Institut für HIV Forschung
SOP #16-00 **(April 2015)**

Viral Inhibition Assay

For Cell Culturing:

Item	Manufacturer	Order Number
RPMI-1640	Sigma	R0883
Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Mediatech	30-001-Cl
L-glutamine (200mM; 29.2 mg/mL)	Mediatech	25-002-Cl
HEPES (1M; 238.3mg/mL)	Mediatech	25-060-Cl
FBS, Heat-inactivated	Sigma	F4135 (own tested lot)
IL2 (1 million units, dissolved per SOP#4)	Hoffmann-La Roche	Ro 23-6019
PBS	Sigma	D8537
Anti-CD3/8 Antibody	Dr. Johnson WONG	

R10: 500ml RPMI, 5ml Pen/Strep, 5ml L-glutamine, 5ml HEPES, 55ml FBS
R10/50: same as R10 + 50u/ml IL-2
1% FBS (wash buffer): 1% FBS in PBS (1000ml PBS, 10ml FBS)

For Thawing/Cell Isolation/ELISA:

Item	Manufacturer	Order Number
Nuclease S7*	Roche	10107921001
Dynal CD8 Positive Isolation Kit (contains both Dynabeads and Detachabeads)	Invitrogen	133-33D
DynaMag-15 Magnet**	Invitrogen	123-01D
Dynabeads Rotator Mixer**	Invitrogen	947-01
HIV-1 p24 ELISA kit	PerkinEmler	NEK050B

*Nuclease is used to help increase yield – see SOP #8 (Thawing)
**Any magnet or rotator capable of holding a 15ml conical can be used

Day -3: Thawing and Cell Separation

Thawing – See SOP#8

1. Thaw (in R10)/separate cells on Tuesday or Friday to avoid weekend harvests if desired
2. Start with at least 20M cells in order to achieve necessary CD8 yield. You may need more depending on how many CD8 cells are needed to fulfill all experimental conditions.

Cell Separation/Isolation (we use a positive isolation kit – CD4s will remain in supernatant while CD8s are attached to the beads)

1. While cells are in their final thawing spin, count cells and wash Dynabeads (mix beads thoroughly before using):
   a. Add 1 mL of 1% FBS for every 10 Million cells in 15mL conical. If dealing with a large number of cells (80M or more) use only 2.5 to 3mL of 1% FCS.
   b. Add appropriate volume of beads: 15µl beads/10M cells, mix thoroughly.
   c. Loosen caps and place in magnet. Let it sit for a minute or until you notice the beads have been safely pulled out of solution.
   d. Pipet or decant wash buffer while tube is still in magnet being careful not to touch beads with pipet; remove tube from magnet.
   e. Add the same amount of 1% FBS and repeat wash.
   f. After second wash, add same volume of 1% FCS to beads and mix.
2. When cells are done spinning, aspirate and resuspend in the remaining liquid. Add 2mL of 1% FBS, mix (you can do this step and keep the cells on ice if you need more time to finish washing the beads).
3. Combine cells and beads and parafilm tops of conicals.
4. Place tubes in rotator for 20-25min. at 4C on setting 11 (slow speed).
   a. CD8+ cells will stick to the beads while CD4+ cells won't.
5. Following spin, remove parafilm, loosen caps and place tubes in magnet, allowing CD8s/beads to be pulled out of solution.
6. Pipet supernatants into new 15mL conicals.
7. Wash beads as above twice (Step 1c-e, use 2mL 1% FBS), each time putting the supernatants into the new conicals (total of 3 transfers).
   a. These are the CD4 cells.
8. Once wash is over add 2mL of 1% FBS to CD8 cells (stuck on the beads). Mix well.
9. Add Detachabead to CD8s and mix (8µl of Detachabead per 10 million cells, mix well before using).
10. Parafilm the CD8 conicals and place in rotator at room temperature for 45 minutes on settting 11 (slow speed).
11. Spin CD4 cell supernatants: 1500rpm, 10min.
12. Resuspend the CD4 cells in 10mL R10/50.
    a. Add 5ul of CD3.8 (for final concentration of 0.5ug/ml) to each sample.
    b. Transfer to a T25 flask and incubate at 37C until infection day (stimulate 3 days).
13. Following 45 minutes spin for CD8 cells, repeat wash and collection procedure outlined above for CD4 cells (Steps 5-7).
    a. Now the supernatants are the CD8s.
14. Spin CD8 cells (1500rpm, 10min) and resuspend in 7-10ml R10. Transfer to T25 flask and incubate 37C until infection day (rest 3 days).

Kommentar [BJ1]: Add some R10 to the CD4 and spin then.... cells don't stick so much

Day 0: Infection of target cells/Setup assay cultures

Infection of Target cells (CD4s)

- All steps must be done in BL2+ now that work is being done with live virus and superinfected cultures. Be sure to use safe technique and dispose of everything properly

1. Prepare/warm R10 media. NEW R10/50 will be needed for the afternoon.
2. Spin down CD4 cells (1500rpm, 10min).
3. Resuspend in 10ml R10. Remove a counting aliquot and spin down.
   a. Count CD4s during second spin.
4. Resuspend in R10. Separate CD4 cells for infection and ones to leave uninfected (for negative controls), depending on setup of assay.
5. Spin down CD4s for infection; uninfected CD4s can be placed in 37C incubator (caps loose) until needed.
6. During third spin, calculate appropriate amount of virus.
   a. MOI = multiplicity of infection and depends on virus stock used. It is best to use the lowest MOI that still yields good infection, so as not to overwhelm the culture with virus). Each virus stock will also have its own titer. *Example calculation:*
   b. Infect 5M cells at an MOI of 0.01 (500,000 will actually get infected = 5M x 0.1)
   c. Titer of virus = 3.3 x 10^6/mL = 3,300/ul
   d. Therefore, we need 500,000/3,300 = 151.5ul of virus
   e. Virus can be diluted in R10 if titer is too high for practical use at stock concentration
7. Following second wash, aspirate, leaving about 200ul of media above pellet and resuspend with a *clean* micropipette.
8. Take out virus and thaw. As soon as completely thawed, add appropriate amount of virus to each sample. Clean micropipette with ethanol to prevent cross-contamination.
   a. Once virus is thawed it must be used immediately. If it remains thawed for too long, the titer will begin to change and the calculations will no longer be accurate.
9. Mix and incubate for 4 hours at 37C (caps loose).
10. Following incubation, wash cells twice in R10 (count during second spin).
    a. Remember that cultures are now superinfected. Disinfect/discard everything appropriately.
11. Resuspend in R10/50 at 1M/ml, incubate until ready.

Preparation of Effector cells (CD8s)

- Uninfected CD4s can also be prepared in same manner

1. Prepare effectors and uninfected CD4s about 1 hr. before end of incubation of infected target cells.
2. Wash cells once in R10 and resuspend at 1M/ml in R10/50 (for 1:1 Effector:Target ratio).
3. Incubate at 37C in conicals or in 96 well plate (according to assay setup) until target cells are ready.

Setting up inhibition assay cultures (Day 0)

1. Design plate setup on round-bottom 96-well plate (eg. Negative control, positive control, 1:1 E:T, 2 wells each).
2. Add 100k cells to necessary wells.
    a. 100k uninfected CD4 cells for negative control
    b. 100k infected target CD4s (infected) to rest of wells
    c. 100k effector cells to wells (don't touch target cells)
    d. Add 100ul R10/50 media to wells that do not have both target and effector cells: final volume for all wells should be 200ul
3. Add 200ul sterile PBS to all wells surrounding the cells to prevent media evaporation.
4. Incubate 37C.

Harvesting

- Should be done D3/D5/D7 (for Friday infection) or D2, D4, D7 (for Monday infection)

1. Label a new 96-well plate. Add 10ul of 5% triton to each well (from p24 ELISA kit).
2. Carefully transfer assay plate to the hood without disturbing the cells.
3. Transfer 100ul supernatant to harvest plate. Be careful not to disturb or pick up cells.
4. Feed cells with 100ul R10/50 and return to incubator.
5. p24 ELISA assay can be preformed on fresh supernatant at this point (SOP#54). Otherwise, cover harvest plate with plate cover sticker (from p24 ELISA kit) and store at -80C. If possible, test D7 positive controls to ensure good infection before running entire set (won't waste the ELISA kit on a sample that didn't get infected).
    a. Dilutions for ELISA: D3 1:10 – 1:100
    b. D5 1:10 – 1:100 – 1:1,000
    c. D7 1:10 – 1:100 – 1:1,000 – 1:10,000