HIV-1 Pseudovirus Neutralization Protocol using Ghost cells

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1. AIM:

This protocol describes the preparation and titration of pseudovirus (PV) and the detection of neutralizing activity of monoclonal antibodies (mAb) as well as sera/plasma obtained from HIV infected individuals using Ghost cells as target cells.

2. MATERIALS AND EQUIPMENT:

- Biological safety cabinet class II
- Humidified 37°C incubator with 5% CO_2
- Centrifuge
- Electric pipettor
- Adjustable pipetters
- Multichannel pipetters
- Microscope
- Disposable plastic pipettes
- 1-250µl pipette tips
- 50ml conical centrifuge tube
- 96-well flat bottom plates with lid
- Microtiter plate sealers
- White 96-well flat bottom plates (Costar)
- Sterile reagent reservoirs
- Pooled normal human plasma (NHP)
- Control serum/plasma from HIV-1 positive individuals
- Trypan blue: 0.4 %
- Transfection kit
- Luminometer (Berthold, LB941 Tristar)

3. PROCEDURES

3.1. Preparation of HEK293T and Ghost cell lines

1. Check cells under the microscope.
2. Pour off the medium (cells upside) gently.
3. Rinse cells by adding 1 ml Trypsine/EDTA (do not pipette on the cells) and pour off gently.
4. Add 5 ml Trypsine/EDTA to the cells (do not pipette on the cells) and incubate 10 min in incubator (37°C; 5% CO_2)
5. Tap the flask gently to detach all cells.
6. Neutralise with 5ml medium (pipet up and down) and bring into a new tube
7. To a 1.5ml eppendorf tube, add 10µl tryphane blue and 90µl of cells. Mix by pipetting up and down. Add ~10 µl to a microscope counter plate. Cells are counted during step 8.
8. Centrifuge the cells 10 min at 1200 rpm (400 g).
9. Take cells from centrifuge and discard supernatant. Loosen the cells by running the tube along the grids of the hood.
10. Resuspend the cells in the appropriate cell medium to a final concentration of 10^6cells/ml.
11. Label a new flask (name of cells, passage N°, Date) add 9ml of appropriate medium and 1ml (10^6) cells. Incubate at 37°C, 5%CO_2 for 3-4 days.

Notes:

1. *All volumes are given for 25cm^2 flasks. When using larger flasks the volumes should be adjusted*.
2. *Depending on the cell line 293T or Ghost cell media is used*

3.2. Preparation of PV

DNA transfections are done in 24 well plates using the Calcium phosphate mediated method according to the description of the manufacturer. (Profection kit; Promega)

Day -1

- Label a 24 well plate
- Add 5 ml (10^6/ml) of 293T cells into 20 ml of 293T medium and mix gently
- Add 1 ml ($2x10^5$ cells) into each well of the 24 well plate
- Incubate overnight at 37°C, 5% CO_2

Day 0

- Bring transfection-kit to room-temperature
- Check cells under the microscope. Confluency should be at 30-60%.
- Put the cells back in the incubator while preparing the transfection mixes.
- Label 2 tubes/construct (e.g. 1 and 1+; 2 and 2+; …).

Add 25µl of the 2x Hepes Buffered Saline (HBS) to the 2° tube (e.g. 1+; 2+):
Add in the 1° tube (e.g. 1; 2)) the following reagents in this order:

1. nuclease free H_2O
2. Env
3. pNL4-3.LucR^-E^-
4. add drop wise the $CaCl_2$

The final volume of this mix should be 25µl

- Add drop wise the content (25µl) of the 1° tube to the tube containing the HBS
- Incubate 30 min at room temperature
- Add the transfection mixture drop wise to the 293 T cells *(North, East, South, West)* in the 24 well plate.
- Incubate overnight in the 37°C, 5% CO_2 incubator

Day 1

- Remove transfection mixture (supernatant) and carefully wash the cells once with 1ml 293T medium
- Add 1ml 293T media containing 1 mM Sodium Butyric Acid and incubate overnight in the 37°C, 5% CO_2 incubator

Day 2 (48 hr Post Transfection)

- Harvest the PV (1ml) without touching the 293T cells using a 1 ml pipette, filtrate over a 0.45µm filter and collect the PV into a labeled Nalgene Cryotube tube containing 10% (~80µl) FBS *(on ice)*.
  Optional: Add again 1ml of 293T medium containing 1mM Sodium Butyric Acid on the cells and incubate overnight at 37°C (5% CO_2) for 2° harvest of PV.
- Store PV at -80°C until used

3.3. Titration of PV

- Take a 96 well plate and label *(name, date)*
- Add 50µl Ghost medium to rows B-G
- Add 100µl PV to the first row (3 wells)
- Make 7 serial 2-fold dilutions by pipetting 50µl of the first row (A) in row B. Mix by pipetting up and down (5 strokes) and bring 50µl from row B to C. Repeat until row G.
- Add to row H only 50µl Medium (negative ctrl) (3 wells)
- Add 150µl Ghost cells (10,000 cells) to each well
- Incubate 48 hr to 72hr at 37°C, 5% CO_2 (total volume is 200µl/well)
- Check the cells microscopically
- Discard 120µl of the supernatant without touching the bottom of the plate.
- Add 70µl of SteadyLite (Perkin Elmer) to each well without touching the plate
- shake gently during 5 minutes (wrapped in aluminium paper)
- Transfer 100µl of each well into a white plate (with white bottom) and measure Relative Light Units (RLU) in the (LB941 Tristar) Luminometer
- Using excel a titration curve is generated and based on this curve the dilution factor to obtain ~10^4 RLU can be calculated. Background (only cells) should be below 50 RLU.

Titration scheme

	1	2	3	4	5	6	7	8	9	10	11	12
A	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV	100µl PV
B	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
C	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
D	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
E	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
F	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
G	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
H	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium
	PV 1			PV 2			PV 3			PV 4		

serial 2-fold dilutions of PV only cells

transfer 50µl from row A to B, B to C, C to D, D to E, E to F, F to G and G to waste

3.4. Neutralization protocol

3.4.1. Using 4 serial 4-fold dilutions of the Antibody (Ab: mAb or plasma)

- Take a 96 well plate and mark the plate
- Add 50µl Ghost medium to rows B-D and G-H
- Add 66.5µl of Ab (2x final concentration) to the wells of row A
- Transfer 16.5µl from row A to row B and mix by pipetting up and down (5x)
- Transfer 16.5µl from row B to row C and mix by pipetting up and down (5x)
- Continue until row D
- Discard 16.5µl of row D (in waste)
- Add 50µl PV (corresponding to ~10^4 RLU) to row A-D and G respectively
- Incubate during 1 hr at 37°C

  During the incubation time the target (Ghost) cells are prepared
- Add 100µl (10,000 cells) Ghost cells expressing CD4 and the appropriate co-receptor (CCR5 or CXCR4)
- Incubate 48 hr up to 72 hr at 37°C and 5% CO_2
- Check the cells microscopically
- Discard 120µl of the supernatant without touching the bottom of the plate.
- Add 70µl SteadyLite (Perkin Elmer) to each well without touching the plate
- Shake gently during 5 minutes (wrapped in aluminium paper)
- Transfer 100µl of each well into a white plate (with white bottom) and measure Relative Light Units (RLU) in the (LB941 Tristar) Luminometer.
  Background (only cells) should be below 50 RLU
- In each experiment a positive (mAb or plasma with known IC) and a negative (only cells) control is included (row H)

Pipetting scheme for four 4-fold dilutions

	1	2	3	4	5	6	7	8	9	10	11	12	
A	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	66.5µl Ab	serial
B	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	4-fold
C	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	dilutions of
D	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	Ab
E													
F													
G	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	no Ab
H	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	only cells
	Sample 1			Sample 2			Sample 3			Sample 4			

transfer 16.5µl from row A to B, B to C, C to D and D to waste

3.4.2. Using 5 serial 2-fold dilutions of the Antibody (Ab: mAb or plasma)

- Take a 96 well plate and mark the plate
- Add 50 µl Ghost medium to rows B-E and G-H
- Add 100µl of mAb (2x final concentration) to the wells of row A
- Transfer 50µl of row A to row B and mix by pipetting up and down (5x)
- Transfer 50µl of row B to row C and mix by pipetting up and down (5x)
- Continue until row E
- Discard 50µl of row E (in waste)
- Add 50µl PV (corresponding to ~10^4 RLU) to row A-E and G respectively
- Incubate during 1 hr at 37°C

  During the incubation time the target (Ghost) cells are prepared
- Add 100µl (10,000 cells) Ghost cells expressing CD4 and the appropriate co-receptor (CCR5 or CXCR4)
- Incubate 48 hr up to 72 hr at 37°C and 5% CO_2
- Check the cells microscopically
- Discard 120µl of the supernatant without touching the bottom of the plate.
- Add 70µl of SteadyLite (Perkin Elmer) to each well without touching the plate
- Shake gently during 5 minutes (wrapped in aluminium paper)
- Transfer 100µl of each well into a white plate (with white bottom) and measure Relative Light Units (RLU) in the (LB941 Tristar) Luminometer.
  Background (only cells) should be below 50 RLU
- In each experiment positive (mAb or plasma with known IC, as well as no Ab) and negative (only cells) controls (row H) are included.

Pipetting scheme for five 2-fold dilutions

	1	2	3	4	5	6	7	8	9	10	11	12	
A	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	100µl Ab	serial
B	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	2-fold
C	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	dilutions of
D	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	
E	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	Ab
F													
G	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	**no Ab**
H	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	50µl medium	**only cells**
	Sample 1			Sample 2			Sample 3			Sample 4			

transfer 50µl from row A to B, B to C, C to D, D to E and E to waste..

3.5. Calculation Inhibitory concentrations

- The 50%, 75% and 90% inhibitory concentrations (IC50, IC75 and IC90) are calculated with a linear interpolation method, using the geometric mean of the triplicate responses.

4. MEDIA

4.1. HEK293T medium

500ml DMEM *(Sigma, D-5546)*
50ml FBS *(Lonza)*
500µl Gentamycine *(Lonza, 17-518L)*
5ml L-Glutamine *(Lonza, 17-605E)*

4.2. Ghost cell medium

500ml DMEM with glucose *(Lonza, 12-604F)*
50ml FBS
5ml Pen/Strep *(Invitrogen, 15140-122)*
5ml L-Glutamine *(Lonza, 17-605E)*
5ml Geneticin *(Invitrogen, 10131-027)*
500µl Hygromycin B *(Roche, 842.555)*
500µl Puromycine 1mg/ml *(Sigma, P-8833)*

Note that Ghost cells are kept in culture up to 15 passages where after they must be replaced with new cells