Chapter 2

MATERIAL AND METHODS
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2.1 Materials

Chemicals
Agarose, ampicillin, ammonium acetate, Tris Base, EDTA, SDS, sodium-acetate, potassium-acetate, boric acid, disodium-hydrogen-phosphate, sodium-dihydrogen-phosphate, sodium chloride ethidium bromide, urea, ammonium persulphate, glycerol, sodium bicarbonate, Triton X-100, dithiothreitol, magnesium chloride, BSA, IPTG, Orange G, DEPC, Tween-20, acrylamide, calcium chloride, trypsin, EDTA, sodium citrate, bromophenol blue, xylene cyanol FF and Cycloheximide were obtained from Sigma-Aldrich Co. (Missouri, U.S.A.). X-gal, NTP and dNTP, sodium chloride, bis-acrylamide, TEMED, PCR buffer and Magnesium chloride for PCR, DNA markers, were from Promega Biotech Co. (Madison, U.S.A.). All other chemicals were at least of analytical grade and were from Qualigens laboratories (Bombay, India) or Merck (New Jersey, U.S.A.) Trizol reagent, DMEM, lipofectin, lipofectamine 2000, antimycotic-antibiotic, gentamicin, RNase-DNase free water, was obtained from Invitrogen-GIBCO/BRL (Maryland, U.S.A.). Fetal bovine serum was obtained from Biological Industries (Beit Haemek, Israel). Luria Bertini medium and Luria Miller agar for bacterial culture were obtained from Difco Laboratories (Detroit, U.S.A.). Prestained rainbow protein markers, nylon and nitro-cellulose membranes, ECL reagent, all were obtained from Amersham Biosciences (Buckinghamshire, U.K.). The p53 inhibitor, Pifithrin-α was obtained from Santa Cruz Biotechnology.

Commercial Kits
Qiaprep spin mini kit and Qiagen plasmid midi kit (West Sussex, U.K.) were used for isolation of DNA. Isolation of DNA fragments from gel was carried out using QiaGel extraction kits or PCR products were purified using nucleotide removal kit from Qiagen (West Sussex, U.K.). PCR core system I was used to amplify the oligonucleotides (Promega Biotech, Madison, USA). pGEMT-Easy cloning vector was
obtained from Promega. BCA protein assay kit was obtained from Pierce Biotechnology (Rockford, IL, U.S.A.). Reverse transcription was carried out using ImProm-II™ Reverse Transcriptase kit from Promega.

**Enzymes**

DNA restriction enzymes were purchased from New England Biolabs (Massachusetts, USA) and Promega Corporation, (Madison, U.S.A.). RNase A was obtained from Qiagen (West Sussex, U.K.). DNA ligase, RNA polymerase, RNAsin, Taq DNA polymerase, T7 RNA polymerase, SP6 RNA Polymerase and alkaline phosphatase was obtained from Promega Biotech.

**Oligonucleotides**

Oligonucleotides used in this study were synthesized by Sigma- Genosys (Missouri, U.S.A.).

**Antibodies**

The primary Abs used were anti-p53, anti-GST, anti-His, anti-HA, anti-GAPDH, anti β-TrcP, anti–IκB-α, anti-BST-2, anti-Bax (Santa Cruz Biotechnology), anti–Ser362/366 phosphorylated p53 (P-362/366 p53; Abcam) and anti-Ser15 phosphorylated p53 (P-15 p53; Cell Signaling Technology). Secondary antibodies used were anti–mouse or anti–rabbit conjugated with HRP (1:10 000 dilution; Jackson Immuno-Research Laboratories Inc).
2.2 Viruses, Plasmids and Cell lines

Viruses
HIV-1 isolate pNL43 (GenBankTM accession number AF324493) and HIV-1 isolate 93IN905 (GenBankTM accession number AF067158) [both obtained from NIH, Maryland, USA] were used for most of the studies. A comparison of the predicted amino acid sequences lead to the selection of the two subtype C virus used in this study. We also used the infectious molecular clone of HIV-1 subtype C, pIndie in some studies. P93905 and p-Indie were chosen based on their predicted Vpu protein length and the presence of conserved and significant sequence motifs. A pNL4-3 virus based viral backbone was designed for intracellular expression of different Vpu constructs (B, C and various primary isolates) upon infection in T-cell line by specifically substituting the wild type Vpu locus with that of natural Vpu variants (Details provided in Chapter 4).

Plasmids

Wild type (B and C), M (mutant) and chimeric (BC and CB) Vpu expressing constructs
Vpu from subtype B (pNL4-3) and C (Indian isolate 93IN905) HIV-1 were cloned in vector pCMV-HA vector (Clontech) to express HA tagged Vpu B and Vpu C proteins. Mutations of S52A and S56A were introduced by site-directed mutagenesis with Pfu Turbo polymerase in pCMV Vpu B-HA to give pCMV M-Vpu-HA (mutant Vpu). In another strategy taking advantage of the fact that most of the variation between subtype B and C Vpu are clustered in the cytoplasmic helix-2 (amino acid position 59 to 81), we created two Vpu chimeric constructs; one possessed the N-terminal half of subtype B (nucleotide position 1-162) and the C-terminal half of subtype C (nucleotide position 168-249) to generate BC-Vpu and the second construct harbored the opposite orientation (nucleotide position 1-168 derived from subtype C and 162-246 of subtype B) to generate CB-Vpu.
The $\beta$-TrcP shRNA expression vector

The shRNA sequence against $\beta$-TrcP that was reported previously (Xia et al., 2009) was cloned in RetroQ-Zs Green Vector.

BST-2-RFP expression plasmid

To clone a BST-2-RFP fusion gene, the cDNA corresponding to BST-2 mRNA was amplified from total RNA isolated from HeLa cells and precisely clone in frame with red fluorescent protein (RFP, at C-terminus) of dsred2-N1 (Clonetech) vector.

Acquired Plasmid

HA-p53 and GST-p53 were a kind gift from Dr. Yukiko Gotoh (The University of Tokyo, Japan) and Dr. Sanjeev Das respectively (National Institute of Immunology, India). p53ΔI expressing plasmid was provided by Dr. Karen Vousden (Beatson Institute for Cancer Research, UK). The pNL4–3 HIV-1 clone as well its mutant M-Vpu+ or Vpu-deficient derivative were kind gifts from Dr. K. Strebel (NIH, USA).

Bacterial strains

*E.coli* strains DH5α and XL-Blue were used for DNA cloning.

Cell lines used

HEK 293T (human embryonic kidney 293 cells), MCF-7 (breast cancer cell line, p53 Wt), HeLa (Human Cervical Cancer line), Tzmbl cells (HIV indicator cells, AIDS Research and Reference Reagent Program, NIH, Bethesda) and H-1299 (non-small cell lung carcinoma, p53 null) cells were maintained in DMEM (Gibco, Invitrogen) supplemented with glutamine, 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C with 5% CO2. MOLT-3, MOLT-4 T cells (T-lymphoblastoid cell line, p53 Wt) and K-562 cells (human erythroleukemia cell line, p53 null) were maintained in RPMI (Gibco, Invitrogen) media supplemented with
glutamine, 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C with 5% CO2.
2.3. Methods

Polymerase chain reaction

The polymerase chain reaction (PCR) was carried out using the PCR Core System I (Promega, U.S.A.). 200ng of template DNA/oligonucleotide and 1µM terminal primers were combined in 25µl reaction volume finally containing 1X Mg free reaction buffer (500mM KCl, 100mM Tris-HCl, pH 9.0, 1.0% Triton X-100), dNTP mix with 0.2mM of each, 1.5mM MgCl₂ and 0.625U of Taq DNA Polymerase. 30 thermal reaction cycles from steps 2-4 were repeatedly carried out, in GeneAmp PCR 2400 machine (Perkin Elmer, USA). PCR amplification was analyzed on a required % agarose gel using a 100 bp ladder or λ Hind III marker (Promega, USA).

Gel elution of DNA fragments

The plasmid DNAs, for cloning, were digested with the respective enzymes, checked on an appropriate percentage of agarose gel along with 100 bp ladder or λ Hind III marker (Promega, USA) and the required fragments were eluted from the gel using the Qiagen Gel Extraction kit (Qiagen, U.K.). According to the manufacturer’s directions, the area of the gel containing the DNA fragment was excised using a clean and sharp blade, minimizing the amount of surrounding agarose excised with the fragment. The gel slice was weighed and placed in a microfuge tube. Three volumes of Gel Solubilization Buffer (QG) was added for every one volume of gel. The gel piece was then vortexed and incubated at 50°C for 10 min. The contents were mixed in between, by inverting the tube few times, to ensure gel dissolution. It was then centrifuged at 13,000 rpm for 1min. The flow through in the discard column was carefully removed. Then 500 µl of buffer PB was added to remove the traces of gel the tube centrifuged at 13,000 rpm for 1 min. The flow through in the discard column was removed and 750 µl of Wash Buffer (PE) (containing ethanol) was added and the tube was centrifuged at 13,000 rpm for 1 min. The flow through was discarded and another spin at maximum for 2 min was given to remove the traces of
Wash buffer. The column was then put on a fresh tube and finally, to elute the DNA, 40 µl of Tris-EDTA buffer (TE) or RNase-DNase-free water was added and then centrifuged at 13,000 rpm for 1 min. The flow through in the eppendorf tube had DNA of interest. The purified DNA fragments were checked on an agarose gel, with an appropriate marker, before setting up the ligation reaction.

**Ligation and Transformation**

The PCR products were ligated with T-tailed vector (pGEM-T Easy from Promega). In general, the ligation was carried out at a vector: insert molar ratio of 1:3 or 1:4 in a 10µl reaction volume finally containing 1X T4 DNA ligase buffer (300mM Tris-HCl, pH 7.8, 100mM MgCl2, 100mM DTT, 10mM dATP) and 1U of T4 DNA ligase (Promega, U.S.A.). The reaction mix was incubated at 16°C for 16 hrs (overnight). Following the reaction, the ligated DNA was transformed into Calcium Chloride treated *E.coli*-DH5α or XL-Blue1 competent cells with a high transformation efficiency. The transformed cells were plated on to Luria-Bertani-Agar plates containing 100µg/ml of ampicillin. The plated cells were incubated at 37°C for 8-10 hours.

**Plasmid Minipreps**

Each single colony was inoculated individually in 5ml Luria-Bertani (LB) medium with 100µg/ml of ampicillin. The cultures were grown for 8-10 hours at 37°C with vigorous shaking (~200 rpm). Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, U.K.). According to the manufacturer’s directions, the pelleted bacterial cells were resuspended in 250µl of buffer P1 and transferred to a microfuge tube. 250µl of buffer P2 was then added and mixed gently by inverting the tube 4-6 times. Further, 350µl of buffer N3 was added and mixed by gently inverting the tube 4-6 times. The microfuge tubes were then centrifuged at 13,000 rpm for 10 min. QIAprep spin columns were placed in 2-ml collection tubes and the supernatant was applied to these columns. These were then centrifuged for 1 min and the flow-through was discarded. The Qiaprep columns were then washed by adding 0.75ml of
buffer PE and centrifuged for 1 min. The flow-through was discarded and an additional centrifugation was given for another minute to remove traces of the wash buffer. The QIAprep columns were placed in a fresh 1.5ml microfuge tube. Finally, to elute out the DNA, 50µl of buffer EB (10mM Tris-Cl, pH 8.5) or RNase-DNase free water was applied to the center of each column and then centrifuged for 1 min after letting it stand for 1 min. The flow-through contained the DNA of interest.

**Plasmid Midipreps**

For large scale plasmid DNA isolation, the bacterial cells were cultured in 100ml of LB medium with 100µg/ml of ampicillin. The cultures were grown for 8-10 hours at 37°C with vigorous shaking (~200 rpm). Plasmid DNA was isolated using the QIAGEN Plasmid Midi kit (100). Briefly, the bacterial cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The bacterial pellet was resuspended in 4 ml of the resuspension buffer P1 (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100µg/ml RNase A). 4 ml of lysis buffer P2 (200mM NaOH, 1% SDS) was added, mixed gently by inverting 4-6 times and incubated at room temperature for not more than 5 min. Further 4 ml of chilled neutralization buffer P3 (3.0 M potassium acetate, pH5.5) was added, mixed gently as before and incubated on ice for 10 min. It was then centrifuged at maximum rpm for 30 min at 4°C. The supernatant containing the plasmid DNA was immediately removed and re-centrifuged at maximum rpm for 15 min at 4°C. The supernatant was now collected in fresh tubes and kept on ice. A QIAGEN-tip 100 was equilibrated with 4 ml of equilibration buffer QBT (750mM NaCl, 50mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton X-100) and the column was allowed to empty by gravity flow. The supernatant was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed thrice with 10ml of wash buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol). The DNA was then eluted with 5 ml of elution buffer QF (1.25M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol). The DNA was precipitated by adding 0.7 volumes of isopropanol to the eluted DNA. It was thoroughly mixed and centrifuged immediately at maximum rpm.
for 30 min at 4°C. The supernatant was carefully decanted. The DNA pellet was washed with 2 ml of 70% ethanol, and centrifuged at maximum rpm for 15 min at 4°C. The supernatant was carefully decanted without disturbing the pellet. The pellet was air dried for 5-10 min and the DNA was dissolved in 200 µl of RNase-DNase free water.

To determine the yield, DNA concentration was determined both by Ultra Violet (UV) Spectrophotometry (DU-65 spectrophotometer, Beckman, U.S.A.) and quantitative analysis on an agarose gel using a UV Transilluminator (UVP, California, U.S.A.). All the putative clones were then screened for the correct recombinant clones by restriction enzyme digestion using appropriate enzymes. The digested samples were checked on an agarose gel along with an appropriate size marker to assess the size of the insert from the putative clones. The clones containing very small fragments were further confirmed by sequencing both strands of the DNA.

**Cell culture conditions and passage**

Cells were incubated at 37 °C in a 5% CO2 atmosphere. Cells were passaged every three to four days when subconfluent, using trypsin EDTA solution (Sigma-Aldrich). Briefly, old media was discarded and the cell monolayer washed gently with 1X Phosphate Buffered Saline (PBS) (Sigma-Aldrich). Cells were dislodged by adding 1 ml trypsin EDTA solution, pouring off the excess, and incubating for ~3 minutes at 37 °C. Cells were then tapped off the flask surface and resuspended to a concentration of 4 X 10⁶ cells in 15 ml complete DMEM in a 75 cm³ flask (Nalge Nunc Int.). Cell concentration was determined by staining cells with 0.4% Trypan Blue solution (Sigma-Aldrich), and assessing the viable cell count on an Improved Neubauer haemocytometer (Merck, Darmstadt, Germany). Briefly, ten microlitres cell suspension was added to 90 µl 0.4% Trypan Blue solution and mixed thoroughly before pipetting onto a clean haemocytometer and allowing diffusion. Viable cells do not take up the dye and appear white under a light microscope. Live cells were counted in all four primary squares and averaged for one square to
denote the number of cells in $1 \times 10^{-4}$ ml. By taking into account the dilution factor of ten, the cell count was calculated per ml of cell suspension and adjusted accordingly. Adherent cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) while suspension cells were maintained in RPMI, with 10% Fetal bovine serum (FBS) and 1% antibiotic-antimycotic (penicillin, streptomycin and amphotericin B). The cells were maintained at 37°C with 5% CO$_2$.

**Storage of mammalian cell strains**

Cells were frozen in a 90% fetal calf serum (FCS), 10% Dimethyl sulfoxide (DMSO) freezing mix, and stored in liquid nitrogen. Cell stocks were prepared as follows: 1 ml aliquots of cell suspension were centrifuged in 1.8 ml Cryovial (Nalge Nunc Int.) at 320 x g for 2 min (Eppendorf centrifuge 5810). The supernatant was carefully pipetted off the cell pellet and the pellet resuspended in 1 ml of the appropriate freezing mix. Cryovials were transferred immediately to a nest box at -80 °C and stored overnight before transferring to liquid nitrogen for indefinite storage.

**Transient transfection**

Transfection of cell lines used was carried out using lipofectamine-2000 reagent (Invitrogen, U.S.A.). In a six well plate $10^5$ cells/ well were seeded in 2ml medium supplemented with serum. The cells were incubated in a CO$_2$ incubator until the cells were 60% confluent. For each transfection, 1-2µg of DNA was diluted in 250 µl serum free media. Also, 10µl of lipofectamine 2000 reagent was diluted in 250 µl of serum free media and allowed to stand at room temperature for 5 minutes. The two solutions were combined, mixed gently and incubated at room temperature for 20 minutes. The cells were washed once with 2ml of serum free medium. For each transfection, 0.8 ml of serum free medium was added to each tube containing lipofectamine –DNA complexes. The complex was mixed gently and overlaid onto cells. The plate was incubated for 4-6 hrs in a CO$_2$ incubator. The medium in each well was replaced with serum containing medium and the cells were further incubated for varying periods of time at 37°C. The concentration of lipofectamine
2000 was used in the ratio 1:2 or 1:3 with DNA. Cells were processed for various assays at 24, 48, and 72 hours post-transfection. For cells assayed at 72 hours, the media was replaced with fresh complete DMEM 24 hours post-transfection. The empty control vector DNA was used to normalize equal amounts of DNA in each transfection.

**RNA isolation from cell lysates**

After transfection, the cells were harvested and RNA was isolated from the cell lysates using Trizol reagent (Invitrogen) and, purified according to the manufacturer’s directions. Briefly, the cells were lysed directly in the culture dish by adding 1ml of Trizol reagent to each well. The homogenized sample was incubated at room temperature for 5 min to permit complete dissociation of the nucleoprotein complexes. For purifying the RNA, 200µl of chloroform was added, the tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 min. Tubes were centrifuged at 12,000 rcf for 15 min at 4°C. The aqueous phase was collected, mixed with 500µl isopropanol and incubated at room temperature for 10 min. Centrifugation was carried out at 12,000 rcf for 10 min at 4°C. The supernatant was carefully removed and the RNA pellet was washed with 1ml of 70% ethanol by vortexing and then centrifuging at 7500 rcf for 5 min. The pellet was air dried and dissolved in water.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

1µg of template RNA and 1µM terminal primers were combined in 5µl reaction volume and the primer/template mix was thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix of volume 15 µl was assembled on ice to contain nuclease-free water, 1X reaction buffer, 1µl reverse transcriptase, 6 mM magnesium chloride, 0.5 mM dNTPs and 1 U ribonuclease inhibitor RNasin. As a final step, the template-primer combination was added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction
was incubated at 42°C for up to one hour. The reverse transcriptase was thermally inactivated prior to amplification and for that the reaction was incubated at 75°C for 15 minutes. The cDNA might be directly amplified by adding the products of the heat inactivated reverse transcription reaction to the PCR mix and proceeding with the standardized thermal cycling.

**Protein isolation from cell lysates**

After transfection, the cells were harvested and protein was isolated from the cell lysates. The cells from each well were pelleted at 2000 rpm for 10 min at 4°C. The supernatant was carefully removed and the pellet was incubated on ice for 1 hr after adding 100µl of RIPA Lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1µg/ml leupeptin) with intermittent vortexing. The tubes were centrifuged at maximum rpm for 10 min at 4°C and the supernatant, containing the proteins, was collected and stored at –70°C. The purified protein fractions were quantitated using the BCA protein assay kit and the O.D. was taken at 562nm.

**SDS-PAGE and Western blot analysis**

Expression of recombinant proteins was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of transfected cell lysates. Cells were transfected and assayed at 48 and 72 hours post-transfection. Mock transfected (empty vector) and untransfected cells were also assayed at these time points. Approximately 1.2 x 10^6 cells were washed and scraped into 1 ml 1X PBS (Sigma-Aldrich), before harvesting at 15 000 x g for 5 min (Eppendorf centrifuge). Cells were resuspended in 100 µl cell lysis buffer and vortexed thoroughly. The cell lysate was centrifuged at 10 000 x g for 10 min at 4 °C, the supernatant transferred to a fresh tube and stored at -80 °C until assayed for protein concentration. Twenty to fifty micrograms of cell lysate was resolved on a
12% SDS-PAGE gel. Briefly, a 4.5% stacking gel and 12% resolving gel were prepared in duplicate, set, and assembled into PAGE apparatus (BioRad) with electrophoresis buffer. Fifteen microlitres each of the sample and the 2X loading dye were added to a 1.5 ml microcentrifuge tube (Eppendorf) and boiled for 5 min before transferring to ice. The mix was loaded onto the gel alongside 10 μl Prestained 6-175 kDa Protein Marker (New England BioLabs). The gel was run at 70 volts for stacking, and then at 100 Volts until the dye front was seen a half centimetre from the bottom of the gel (~2.5 hours). Proteins were resolved with PAGE and transferred to Immobilon membrane (Millipore). The membrane was then blocked for an hour in 50 ml 5% fat free milk powder dissolved in T-TBS to prevent non-specific antibody binding. The membrane was then rinsed three times with T-TBS, prior to primary antibody binding. The membrane was incubated for one hour with 2 ml of the recommended dilution of the primary antibody before washing three times for 5 min in T-TBS. The membrane was then incubated for one hour in 20 ml of a 1:10,000 dilution of the Horseradish Peroxidase (HRP) conjugated secondary antibody, and washed three times as before to remove any unbound HRP-conjugate. Enhanced chemiluminescence (ECL) detection of the protein was performed using the EZWestern HRP substrate (Biologic Industries). GAPDH was used as a loading control in all cases.

**Cell death and Apoptosis Assay**

HEK 293T cells were removed by treating with cell dissociation buffer. Cells were centrifuged at 1200 rpm at 4°C, the supernatant decanted and the cells were washed in 1X PBS. Finally, the cells were resuspended in 1X PBS containing Propidium iodide (at a final concentration of 10 μg/ml). The cells were analysed on the flow cytometer for propidium iodide incorporation to measure cell death. For analysis of apoptosis, Vpu transfected cells were harvested, stained with Annexin V-FITC/propidium iodide by using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to manufacturer’s protocol, and analyzed on a BD FACS caliber system (BD Biosciences).
Infection by HIV-1 PNL4-3 or HIV-1 mutants

Infection of various cell lines was accomplished by incubating the cells for 4 hours with equal amounts of infectious virus (1 MOI) assessed by β-galactosidase staining with the use of HIV-1 indicator Tzmb1 cells. The infected cells were harvested 48 hours after infection and lysate subjected to immunoblotting with indicated antibodies. Another set of cells was stained with PI (10 μg/mL) for determining the total cell death. Reporter virus pNLΔGFP (obtained from NIH) was used to ensure uniform infection in different cell lines.

Beta Galactosidase (β-Gal) staining

The cells were washed twice with ice cold 1X PBS buffer. Cells were then fixed with fixation buffer (0.25% Glutaraldehyde in 1X PBS) for 10 minutes at room temperature while gently rocking the plates. The cells were washed twice with ice cold 1X PBS and stained with freshly prepared staining buffer (5mM K₄Fe(CN)₆·3H₂O, 5mM K₃Fe(CN)₆, 1mg/ml X Gal solution, 2mM Mgcl₂ for 2-20 hours at 37°C.

Beta galactosidase assay

After 48 hours of transfection, cells were harvested and lysate was prepared using Reporter Lysis buffer (Promega). Bradford assay was performed to determine overall protein concentration of each lysate. 25 μg of each lysate was used for the assay in a total reaction volume of 300 μl containing 3μl of 1M Mg buffer containing 14 M β mercapto-ethanol and 66μl O-nitrophenyl-β-D galactopyranoside. The volume was made up to 300μl with 0.1 M phosphate buffer (pH 7.5). The reaction tubes were incubated at 37°C for 2 hrs and the absorbance read at 420 nm.