Institut für HIV Forschung
SOP #18-00 **(April 2015)**

HIV Virus Cloning and Titering

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Supplies:

Fluid-resistant disposable lab coat
Exam gloves (two pairs when working in hood)
Protective eyewear if desired
Regular *sterile* P200 and P1000 tips
Sterile 25 cm^2 and 75 cm^2 flasks (T25 and T75 flasks); sterile round-bottom 96-well plates
Uninfected PBMCs (from donation or negative buffy-coat)

5% Triton: Using the Eppendorf repeater with a suitably sized tip, which makes expelling the right amount of Triton X-100 much easier, slowly draw up 2.5 mL Triton X-100. Expel slowly into a 50-mL tube containing 47.5 mL Milli-Q purified water. Mix by vortexing and shaking until the viscous Triton disappears, leaving the solution uniform. Or just use the dyed 5% Triton that comes with the p24 kit.

0.5 mg/mL polybrene: Weigh a reasonable amount of polybrene, keeping as things clean as possible. In the cell culture hood, dissolve it in sterile PBS, filter-sterilize it (0.22 µ pore size), & make sterile aliquots. Freeze at –20 Celsius.

R20: 500 mL RPMI-1640
5 mL Pen/Strep
5 mL L-glutamine
6.2 mL HEPES
110 mL FBS

R20-50: One 280- µL aliquot IL2/bottle R20

Cloning Medium (CIMDM):
500 mL IMDM
5 mL Pen/Strep
5 mL L-glutamine
55 mL FBS
5 mL 0.5 mg/mL polybrene (Med. thus 5 µg/mL)
One 280- µL aliquot IL-2, as for R20-50

NOTE: CIMDM was recommended for cloning by Jim Mullins' group in Seattle, Washington. I have not found it necessary; I have cloned successfully in the same R20-50 I use for viral isolation (SOP#82).

Item	Manufacturer	Order Number
RPMI-1640	Sigma	R0883
IMDM (Iscove's Modified Dulbecco's Med.)	Sigma	I3390
Polybrene (Hexadimethrine bromide)	Sigma	H9268
Pen/Strep (10,000 IU Pen/10,000ug/mL Strep)	Sigma	P4333
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 (of lot tested for here)
IL2 (1 million units, dissolved per SOP#4)	Hoffmann-La Roche	Ro 23-6019
PBS	Sigma	D8537
PHA (2 mg, dissolved per SOP#5)	Remel	HA16/30852801
Anti-CD3/8 Antibody—see Alicja		
Triton X-100	Fisher	BP151-500

NOTE: At any step calling for 2- to 4-day PHA blasts, PBMCs stimulated for 2 to 6 days with anti-CD3/8 antibody may be used. If the antibody is available, it's better than PHA blasts for cloning and titering, particularly when the original viral isolate was grown using PBMCs stimulated with it.

Stimulation with antibody: Add 2/3 µg antibody/mL to PBMCs at 5 million PBMCs/mL in R20-50. Feed after 3-4 days; ***cells are best used 5 to 6 days after stimulation***. Count and use as for PHA blasts. Remember to allow for a loss of 3/5 to 2/3 of your starting count; you are, after all, stimulating only CD4 cells. And, remember to *stimulate 5-7 days before use*, not 2-4.

Also, if less than 25-30 mL of clonal virus-containing supernatant is needed, scale back the volumes of medium suggested in from Step 5 on—for example, if only 15 mL are needed, Step 5 becomes like Step 4, except that a p24 sample is taken; transfer to a larger flask isn't needed.

Cloning:

1. Day –2 or –4: Two to 4 days before starting the culture, obtain *UNIRRADIATED* PBMCs and make PHA blasts by seeding PBMCs at 2 to 5 million/mL in R20-50 and adding 1 or 1.5 µL PHA/mL (250 µg/mL stock PHA, so 250 ng/mL final concentration)**.** Count the PHA blasts just before use and use the indicated number—make some extra, since some die during stimulation. Alternatively, thaw frozen 2- to 4-day PHA blasts on Day –1 and let rest in R20 (no IL-2) overnight—thawing even on the day of use may be used at any later stage calling for 2- to 4-day old PHA blasts, though you should probably not use thawed PHA blasts for cloning, just for amplification of the clonal virus. For titering, I've used frozen PHA blasts and also ones up to 7 days old.

2. Day 0:

- **a.** Keep viral isolates to be cloned on ice. Thaw isolate and dilute 0.1 mL to 2.5 mL with R20-50 or CIMDM.
- **b.** Set up 96-well plate with 100 µL R20-50 or CIMDM/well
- **c.** Add 20 µL diluted viral isolate to each of the first 16 wells (columns 1 & 2).
- **d.** Using the non-electronic multichannel pipettor set for 20 µL, mix col. 1 by pipetting up and down several time. Expel all liquid, draw up 20 µL, and transfer to wells in col. 3.
- **e.** Repeat Step d with col. 3 and transfer to col. 5.
- **f.** Repeat Step d with col. 5 and transfer to col. 7.
- **g.** Repeat Step d with col. 7 and transfer to col. 9.
- **h.** Repeat Step d with col. 9 and transfer to col. 11.
- **i.** Repeat step d with col. 11 and dispose of the tips containing the 20 µL in the hood bucket.
- **j.** Using fresh tips, repeat Steps 2-d through 2-i starting with col. 2 and transferring to the corresponding even-numbered columns. This produces serial dilutions of 1:6 across the plate, 2 columns/dilution. The initial dilution was 1:25, so cols. 1&2 are now at 1:150. When 100 µL of cells is added, final dilution is 1:300; serial 1:6 dilutions across the plate produce a final dilution of 1:2,332,800.
- **k.** Count PHA blasts of Step 1. Resuspend 10.5 million in 10.5 mL R20-50 or CIMDM.
- **l.** Using the electronic pipet with 8 tips, mix well and draw up 600 µL and dispense 100 µL/well into cols. 12, 10, 8, 6, 4, and 2 in that order. Eject tips.
- **m.** Repeat Step 2-l for columns 11, 9, 7, 5, 3, and 1, in that order. This means that you are adding from the most dilute to the least dilute well, and thus minimizes accidental carryover of virus.

n. Wrap plate (or stack of plates, if you are cloning more than 1 viral isolate—a stack of 6 or so plates is about as high as you want to get) in Saran Wrap fresh off the roll and incubate for 1 week. (37^{o} C, 5%-6% CO_2)

3. Day 7: first feeding

a. After 1 week, transfer Saran-Wrapped stack of plate(s) to hood, unwrap (you may want to re-use the wrap, so you can wipe it off with Expose or alcohol, being careful not to get that liquid onto the plates or back into the incubator), and feed culture by removing 100 µL/well of supernatant with the non-electronic multichannel pipettor. Use 12 tips and remove one row at a time, expelling into the hood bucket except for the last row, which should be expelled into a row of a fresh 96-well plate containing 10-25 µL/well of 5% Triton. Eject tips; use a fresh set of tips for each cloning plate.

b. Using the electronic pipettor with 8 tips, draw up 600 µL and dispense 100 µL/well into cols. 12, 10, 8, 6, 4, and 2 in that order. Eject tips.

c. Repeat Step 3-b for columns 11, 9, 7, 5, 3, and 1, in that order. This means that you are adding from the most dilute to the least dilute well, and thus minimizes accidental carryover of virus.

d. Rewrap plate(s) and return to incubator for one week.

e. Run a p24 on the saved row to be sure that the virus is growing and has been diluted sufficiently to make cloning likely. If some wells are positive and some are negative, you don't need to do anything more. If all wells are positive, even the most dilute (11 & 12), you will need to reclone at a higher dilution—perhaps making an initial dilution of 1:250 or more instead of 1:25 as in Step 2-a. If all wells are negative, even the most concentrated (1 & 2), you will *probably* need to reclone at a lower dilution (you *might* find a positive well after the second week of incubation). It might be appropriate to make an initial dilution of 1:2.5 instead of 1:25 as in Step 2-a, or even not to dilute the viral isolate before pipetting it into columns 1 and 2. A positive well is one that has an O.D. of 0.100 or higher when read after a 30-minute final incubation (I follow the protocol printed in the p24 kit manual, rather than stopping at 15 minutes); this is arbitrary but is just below the lowest (6.25 pg/mL) standard, corresponding to about 5 pg. If you know that the kit is old and the negative control O.D. is significantly higher than 0.007 to 0.015, a slightly higher cutoff may be used, based on the lowest standard. There is no need to actually run the standard curve; all you want is a yes-or-no answer. Incubated medium, incidentally, tends to give slightly higher O.D.—0.020-0.030. The negative control, however, will still be in the range cited above.

4. Day 11 or 12: Fresh PHA blasts

Set up PHA blasts to be ready by Day 15; you need 10 million for each subcloning you expect to do and 5 million for each clone you need to expand. Make extra, just in case. If you are using antibody-stimulated cells, set them up earlier—about Day 9 or 10.

5. Day 14: Harvest

a. After the second week of incubation, transfer Saran-Wrapped stack of plate(s) to hood, unwrap (you may want to re-use the wrap, so you can wipe it off with Expose or alcohol, being careful not to get that liquid onto the plates or back into the incubator), and harvest by removing 100 µL/well of supernatant with the non-electronic multichannel pipettor. Use 12 tips and remove

one row at a time, expelling into the corresponding row of a fresh, labeled, 96-well plate containing 10-25 µL/well of 5% Triton. Eject tips; use a fresh set of tips for each row.

b. Rewrap plate(s) and return to incubator.

c. After samples have stood 10 minutes in the 5% Triton, run a p24 on selected columns: based on the p24 run on Day 7, run the last pair (e.g., 5 and 6—the same dilution) of columns that were positive in the row tested and 2 to 4 columns more. Cover the remaining samples of each plate with a sticky top from the p24 kit (we never use all of them), press firmly, cover with the plastic plate lid, and freeze at –80°—you may need to use some of them later.

d. When there is only one positive well of the 16 at a given dilution, that well is considered clonal. When 2 or more are positive at a given dilution, they are probably not clonal, particularly if most or all wells of earlier dilutions are positive. Run both columns of any given dilution at the same time; you may need to run more samples to be sure that all samples of at least some dilution are negative. Occasional outliers may result from the way the plate has been diluted; they may be treated as clonal if they appear at the second lower dilution from the last positive well that diluting tip was in, and if there aren't other positives in the same dilution which aren't outliers. On occasion—particularly when dealing with isolates subcloned more than once, more than one well at a given dilution may be considered clonal.

6. Day 15: Subcloning and expanding clones

a. On the basis of the Day 14 p24 values and any extra p24 runs that needed to be made, transfer the entire contents, cells and all, of each well considered clonal into a T25 flask containing 5 million PHA blasts in 5 mL R20-50 or CIMDM.

b. Subclone the most dilute strongly positive well of those isolates which produced no clones, diluting 15 µL to 375 µL for the initial dilution and then following Steps 2-b to 2-n. Continue as for the initial cloning, making allowances for the fact that this is a subclone (Step 5-d).

c. As a precaution, seed a fresh plate with 10^5 PHA blasts in 100 µL/well fresh medium; transfer at least one column containing at least some highly positive wells of the most dilute possible for each of the initial cloning plates to this plate, taking the entire contents of the wells, cells and all. Wrap and incubate as for cloning plates; this plate provides partially cloned virus in case a well considered clonal fails to grow or in case a subcloning was done at too great a dilution.

d. Feed, expand, and harvest the clones in flasks much as you did when making isolates (SOP#82).

Titering virus stocks:

NOTE: This procedure mostly follows the Red Book methods, but the titering is done in PHA blasts or antibody-stimulated PBMCs because few, if any, of my viral isolates grow in C8166 cells. If you have a virus to titer that *does* grow in C8166 cells, you can use them at ¼ the concentration of PHA blasts and your endpoint will be the visual detection of cytopathic effects (syncytia, primarily) instead of a positive p24 assay for the well.

Also, as at least a way of estimating the titer of a virus, you can use a formula derived from regression analysis of 111 titers I obtained with viral isolates and clones: Log10(Titer)=(4.60E-06)pg/mL p24+4.88, where *Log10(Titer)*=y, *4.60E-06*=m, *pg/mL p24*=p24 value in pg/mL determined for the isolate or clone, and *4.88*=b in the standard equation y=mx+b.

1. **Day –2 to –4:** Prepare PHA blasts. You will need 16 million PHA blasts for each viral isolate or clone. I have used PHA blasts as old as 7 days for titering.

2. **Day 0: Setting up the titering plate**

   a. Take one 96-well plate for each viral isolate or clone you wish to titer. Add 200 µL/well of sterile PBS to rows A and H; add 133 µL/well of R20-50 to the remaining wells of column 1, and add 150 µL/well of R20-50 to the remaining wells of columns 2 through 12.

   b. Plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
C	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
D	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
E	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
F	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
G	133 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL	150 uL
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

   c. Into each of the 6 wells with R20-50 (1B through 1G) in column 1, pipet 67 µL of the isolate or clone you wish to titer.

   d. Using the non-electronic multichannel pipettor, mix the 6 wells of column 1 with virus by pipetting up and down a few times. Expel all liquid back into the wells, draw up 50 µL, and expel it into the corresponding wells of column 2. Eject the tips.

   e. Repeat Step 2-d for the remaining columns, expelling the 50 µL withdrawn from column 12 wells into the hood bucket along with the ejected tips.

   f. Count PHA blasts and resuspend 16 million in 4 mL R20-50. Using 6 tips and the electronic pipettor, mix well and draw up 600 µL. Expel 50 µL/well, starting with column 12 and going in descending order to column 1—this goes from the most dilute to the least dilute, minimizing the chance of carryover.

   g. Cover plate and incubate for 3-4 days, at 37° C, 5%-6% CO_2.

3. **Day 3 or 4:** feed plate by withdrawing 100 µL/well with 6 tips of the non-electronic multichannel pipettor from column 12, expelling it into the hood bucket, and repeating in descending order of columns. *Be very careful not to spatter or otherwise contaminate the wells with other wells' virus.* If you are using C8166 cells, you can start checking for syncytia; your endpoint is having 2 successive days with no new wells with syncytia, up to 10 days of incubation. It's not worth running the p24 at this point on PHA blast titers.

4. **Day 7:** harvest

   a. Set up a 96-well plate with 10-25 µL/well 5% Triton; if you have several plates, you may be able to use fewer harvest plates than titer plates, since only 6 of the 8 rows on the titer plate will be harvested—but be careful not to mix things up.

   b. Using the non-electronic multichannel pipettor with 12 fresh tips, remove 100 µL/well from row B and place in the correct row of the harvest plate, mixing the Triton with the sample by pipetting up and down a few times. Eject the tips.

   c. Repeat for the remaining rows (C through G) of the plate.

   d. Return the plate to the incubator in case of accident with the p24.

e. Either cover the harvest plate with a sticky top and freeze at –80° C until you can run the p24 or run a p24 on selected columns of the harvested plate, cover the remaining samples with a sticky top, and freeze at –80° C. After you have run several of these, you'll be able to guess fairly well which columns to use. You want both the last column with all 6 wells positive and the first column with all 6 wells negative, as well as the ones between those two, for the calculation of titer. Run columns until you have this information, even if you have to do more p24 assays. However, ignore outliers here, unlike the situation in cloning—outliers almost certainly are contaminated wells, since you changed tips every time you diluted. (You want the same information for a C8166 titer, the difference is only that your endpoint is visual instead of p24.)

f. When you are satisfied that you have sufficient p24 data, or that you've seen all the positive wells in a C8166 titer plate, dispose of the plate.

5. Calculate the titer as in the following example:

Take farthest-right well with p24 O.D.>= 0.1 unless noted
(But ignore outliers)

205: SK033 B1, Clone A10
1,923 Day 7

Column	Dilution	p24+ wells	p24- wells	Total Infected	Total Uninfected	% Infected (% mortality)
1	4^-1	6	0			
2	4^-2	6	0	10	0	100.00
3	4^-3	4	2	4	2	66.67
4	4^-4	0	6	0	8	0.00
5	4^-5	0	6			
6	4^-6	0	6			
7	4^-7	0	6			
8	4^-8	0	6			
9	4^-9	0	6			
10	4^-10	0	6			
11	4^-11	0	6			
12	4^-12	0	6			

% mortality at dilution next above 50%	66.67	
% mortality at dilution next below 50%	0.00	
Dilution next above 50% mortality	-3	
Log4(50% endpoint dilution)	-3.25	
Log10(50% endpoint dilution)	-1.9565	(=Log10(4)*(log4 (50% endpoint dilution))=0.602*(log4 (50% endpoint dilution))
Log10(50% endpoint titer)	1.9565	
50% endpoint *titer* =	10 to the	1.9565
TCID50/mL =	20*10 to the	1.9565
which is 10^1.301*10 to the	1.9565	
which is 10 to the	3.2575	
which is	1809.25591	
which is approximately	**1.8*10^3**	

a. Enter the number of positive wells and negative wells in each column in the proper place of the spreadsheet.

b. Determine the last column with all wells positive and the first column with all wells negative.

c. For those columns and all columns in between, calculate the total infected by adding up all the infected wells in the current column *plus* all infected wells in the *later* columns. Thus, for column 2, the total is 6+4+0=10; for column 3, the total is 4+0=4; and for column 4, the total is 0=0.

d. Calculate the total uninfected by adding up all the uninfected wells in the *latest* column *plus* all the uninfected wells in any *earlier* columns. Thus, for column *4*, the total is 6+2+0; for column *3*, the total is 2+0=2; and for column *2*, the total is 0=0.

e. Calculate the % infected by dividing the total infected at each column by the sum of the total infected and the total uninfected for that column.

f. Then take these figures and calculate the $\log_4$(50% endpoint dilution) by taking the % mortality at the dilution next *above* 50% mortality, subtracting 50, and dividing that figure by the difference between the % mortality at the dilution next *above* 50% mortality and the % mortality at the dilution next *below* 50% mortality. In the example, this becomes (66.67-50)/(66.67-0), or 16.67/66.67=0.25. This is the mantissa of the $\log_4$(50% endpoint dilution). Since these are serial 1:4 dilutions, the first column is 4^{-1}, the second column is 4^{-2}, and so on with reference to the original concentration of virus in your stock. Therefore, the column number of that dilution next *above* 50% mortality gives characteristic of the $\log_4$(50% endpoint dilution). In the example, this is –3. To get the full $\log_4$(50% endpoint dilution), add the absolute value of these two and put a minus sign in front: -3.25.

g. To calculate the $\log_{10}$(50% endpoint dilution), multiply $\log_{10}(4)$x($\log_4$(50% endpoint dilution))=0.602x($\log_4$(50% endpoint dilution))= -1.9565 in this example.

h. Then $\log_{10}$(50% endpoint titer) is the same as $\log_{10}$(50% endpoint dilution), except that it becomes positive: 1.9565.

i. Since you obtain the TCID50/mL by multiplying the 50% endpoint titer by 20 (I believe that this factor is the result of transforming the figures from per μL figures to per mL figures—20x50=1000), simply add the $\log_{10}$(50% endpoint titer) and $\log_{10}(20)$: 1.9565 + 1.301 = 3.2575.

j. Take the antilog for your final titer (TCID%): 1.809×10^3.

k. Remember that this titer may well be an underestimate; you are only incubating the plates for 7 days and a longer incubation might show more positive wells, particularly if your virus grows slowly.