the protein expression level were analysed through SDS/western blot. Another two more dishes are transferred to T25 tissue culture flask and proceed to stable transfection with selection on G418 antibiotic

Selection on G418 for stable transfection/genome integration
Stable transfection is long-term expression of Nef gene when pQBI-nef-6His directly integrated with genome of HEK 293 cells. Integration into HEK 293 cells genome by Nef gene was introduced into the cell by the plasmid DNA subsequently into the nucleus, and finally, it was integrated into chromosomal DNA of HEK 293 cells. Stably-transfected cells were selected by the addition of G418 antibiotic as a selection marker to the culture medium, since the expression plasmid carries gene neomycin resistance (NEoR) system. Cells differ in their susceptibility to G418, kill curve for this HEK 293 cells transfected with pQBI-Nef-GFP was determined as 0.7 mg/ml concentration of G418 to be used for selection. The clonal events observed for 14 days shown below in Figure-6.



M: 5 µl of prestained protein ladder.

L1: Unbound protein (Pellet) 9 µl + 3 µl Sample buffer
L2: Washed protein (Pellet) 9 µl +3 µl Sample buffer

Fig.-6: Post stable transfection on G418. **A:** Day 4 post-transfection (40x) **B:** Day 7 post-transfection (40x)**, C:** Day 9 post-transfection (40x) **D:** Day 14 post-transfection (40x)

Purity determination by SDS-PAGE/western blots
Analysis of transient transfection nef expression on SDS-PAGE/western blots
Various studies showed that nef can secreted out from transfected cell. In the study of Tamika *et al.*[18] showed that nef was associated with membrane, ultimately was secreted into vesicles in the form of

exosomes. They also showed that nef was secreted by HEK 293 cells. In Campbell *et al.*[10] study also showed that nef is secreted from nef-transfected cells in a high molecular weight form and vesicle was found in this high molecular weight differential centrifugation pellet. It was showed that majority of the nef protein was shown to be on the luminal side of these vesicles, although there was clearly nef protein on the vesicle surface as well. Nef has been shown to be secreted from infected cells and in one report nef was found to be secreted in association with small membrane-bound vesicles[19-22].

These identification was supported this study to analysis supernatant which may contains small membrane bounded vesicles contains nef protein. As shown in Figure-7, 48 hours of post-transfection of HEK 293 cells by pQBI-nef-6his was typsinized and the pellet and supernatant was collected. The supernatant subjected to ultracentrifuge 120000xg for 70 minutes for 4^0C and the proteins were collected. The collected protein from supernatant and cell pellet lysis was purified by Hispure cobalt column purification. On the purification process unbound, washing and elution was collected for both ultracentrifuge contents and cell lysis pellet. Figure-7 shows result of SDS-PAGE for all the collection components from purification procedure.

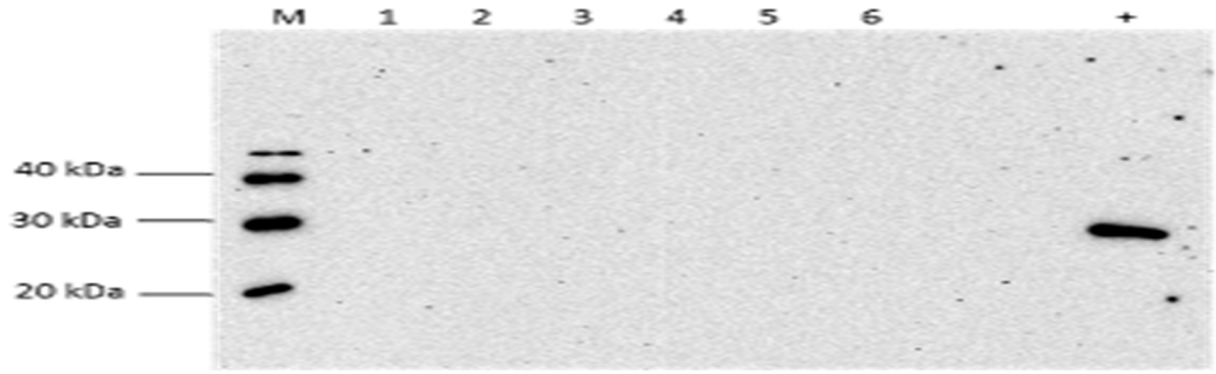


Fig.-7: SDS-PAGE for purified protein from HEK 293 cells transiently transfected by pQBI-nef-6His

Lane M in Figure-7 was prestained protein ladder. Lane 1 – Lane 3 was components from pellet and Lane 4 – Lane 6 was components from ultracentrifuged supernatant. Purified 27 kDa nef-6his was loaded as positive control. The result revealed that targeted band of 27 kDa nef was unable to be detected in SDS-PAGE. However a very faint band is detected in lane 6 which was a portion of eluated protein from HisPur cobalt column. It shows that targeted 27 kDa nef-6his protein was not efficiently expressed either in supernatant or cell pellet. It gave us suggestion that targeted nef-6his protein was expressed in very low quantity due to low transfection efficiency. The presence of very faint band of targeted nef-6his on lane 6 of Figure-7 was confirmed by western blot which shown in Figure-8.

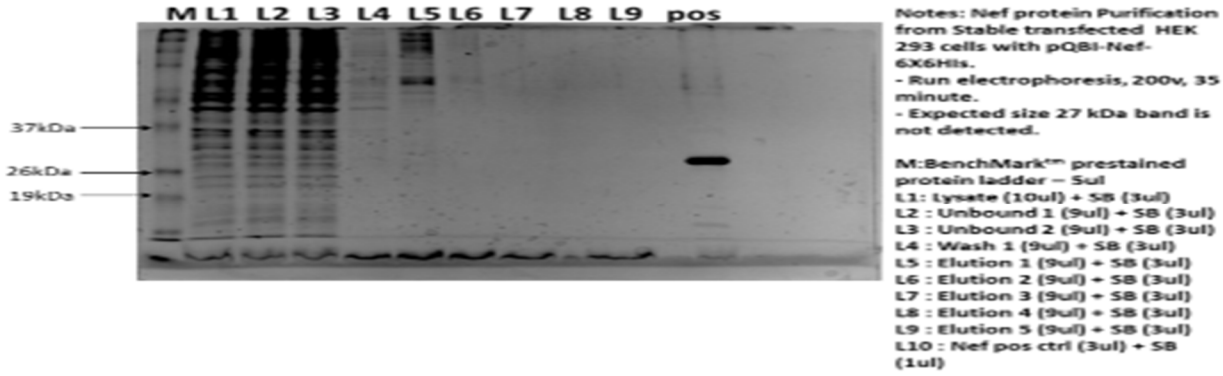


Fig.-8: Western blot for purified protein from HEK 293 cells transiently transfected by pQBI-nef-6His

In Figure-8, the primary antibody was anti-nef (anti-mouse-Merck Millipore) with dilution of 1:2 000. It was used to recognize a single 27 kD protein band in western blots and the reactive epitope maps to the COOH terminus of the full length nef protein. The secondary antibody was anti-mouse IgG with the dilution of 1:20 000. In contrast to our expectation, there was no detected of single band of 27 kDa in any of the lane but positive control of purified 27 kDa nef-6his protein gave signal against anti-nef was use. It indicates the antibody was used efficient enough to detect epitope maps to the COOH terminus of the full length positive nef-6his protein. We concluded that transient transfection either produce insignificant amount of protein or did not express targeted protein. For further identification, it was continued with stable transfection selection on G418 selective marker.

Optimization of stable transfection Nef expression on SDSPAGE/Western Blot

Stably transfected HEK 293 cells by pQBI-Nef-6His was selected on selection medium contains G418 antibiotic. The clonal event was stable and productive on the day 14 of the selection with lesser dead cells and stable clones were observed as shown in Figure-8. Four petri dishes which contain stably transfected selected clones of HEK 293 cells with pQBI-Nef-6His contain 1.3 X 10^7 cells / 13 ml and the viability was 100%. Lysate of HEK 293 transfected with pQBI-Nef-6XHis was prepared by M-PER and the lysate was purified by cobalt spin column. Total of 109.7 mg pellet was collected and added with 1.1 ml M-PER, 11 µl PMSF and 2.2 µl DNase I. The lysate was purified by Hispure cobalt spin column. The lysate, unbound 1, unbound 2, wash 1, elution 1, elution 2, elution 3, elution 4 and elution 6 was prepared and resolved on SDS/PAGE. The result has been shown in Figure-9.

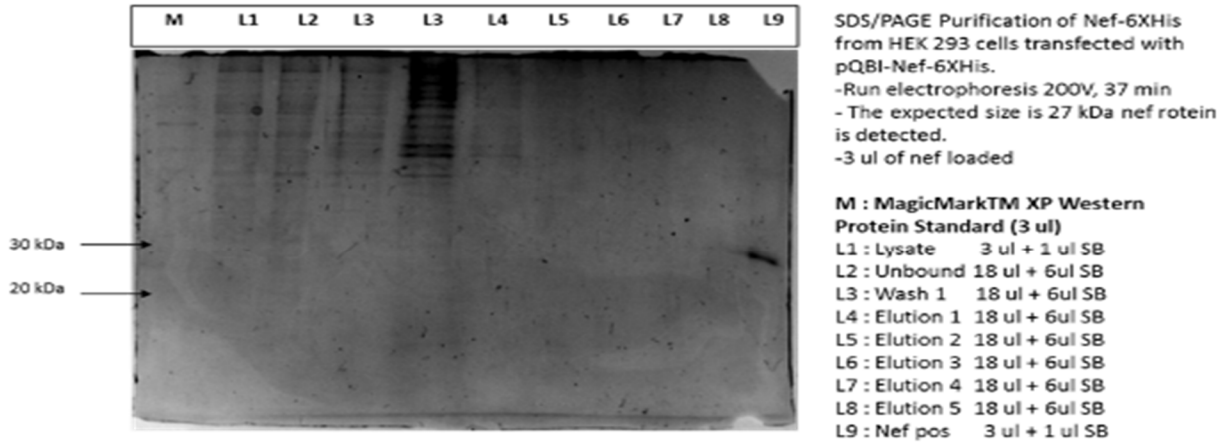


Fig.-9: SDS-PAGE for purified protein from HEK 293 cells stably transfected by pQBI-nef-6His

The result reveals that the expected size of 27 kDa nef protein was not detected through SDS-PAGE in lysate and also in purified compound of unbound, washed and eluted. Even though we was not able to determine the transfection efficiency but the stable transfection but clonal event indicates successful transfection that cells which was transfected with pQBI-Nef-6His due to the presence Neomycine resistance gene was included in that particular plasmid DNA. Moreover the result of gene sequencing for this plasmid DNA was matched when analysed through BioEdit software. The protein which was collected quantified to ascertain the quantity of the protein. The amount of protein in lysate and in the entire purified fraction was quantified by using Qubit meter. The lysate, unbound 1 and 2 diluted in the ratio of 1: 10. The results was: 1 : 2.6 mg/ml 2 : 2.9 mg/ml, 3 : 4.04 mg/ml, 4 : 0.18 mg/ml, 5 : 0.188 mg/ml, 6 : 0.178 mg/ml, 7 : 0.171 mg/ml, 8 : 0.188 mg/ml and 9 : 0.172 mg/ml. All fractions were subjected to SDS/PAGE as shown in Figure 23 and continually were proceed to Western blot as shown in Figure-10.

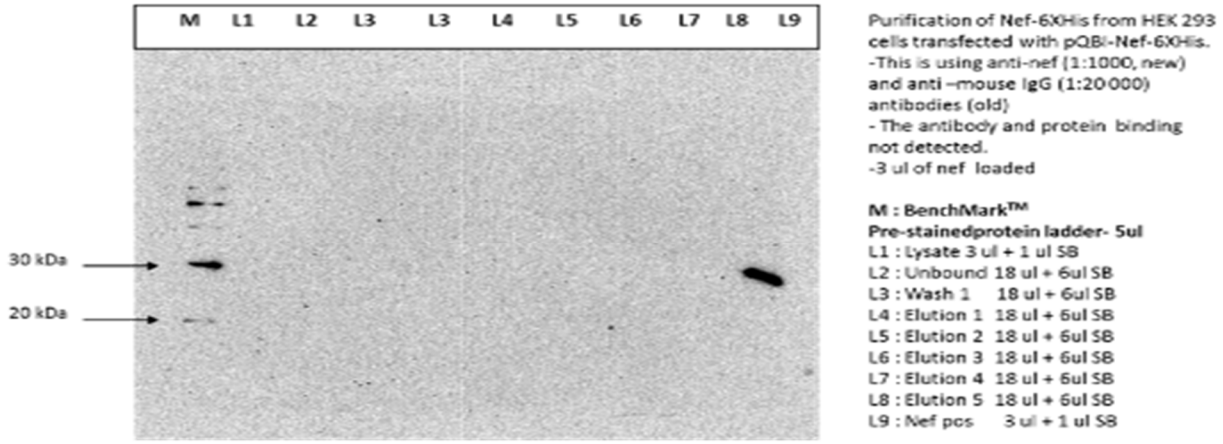


Fig.-10: SDS-PAGE for purified protein from HEK 293 cells stably transfected by pQBI-nef-6His.

The Western blot was obtained after primary antibody (anti Nef – Merck Millipore antimouse with dilution 1:2000) was incubated for overnight. The secondary antibody was IgG antimouse with the dilution of 1:20000. The Western blot results shows negative for all the sample and positive signal indicates on positive control (27 kDa nef-6his). We have confirmed the absence of protein in western blot where the targeted protein was not detected by anti- Nef (anti-mouse from Merck milipore) but it can efficiency detect the positive control. By considering the targeted protein was not detected, HEK 293 cells stably transfected with pQBI-Nef-GFP was chosen to compare the expression of nef protein and nef-GFP protein. This is because the vector, nef insert, cloning method, transfection method, concentration DNA, concentration of antibiotic G418 and selection time for this pQBI-Nef-GFP was similar the plasmid pQBI-Nef-6His. Moreover the transfection efficiency was determined by intensity of GFP fluorescence. The HEK 293 cells transfected with pQBI-Nfe-6His and pQBI-Nef-GFP was trypsinized, the cells was counted and after centrifuge the pellet was weighed to determine the amount of MPER to be added. The cell counting and weight of the pellet are 51.4 mg of HEK 293 transfected with pQBI-Nef-6His, while 65.9 mg of HEK 293 transfected with pQBI-Nef-GFP.

Both pQBI-nef-6his and pQBI-nef-GFP transfected HFK 293 cells was processed to cell lysis by adding mammalian protein expression reagent (MPER), phenylmethanesulfonyl fluoride (PMSF) and DNase. HEK cells transfected with pQBI-Nef-GFP added with 659 µl of MPER, 6.59 µl PMSF and 1.318 µl of DNase. On the other hand, HEK 293 cells transfected with pQBI-Nef-6His added with 514 µl MPER, 5.14 µl PMSF and 1.028 µl DNase. Upon to the cell lysis, the lysate contains Nef-GFP and Nef -His was resolved in SDS-PAGE and proceed to western blot with three different type of anti-Nef. The proteins also were proceeding to western blot by anti-6His. The sample for all the SDS-PAGE and western blot was standardized which composed of Lane M, Lane 1, Lane 2 and Lane positive as shown in Figure 25, 26, 27 and 28. Lane M was Magicmark^{tm} XP western protein standard, Lane 1 was nef-GFP, lane 2 was nef-6His and purified Nef-6His was included as positive control in lane positive.

Figure-11 shows result of primary antibody anti-Nef (anti-mouse) from Merck Millipore manufacture with dilution 1:2000 and secondary was anti-mouse IgG with dilution of 1:20 000. The anti-nef was not detect the targeted myristoylated 27 kDa nef-6His protein in lane 2 and 53.9 kDa nef-GFP in lane 1. Moreover, the anti-Nef was not detect nef-6His positive control which was purified 27 kDa purified Nef even though it was detected in SDS-PAGE shown in Figure-13.

Secondly Figure-13 shows positive result towards Nef-GFP protein in Lane 1, negative result Nef-6His in Lane 2 and positive signal towards purified Nef-6His detected by anti-Nef (anti-serum from rabbit purchase from NIH AIDS Research and reagent program) as primary antibody with dilution 1:2000 and secondary anti-rabbit was IgG with dilution of 1:20 000.

This antibody only gave moderate signal to 53.9 kDa Nef-GFP which can be seen in Lane 1 but did not give any signal to targeted myristolylated 27 kDa nef protein. In contrast to Merck manufacture anti-Nef

was used in Figure-12, this antibody has strong binding efficiency towards purified 27 kDa Nef-6His protein as positive control but at the same time the antibody was not specific enough to nef protein. The non-specificity of the antibody towards nef by multiple bands for purified Nef-6His showed positive control.

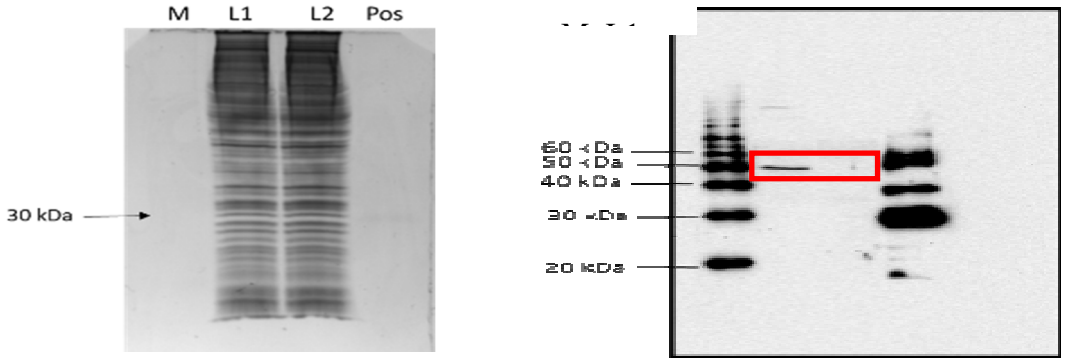


Fig.-11: SDS-PAGE/Western blot by lysate of pQBI-Nef-6His transfected HEK 293 cells Anti-Nef (Merck millipore)

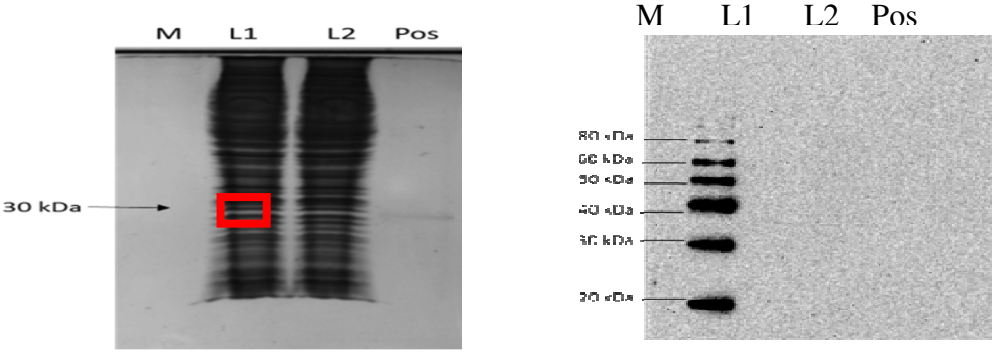


Fig.-12: Western blot for purified protein from HEK 293 cells stably transfected by pQBI-nef-6His.

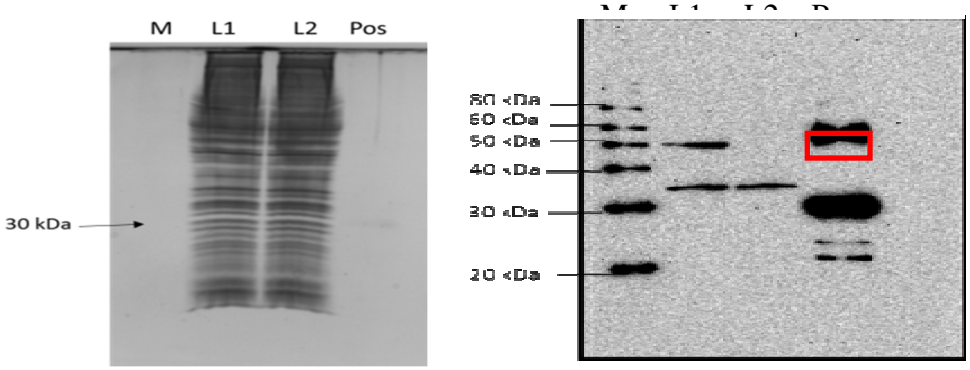


Fig.-13: SDS-PAGE/Western blot Anti-Nef ratio 1:2000 (anti-serum from rabbit of NIH AIDS Research and reagent program)

Thirdly, Figure-14 indicates positive result nef-GFP (Lane 1) and nef-6His (Lane 2) and purified nef-6His as positive was detected by anti-Nef (anti-mouse) dilution 1:500 from thermo scientific as primary antibody whereas anti-mouse IgG as secondary antibody with dilution ratio 1:20000. This was the only antibody efficiently bind to 53.9 kDa nef-GFP and nef-6his. Nef-GFP in lane 1 form two bands, one

around 50 kDa which indicates signal of protein nef-GFP and the band around 30 kDa was non-targeted nef-6his. This condition appears might be the nef protein has cleaved from Nef-GFP recombinant protein. In lane 2 as said earlier it shows strong signal band around 30 kDa, but, unfortunately that was not targeted size of nef-6his. This antibody also has strong binding efficiency towards purified 27 kDa Nef-6His protein as positive control but at the same time the antibody was not specific enough to nef protein. The non-specificity of the antibody toward nef showed by multiple bands for purified Nef-6His positive control.

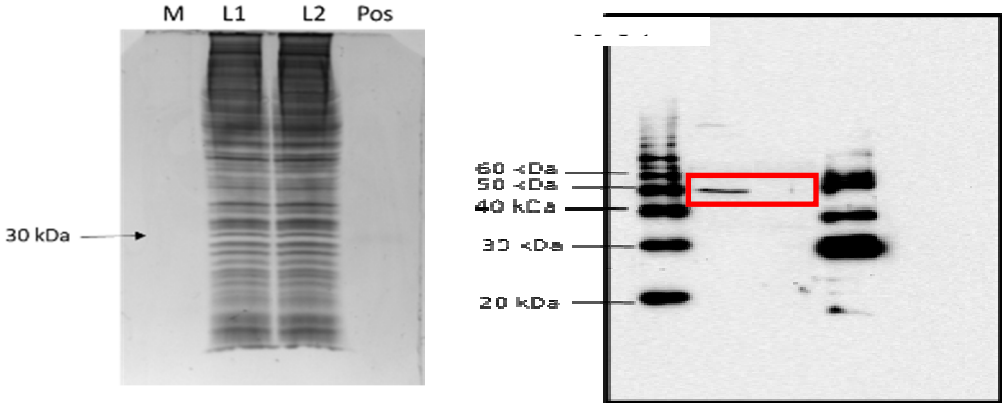


Fig.-14: SDS-PAGE/Western blot by lysate of pQBI-Nef-6His transfected HEK 293 cells Anti-Nef (Merck millipore)

The nef gene was cloned in pQBI-Nef-GFP should also expressing 27 kDa Nef protein but same band size (more than 30 kDa) was detected in lane 2 as well which is Nef-6His. This evidence suggests that Nef-GFP and Nef-6His was expressed by genome integration in stable transfection of HEK 293 cells but not in targeted size. Since Nef was collected from the crude sample and it was expressed in mammalian system, there is a possible Nef may be associated with membranous structure. Moreover Nef expressed in this system were subjected to post translational modification due to Nef is a myristoylated protein which as elaborated in introduction about Nef structure. Improper folding on post translational modification in the process of expression also can be lead to this high molecular weight form recombinant Nef protein.

The binding of anti-Nef to the epitope site found on Nef-GFP and GFP shows that the Nef gene sequence verification through BioEdit could be validated. However, the objective was to detect and isolate the targeted size of myristolylated 27 kDa Nef-6His protein form HEK 293 cells transfected with pQBI-Nef-6His was not successful. Since we have obtained not significant size of Nef protein, the sample was investigated for the presence of 6His tag in non-targeted expressed Nef protein.

We have investigated the presence of 6His tagged protein in Nef-6His by anti-6His (anti-mouse) from Thermo scientific with the dilution is 1:2000 as a primary antibody and anti-mouse IgG dilution 1:20000. The anti-6His was unable to detect 6His protein in Nef-6His recombinant protein which was expressed from HEK 293 cells. The result was validated by comparing negative control Nef-GFP and positive control purified 27 kDa Nef proteins. The Nef-6His positive control detected which shows that anti-6his is efficient enough to detect 6his protein and Nef-GFP did not give any signal because it does not contain any 6His protein. It can be conclude that either 6his was not expressed or very poorly expressed on the stable transfection process. This could be one of the reason why purified Nef protein from transfected cells did not give any signal to western blot despite the fact there is faint band detected in SDS-PAGE Figure 5. Since 6His tag was not detected in expressed Nef we are unable to purify the non-targeted expressed Nef. Unsuccesful to express the targeted myristoylated 27 kDa nef protein by HEK 293 cells transfected with 4 ug/ml pQBI-Nef-6His by calcium phophate method eventhough the cells were stably selected under selection medium 0.7 mg/ml of G418 antibiotic can be due to various factors.

First was inability to perform optimization on DNA concentration to be transfected, concentration of selective antibiotic and its concentration, concentration of FBS, transfection method, transfection reagent,

transfection time by using pQBI-Nef-6His. As stated earlier all these optimization was done by using pQBI-Nef-GFP. Even though both these DNA has the same vector and insert that targeted but the specificity could be different in the molecular structure. This was done due to time limitation; where only able use the available data in the lab at the point of starting this dissertation project. The available has to be used due to time limitation. Moreover this was the first project to be conducted to express recombinant nef protein by using mammalian system.

Second was unable to determine transfection efficiency due absence of fluorescence protein such as GFP in the constructed mammalian expression vector. Normally, the transfection efficiency influence by cell health, cell confluence, number of passages DNA quality and DNA quantity. Transfection seem to be successful as microscopic observation on increases of clonal event on selection medium G418 and percentage of death cells was less than 10%. However the percentage of genome integration and ability to express the targeted protein notable determined. This was the one of the limitation which was reason to detect the targeted protein. As we can see from the result in Figure 27 there is a detection signal by anti-nef but not the targeted size. It could be happen because of improper post-translational modification or improper genome integration by mammalian expressing vector. Apart from these factors we also unable to express the nef frame in different mamalian expressing vector and different one step purification tag. As discuss earlier even the 6his tag was unable to detected by anti-6his in Figure-15. Campbell et al., 2008[10] showed that Nef can be found in the extracellular medium taken from Nef-transfected HEK 293 cells with an expression vector PCDNA created by cloning the nef frame from the viral clone pNL4-3.

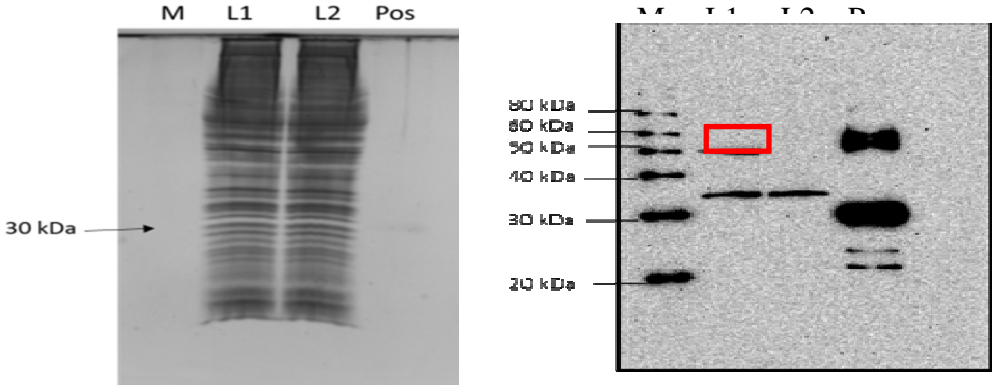


Fig.-15: SDS-PAGE/Western blot Anti-Nef ratio 1:2000 (anti-serum from rabbit of NIH AIDS Research and reagent program)

They also detected the released nef by using polyclonal rabbit nef antiserum from AIDS reagent Program. Additionally[23-25] study showed that Jurkat cells transfected with pQBI-Nef-GFP exressing full length of HIV-1 NL4-3 Nef protein in the form of exosome. It shows that, if all these limitation can be overcome, the myristoylated 27 kDa nef-6His recombinant protein can be succuesfully express by HEK 293 cells

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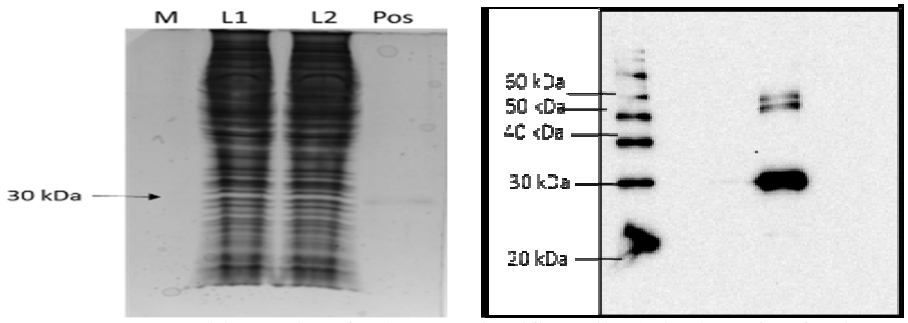

Fig.-16: SDS-PAGE/Western blot Anti-Nef (Thermo scientific antibody in the ratio of 1:500)-Anti mouse

CONCLUSION

It can be conclude that protein that was detected not identically synthesis through post-translation modification though it was expressed in the cytoplasm of HEK 293 cells. 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

ACKNOWLEDGEMENT

The authors would like to thank University of Malaya, Malaysia (RG428/12HTM), Baghdad University and Al-Nahrain University for the financial support and technical assistance on this work.

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[RJC-1568/2017]