Appendix

Important protocols commonly used in different experiments

Separation of PBMCs from whole blood

Procedure:

PBMCs were separated by Ficoll-hypaque density gradient centrifugation as described below:

1. Blood was layered on the top of 4 ml of Histopaque-1077 (Mixture of polysucrose: 5.7 g/dl and sodium diatrizoate: 9.0g/dl with density of 1.077 ± 0.001 g/ml from Sigma) in a 15ml conical centrifuge tube.
2. Tube was centrifuged in swinging bucket rotor at 400xg for 30 minutes at room temperature. During centrifugation, erythrocytes and granulocytes sediment at the bottom whereas mononuclear cells remained at plasma-histopaque interface.
3. After centrifugation, PBMC layer at plasma: histopaque interface was transfered to a sterile 15 ml conical centrifuge tube.
4. Cells were washed with 10 ml wash medium (RPMI: Gibco, USA) by centrifuging at 250xg for 10 minutes. This step was repeated twice and cell pellet was resuspended in 10 ml 10%RPMI.
5. Viable number of PBMCs was counted as follows: 20µl cell suspension was mixed with equal volume of trypan blue (Gibco, USA) and 10µl of this mixture was filled in counting chamber of hemacytometer (Hausser Scientific, USA). Viable cells (that are not stained by trypan blue) were counted in four WBC chambers (each chamber: $0.1mm^3$) under light microscope. PBMC count (cells / ml) was determined as:

$$\text{Cells/ml} = (\text{Average number of cells in four WBC chambers}) \times \text{dilution factor} \times 10^4$$

Preparation of Monocytes from PBMC by Dynabeads (Invitrogen, Inc.)

Procedure:

1. PBMC suspension was prepared from blood as described above.
2. Cells were resuspended at 1×10^8 PBMC per ml in Isolation buffer [Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% BSA and 2mM EDTA].

3. PBMC (500µl; 5 X 10^7 cells) were transferred in Isolation buffer to a tube and 100µl blocking reagent [Ca^{2+} and Mg^{2+} free PBS supplemented with 2% FBS] was added.
4. Antibody mix (100µl) provided with the kit was added, mixed well and incubated for 20 min at 2-8°C.
5. Cells were washed by adding 10µl Isolation buffer. Mixed well by tilting the tube several times and centrifuged at 350 x g for 8 min at 2-8°C. Supernatant was discarded.
6. Cells were resuspended in 500µl Isolation buffer and 500µl pre-washed Depletion MyOne SA Dynabeads provided with the kit were added. Incubated for 15 min at 2-8°C with gentle tilting and rotation.
7. Bead-bound cells were mixed vigorously by pipetting at least >10 times.
8. Isolation buffer (5ml) was added and tube was placed in the magnet for 2 min.
9. Supernatant containing the human monocytes was transfered to a new tube.
10. Remaining monocytes bound to beads were isolated by adding 5ml Isolation buffer to tube containing the Dynabeads, mixed vigorously and the tube was placed in magnet for 2 min.
11. Two supernatants were combined. NOTE: During isolation of monocytes it is necessary to keep the buffers and cells cold.
12. To generate macrophages from monocytes, monocytes were cultured in 10% DMEM with GM-CSF (250 ng/ml) for 6-7 days. Every third day media was exchanged with new medium.
13. After 7 days adherent macrophages were used for infection.

Isolation of $CD4^+$ T Cells from blood by RossetteSep method (StemCell tech, Inc.)

Procedure:

1. RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail (50 µl/ml of whole blood) was added (e.g. for 2 mL of whole blood, add 100 µL of cocktail). Mixed well.
2. Incubated for 20 minutes at room temperature.
3. Sample was diluted with an equal volume of PBS + 2% FBS and mixed gently.

4. 10ml of diluted sample was layered on top of the 15 ml Histopaque-1077 density medium (Sigma) carefully in 50 ml falcon tube. NOTE: Be careful to minimize mixing of density medium and sample.
5. Tube was centrifuged for 20 minutes at 1200 x *g* at room temperature, with the brake off.
6. Enriched cells were removed from the density medium: plasma interface and transferred to fresh sterile 15 ml tube.
7. Enriched cells were washed with PBS + 2% FBS at 2000 rpm for 5 min. Step was repeated.
8. Cells were resuspended in 10% RPMI and viable cells were counted using Hemacytometer (Hausser Scientific, USA) and Trypan blue dye (Gibco, USA).

Calcium-Phosphate transfection method (Promega, Inc.)

Procedure:

1. Cells were seeded in 6-well trays ($3.5x10^5$ cells per well) one day prior to transfection and propagated overnight in DMEM-10%FBS containing antibiotic mix at 37^oC in CO_2 incubator.
2. Three hours prior to transfection, medium of cells was replaced with fresh DMEM-10% FBS.
3. A mix of plasmid DNA and transfection reagents was prepared in an eppendorf (2.5µg plasmid DNA+10.4µl $CaCl_2$+ XµL sterile water). NOTE: Volume of water was adjusted according to volume of plasmid DNA to make total volume 83.4µl.
4. This mix was added in another eppendorf containing 83.4µl HEPES buffer dropwise with continuous vortexing.
5. The mix was incubated for 30 min at room temperature. After incubation this mix was added dropwise on cells to be transfected.
6. Transfected cells were incubated overnight at 37^oC in CO_2 incubator. Next day, media of transfected cells was changed and replaced with fresh DMEM-10% FBS.
7. After 48 h, cell supernatant containing virus was harvested and stored in -80^oC for future use.

Staining of TZM-bl cells

Procedure:

1. Removed growth media (DMEM) from infected cells (48 h post-transfection).
2. Fixed cells with 50µl 0.5% glutaraldehyde in PBS for 10 min at room temperature.
3. Washed cells twice with 1X PBS (pH 7.4) and add 200µl X-Gal: yellow PBS (1:80) and incubated for one to two hours in dark at room temperature until colour develops. NOTE: X-Gal powder was dissolved in dimethylformamide (DMF); 0.4mg/ml.
4. Blue cells were counted under normal light microscope. Blue cells represented foci of infection and infectivity was estimated in terms of FFU.

Reverse Transcriptase ELISA (Lenti RT Activity Kit; Caviditech, Inc.)

Principle:

The Lenti RT Activity kit is used for detection or quantification of the activity of the enzyme Reverse Transcriptase (RT) from lentivirus. The procedure consists of two steps, the DNA synthesis and the DNA quantification. The 96-well Poly A Plate contain RNA template bound to the bottom. A reaction mixture, containing primer and a nucleotide (BrdUTP), is added to the plate as well as the sample to be analysed. The RT in the sample will synthesise a DNA-strand. An alkaline phosphatase (AP) conjugated α-BrdU antibody binds to the double stranded DNA/RNA molecule. The product is quantified by addition of a colorimetric AP substrate. The AP activity is proportional to the RT activity in the sample.

Procedure:

Day 1

A. **Kit Components** :

1. Poly A plates.
2. Sample Dilution Buffer (bottle B)
3. RT reaction component (vial C1)
4. Reconstitution Buffer (bottle C2)
5. HIV-1 r RT-standard (vial D)

6. Concentrated washing buffer (bottle E)
7. Adhesive tapes

Note: Thaw the frozen reagents at 37°C

B. **Preparation of reaction mixture**

1. Added 12ml of reconstitution buffer (C2) to vial containing RT reaction components (C1). Dissolve completely.
2. Transferred the contents of both C1 vials to a 50 ml glass beaker.
3. Added 6ml of pure distilled water and mix thoroughly.

C. **Preparation of Poly A plates**

1. Plate was taken out from the foil pouch.
2. Added 150µl of reaction mixture (beaker) to each well.
3. Plate was sealed with adhesive tape and incubated at 33°C for 20-60 min.

D. Dilution of HIV-1 r RT Standard

1. Added 1.5 ml of Sample Dilution Buffer (B) to the vial containing lyophilized HIV-1 r RT-standard (D). Dissolved completely. NOTE: Do not shake and mix gently.
2. Added 125µl of Sample Dilution Buffer (B) to 12 wells of a 96-well plate.
3. Added 50µl of HIV-1 r RT Standard (D) to the first of the 12 wells.
4. Transferred 100µl from the first well to the second.
5. Transferred 100µl from the second well to the third and so on until a series of 12 dilutions was obtained.

   Note: While preparing the Standard Dilutions, mix thoroughly and change pipette tips after each transfer.

E. **Preparation of Samples**

1. Samples to be analyzed were collected from culture supernatants, avoiding aspirating cells.
2. Samples (Culture Supernatants) were diluted in Sample Dilution Buffer.

F. **Start RT reaction**

1. Poly A plate was taken out from the incubator.
2. Transferred / Added 50µl of each sample dilution from Sample Prep plate to each corresponding well. Marked to identify throughout the process till end.
3. Added 50µl of Sample Dilution Buffer to 2 wells (to work as Blanks).

4. Added 50 µl of Standard Dilutions (r RT Standards) to 12 wells. Starting with the highest dilution and continued to least dilution to avoid changing tips.
5. Sealed the poly A plate with adhesive tape and incubated overnight at 33°C.

DAY-2

G. Preparation of Wash Fluid:

1. Slowly poured 7.5 ml of Triton X-100 into 500ml of distilled water.
2. Dissolved by putting a Magnetic Strirrer to dissolve the detergent dissolves completely.
3. Added 2.5 ml of Concentrated Washing Buffer (E) to 1 litre container
4. Added the dissolved Triton X-100 to the 1 litre container.
5. Adjusted the volume to 1 litre with distilled water and mix thoroughly.

   NOTE: The residual concentrated wash buffer (E) should be stored in refrigerator until use.

H. Preparation of RT Product Tracer

1. RT tracer (Bottle O) was reconstituted by adding 12ml of distilled water containing 1% Triton X-100.
2. Mixed vigorously using a vortex to ensure the contents have fully dissolved. The mixture was kept undisturbed for about 20 min before use.

I. Stop RT Reaction

1. Poly A plate was washed 2 times with wash buffer using automated washer.
2. Residual fluid in the wells was removed by tapping the plate upside down gently on blotting paper.

J. Addition of RT Product Tracer

1. Added 100ul of Tracer to each well.
2. Sealed the plate with adhesive tape and incubated for 90 min at 33°C.

K. Preparation of AP (Alkaline Phophatase) Substrate solution

1. Added AP substrate Tablet (P1) to the AP Substrate Buffer (P2).
2. Bottle was occasionally shaked to dissolve the tablet completely and stored the bottle in dark place.

L. Remove Excess Tracer

1. After 90 min incubation, excess tracer was removed by washing (4 times) with the washing buffer.
2. Residual fluid was removed by tapping the plate upside down.

M. Start Alkaline Phosphatase reaction

1. Added 125ul of AP substrate (P2) solution to each well.
2. Plate was covered with plastic lead (no adhesive tape here) and incubated at room temperature under dark upto 30 min and /or until colour development (yellow).

N. Read the plate

1. Plate was read at 405 nm absorbance.

O. Process the Data

1. Absorbance at 405 of each standard dilution of HIV-1 r RT against concentration (See table below) was plotted.
2. RT activity of each well was determined with the aid of regression line.
3. RT activity for undiluted sample was calculated by compensating for the dilution used in the assay.
4. Mean RT activity of the sample was calculated. Values obtained from these dilutions were excluded from the calculation.

HIV-1 r RT Standard (Quantification of RT activity)

Dilution step	1	2	3	4	5	6	7	8	9	10	11	12
pg/well	93.5	41.6	18.5	8.21	3.65	1.62	0.721	0.320	0.142	0.0633	0.0281	0.0125
pg/well	1870	831.0	369.4	164.2	73.0	32.4	14.4	6.41	2.85	1.27	0.562	0.250

SDS-PAGE and Western blot

Reagents for SDS-PAGE and Western Blot

1) 30% Acrylamide/Bis

Acrylamide - 5.84 gm

Bis-acrylamide- 0.16 gm

MilliQ H_2O - Make volume to 20ml.

2) 1.5M Tris

Tris base - 3.63 gm

MilliQ H_2O- Make volume to 20ml

pH - 8.8

3) 0.5M Tris

Tris Base - 1.2 gm

MilliQ H_2O- Make volume to 20 ml

pH - 6.8

4) Electrode Running Buffer 10X

Tris Base - 30.3 gm

Glycine - 144 gm

SDS - 10 gm

MilliQ H_2O- Make volume to 1 Litre

5) 2x Gel loading buffer/Sample buffer

Tris Base - 0.00121 gm

SDS - 0.4 gm

BPB - 0.02 gm

Glycerol - 2 ml

MilliQ H_2O- 10 ml

pH - 6.8

Note: Add 10% β-mercaptethanol to loading buffer just before loading

6) TBS (Tris Buffer Saline) 10X

Tris - 24.23 gm

NaCl - 80.06 gm

MilliQ H_2O- Make volume to 1000 ml

pH - 7.6

Make 1X working buffer

7) TBS-T (TBS with Tween-20)

10X TBS - 100 ml

MilliQ H_2O- 900 ml

Tween-20 - 1 ml

8) 5% Blocking Buffer

Milk Powder - 5 gm

TBS-T - Make volume to 100 ml

9) 10X Towbin's buffer

Tris Base - 30.3 gm

Glycine - 144 gm

MilliQ H_2O - Make vol. to 1Litre

10) 1X Towbin's Electrotransfer buffer

10X stock - 100 ml

Methanol - 200 ml (20%)

MilliQ H_2O - Make vol. to 1lt

20% SDS - 1 ml (0.02%) optional

11) Stripping buffer (100ml)

Tris - 12.5µl

10% SDS - 20ml

MilliQ H_2O - 66.7µl

β-Mercaptoethanol - 800µl

SDS-PAGE Gels

Resolving Gel:

Components	**10%**	**12%**
30% Acrylamide/Bis	1.9ml	3.4ml
1.5 M Tris (pH 8.8)	1.3ml	4ml
MilliQ H_2O	1.7ml	2.5ml
10% SDS	50µl	50µl
10% APS	50µl	50µl
TEMED	5µl	5µl

Stacking Gel:

Components	**4%**
30% Acrylamide/Bis (29:1)	1.4ml
0.5 M Tris (pH 6.8)	250µl
MilliQ H_2O	330µl
10% SDS	20µl
10% APS	20µl
TEMED	2µl

Procedure for SDS-PAGE:

Preparation of Gel

1. Prepared 10%/12% gel in a tube as per composition and poured between the two glass slabs separated by spacers for casting.
2. First resolving gel was added. Allowed it to polymerize for 20 min and then stacking gel was poured on top of resolving gel.
3. Comb was inserted and gel was allowed to solidify for 30 min.
4. Casted gel slab was kept in SDS-PAGE assembly and electrode running buffer was added in the apparatus.
5. Assembly was connected to Bio-Rad powerpack.

Preparation and loading of samples in gel

1. Samples were prepared by mixing viral or cell lysate with 2X sample reducing buffer in 1:1 ratio.
2. Samples were boiled in sample reducing buffer for 5 min.
3. Samples were loaded in the gel and run was started at 75V-120V until the blue dye front reaches the bottom of the gel.

Western Blot: Transfer of proteins onto PVDF membrane

1. PVDF membrane was cut according to the size of gel and activated with methanol for 10 min.
2. The membrane and gel were sandwiched between pre-wet sponge dipped in transfer or Towbin's buffer.
3. Transfer was done for 1 h at 65mA constant current.

Blocking

1. Membrane was blocked for 1 h in TBS-T buffer + 5% non-fat dry milk on a rocker.

Primary and Secondary Antibody

1. Membrane was incubated with primary Ab (diluted in TBS-T + 0.5% non-fat dry milk) for 4 h at 4^{o}C on a rocker.
2. Membrane washed three times for 5 min with TBS-T buffer on a rocker.
3. Incubated the membrane with appropriate secondary Ab conjugated to HRP for 30 min to 1 h at room temperature in TBS-T buffer.
4. Membrane was washed three times for 5 min with TBS-T buffer on a rocker.

Detection and develop

1. Protein bound to membrane was detected with SuperSignal West Dura kit (ThermoScientific, Inc.).
2. In a separate tube solutions of white and brown bottle were mixed in 1:1 ratio (1ml/membrane).
3. Solution mix was poured on the membrane. NOTE: Wait for one minute till signal appears.
4. Membrane was wrapped in plastic and exposed to film in dark.

p24 Immunostaining

Procedure:

1. Infected cells were washed with 1X PBS 72 h post-infection and fixed with chilled methanol-acetone (1:1) for 10 min at room temperature.
2. Cells were washed twice with PBS and incubated with blocking buffer (PBS-2% FBS) for one hour at room temperature.
3. Blocking buffer was removed and cells were icubated with HIV-1 anti-p24 mAb (183-H12-5C) as hybridoma cell culture supernatant (1:50 dilution in PBS-1% FBS) for 2 h at room temperature.
4. After incubation cells were washed twice with PBS-1% FBS and incubated with goat anti-mouse β-galactosidase antibody (1:1000 dilution in PBS-1% FBS) for 1 h at room temperature.

5. Cells were washed twice with PBS-1% FBS and incubated with X-Gal substrate (1:80 dilution in yellow PBS [3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride]) for 1 h at room temperature in dark.
6. Resultant blue stained cells regarded as foci of infection were counted under microscope, and the virus infectivity was estimated as focus-forming units (FFU) per ml.