

HIV Virus Culturing from Plasma, Spin Protocol

(From Bill Rodriguez in 2002, modified)

Ref.: A. B. Bahnson, et al., *J. Virological Methods* 54 (1995) 131-143
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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 cm² and 75 cm² flasks (T25 and T75 flasks)
Uninfected PBMCs (from donation or negative buffy-coat)
Anti-CD3/8 antibody
R20: 500 mL RPMI-1640
5 mL Pen/Strep
5 mL L-glutamine
6.2 mL HEPES
110 mL FBS

R20-50: Add one 280- μ L aliquot of 1×10^5 units/mL, 0.22 μ filter-sterilized IL2 to one bottle R20

Item	Manufacturer	Order Number
RPMI-1640	Sigma	R0883
Pen/Strep (5000 IU Pen/ 5000ug/mL Strep)	Mediatech	30-001-CI
L-glutamine (200mM; 29.2 mg/mL)	Mediatech	25-002-CI
HEPES (1M; 238.3mg/mL)	Mediatech	25-060-CI
FBS, Heat-inactivated	Sigma	F4135 (a 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		
Round-bottom 5-mL polypropylene FACS tubes	Falcon	352063
Round-bottom 5-mL polystyrene FACS tubes	Falcon	352058

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 will yield many more positive cultures than PHA blasts—the success rate on samples from non-local sites (Peru, China, Thailand) approximately tripled when antibody-stimulated cells were used. I got 13 isolates out of 90 Peruvian samples with PHA blasts and 13 out of 30 Thai samples with antibody-stimulated cells—and some Peruvian and Chinese samples negative with PHA blasts were positive with antibody-stimulated cells.

Stimulation with antibody: Add $\frac{2}{3}$ μ g 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 $\frac{3}{5}$ to $\frac{2}{3}$ 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 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.

Procedure:

1. **Day -2 to -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 μ L PHA/mL (250 μ g/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 the initial spin-infection should probably not use thawed PHA blasts.
2. **Day 0: Warm Centrifuge to 37 degrees, at least 1 hour before use.**
 - a. To start culture, spin down 2×10^5 PHA blasts (in a 5-mL FACS tube for the round bottom; polypropylene is preferable to polystyrene to reduce the number of cells that stick to the bottom) and resuspend gently in 0.2 to 0.4 mL plasma, freshly thawed or just received (keep plasma stock tubes on ice, though not the FACS tubes).
 - b. Transfer half this suspension to a second 5-mL FACS tube. Carefully balance centrifuge buckets with samples and lid and spin at 3000 rpm for 3 hours at 37 degrees.
 - c. During this spin, prepare one T25 flask per plasma sample with 4.8 mL R20-50 and 4.8 million more PHA blasts.
 - d. Gently transfer the contents of both FACS tubes to a T25 flask with 4.6 to 4.8 mL. Rinse, using what's in the prepared T25 flask, into that flask and incubate (37° C, 5%-6% CO₂) for 3 to 4 days (the flask should finally contain 5 mL R20-50 and 5 million PHA blasts).
 - e. **Immediately cool centrifuge for other uses.**
 - f. Start more PHA blasts for the next step.
 - g. Even more than 2 tubes may be used for the spin-infection step, but no tube should contain more than 1×10^5 PHA blasts. More than 0.2 mL plasma per tube doesn't improve infection success; if there is very little plasma, even 50 μ L may be used/tube.
3. **Day 3:** After 3 days, check culture for gel formation and break it up if present. Harvest PHA blasts made in Step 2 (day 0) and resuspend in 10 mL fresh R20-50 per 5 million cells; add 10 mL resuspended PHA blasts per culture and mix gently. Incubate another 4 days.
4. **Day 7:** Remove half the supernatant (7.5 mL) and add back another 7.5 mL of R20-50.
5. **Day 11:** Make PHA blasts as in Step 1. Remove 5 mL of the supernatant from the virus culture and keep 1 mL to measure p24 production. Transfer culture to T75 flask with 10 mL fresh R20-50—if the p24 is positive, add an extra 10 mL fresh R20-50, so that negative cultures have a volume of 20 mL and positive cultures have a volume of 30 mL. 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.
6. **Day 14:** Remove half (15 mL) the supernatant from the virus culture positive on Day 11 and save 1 mL for p24 check. Remove 5 mL of the supernatant from the virus culture negative on Day 11 and save 1 mL for p24 check. Spin down and add 5 million PHA blasts from those made on Day 11 to

each virus culture; add a total of 15 mL fresh R20-50 to each culture. Culture volume is now approximately 30 mL for all cultures; it should not rise higher than this.

7. **Day 17:** Remove half (15 mL) the supernatant from the virus culture, saving 1 mL for p24. Add the same volume back (R20-50).
8. **Day 21:** Remove 0.5-1 mL culture supernatant for p24. Split culture by mixing it and removing half; add 2 to 4 day-old PHA blasts (5 million per culture). Add a volume of fresh R20-50 equal to what remains in the flask to the culture.
9. **Day 24:** If p24 is satisfactory, harvest the culture by spinning 20 minutes at 1500 rpm, removing the supernatant with a pipet (be careful not to disturb the pellet), aliquoting into sterile polypropylene tubes, and freezing at -80 Celsius.
10. **In general:**
 - a. From Day 11 on, monitor the p24 to be sure to harvest while it is increasing. When p24 level reaches 1×10^5 pg/mL, and there is sufficient volume in the culture for your needs, harvest—even if it's earlier than Day 24. I've harvested as early as Day 11 and as late as Day 32, though the latter culture was clearly positive, but slow-growing, well before that date.
 - b. If the p24 levels off or starts declining, harvest even if p24 isn't up to 1×10^5 pg/mL, so long as it's at least 1×10^3 pg/mL and you have sufficient volume for your needs.
 - c. If you need the virus at a higher titer than you are able to get on the initial harvest, you can regrow it by repeating the culturing procedure from step 1 using your initial harvest instead of plasma. Few isolates get to p24 values of more than 1 or 2×10^5 pg/mL, however. Another method of boosting p24 values is to add 10 million cells instead of 5 million at each time cells are added, and even at times when the culture is normally simply fed with fresh medium. This can't be done for very long, though, because of the buildup of dead cells and debris, which will slow down viral growth.
 - d. If p24 is below 1×10^4 pg/mL, keep on going with culture for another 2 weeks (perform Steps 8 and 7 on days 27 and 31, respectively).
 - e. If culture remains negative by p24 assay on Day 35, dispose of it; it didn't work.