Propagation of Primary HIV-1 Isolates

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The techniques described in this protocol are used to prepare virus supernatant for primary HIV-1 isolates obtained through the UNAIDS Network for HIV Isolation and Characterization.

RESCUE CULTURE PROTOCOL

The rescue protocol is useful when the concentration of virus in the starting vial is very low, i.e., less than 50 ng p24/ml. It allows one to increase the titer and the amount of stock, which can then be used for a large scale expansion.

Preliminary Setup

1. Prior to setup, prepare target cells.

2. Label two slant tubes with the sample number, tube A or B, and the date.

Culture Setup (Day 0)

1. In a 15 ml conical tube pellet 4 x 10^6 cells by centrifuging at 1000 RPM for 10 minutes. Note: These should be day 3/4 PHA-stimulated, polybrene-treated cells.

2. Decant supernatant. Quickly thaw original vial of virus stock in a 37°C water bath.

3. Remove a 25 µl sample for antigen capture assay and dilute accordingly. Record total volume of virus stock and resuspend the cell pellet with remaining stock.

4. Divide the cell suspension equally between the two slant tubes (A & B).

5. Incubate at 37°C for ~30 minutes.

6. After incubation add 2.0 ml Co-culture Medium (page 2) to each tube so the final volume in each tube is ~2.5 ml.

7. Return to incubator until day 3/4.

Feeding

Day 3/4 - Follow these steps for each slant tube:

1. Label a cluster tube (Costar) and a cryovial with the specimen number; tube A or B, day 3 or 4, and the date.

2. Remove 2.0 ml of supernatant only and place 1.7 ml in the cryovial and the remaining 0.3 ml in the cluster tube.

3. Add 200,000 Leukopack cells in 200 µl.

4. Add 2 ml of fresh Co-culture Medium (page 2), being sure that the cell pellet has been resuspended.

5. Return the slant tubes to the incubator. Freeze the cryotubes in the liquid nitrogen freezer. The cluster tube material should be used to make serial dilutions of the supernatant for p24 analysis. Store the cluster tubes at 4°C until the rescue has been completed.

Refeeding Schedule

- Follow the steps used in the day 3/4 feed procedure for days 7, 14, 21, and 28.

- For days 10, 17, 24, 31, and 35 follow the day 3/4 procedure but do not add any fresh Leukopack cells.

Generally speaking, the cultures are fed twice weekly and will go for a period of 35 days. However, if the virus is a slow grower (determined by p24 data), then the culture can be maintained for a longer time period by adding cells once weekly.

VIRUS EXPANSION CULTURE PROTOCOL

The preparation and storage of infectious virus stocks are important for numerous in vitro studies, such as evaluation of antiviral agents and characterization of immune responses.

The procedure described here represents one method to prepare infectious HIV-1 virus stocks. Expansion cultures are established by infection of PHA-stimulated normal donor peripheral blood mononuclear cells (PBMC) with infectious culture supernatant (minimum p24 ELISA ~ 15 ng p24/ml; however, this is a relatively low p24 and one should consider doing the Rescue Protocol before this virus expansion) to generate virus stock. Positive growth of HIV-1 is detected by the appearance of p24 gag protein in the culture supernatant. The described procedure is employed by laboratory scientists, or others working in that capacity, to establish virus stocks of HIV isolate(s) by infection of PHA-stimulated normal donor PBMC with clarified infectious culture supernatant.

Reagent Preparation

Prepare all media/stock solutions at room temperature in a Class II biological safety cabinet. Unless otherwise stated, aseptic techniques should be used.

Co-culture Medium

RPMI 1640 supplemented with:

Fetal bovine serum, heat-inactivated at 56°C	15%
Il-2	20 Units/ml. (IL-2 is available from the AIDS Reagent Program or may be purchased from a commercial source.)
L-Glutamine	2 mM
Polybrene	2 µg/ml, prepared from 500 µg/ml Polybrene Stock Solution (page 3)
Penicillin	100 Units/ml
Streptomycin	100 µg/ml

Combine the above components and filter sterilize using a 0.45 µ filtration unit. Label as "Co-culture Medium" with date prepared and expiration date. Store at 4°C ± 2°C. Discard after 10 days.

Freeze Medium

RPMI 1640 supplemented with:

Fetal bovine serum, heat-inactivated at 56°C	20%
DMSO	10%
Penicillin	100 Units/ml
Streptomycin	100 µg/ml

Combine the above components and filter sterilize using a 0.45 µ filtration unit. Label as "Freeze Medium" with date prepared and expiration date. Store at 4°C ± 2°C. Discard after 14 days.

Polybrene Stock Solution (500 µg/ml)

Dissolve 100 mg of polybrene into 200 ml RPMI 1640. Filter sterilize using a 0.2 µ filtration unit. Aliquot into labeled ("Polybrene Stock") 15 ml centrifuge tubes. Aliquot volumes should be calculated to achieve a final concentration of 2 µg/ml when added to 1 liter Co-culture Medium (page 2). Store aliquots at 4°C ± 2°C. Aliquots are stable for 1 year.

Wash Medium

RPMI 1640 supplemented with:

Penicillin	100 Units/ml
Streptomycin	100 µg/ml

Label as "Wash Medium" with date prepared and expiration date. Store at 4°C ± 2°C. Discard after 14 days.

Virus Expansion Cultures

- All tissue culture media or reagents should be brought to room temperature prior to use.
- Use a standard protocol for stimulation of normal donor PBMC and day 3 polybrene treatment.
- The number of cells infected depends on the desired final volume of virus stock. For every 100 ml supernatant 40 x 10^6 cells are infected. This type of expansion can be completed by using a one or two cycle infection process and this should be determined by the study director based on the availability and quality of virus inoculum. The following procedure will describe a two cycle 100 ml expansion.
- Four vials of starting virus is recommended. However, if only 2 vials are available, split them between two virus absorption cycles rather than using them for a single one.

Day 0

1. Culture Set-up

   Aliquot 40 x 10^6 (expect a 100 ml harvest) of Day 3 PHA-stimulated polybrene-treated PBMC into a 50 ml conical tube and centrifuge for 10 minutes at 1000 rpm.

   Retrieve the 4 vials of virus stock from the liquid nitrogen and place on dry ice.

2. Virus Adsorption Cycle #1

   After the cells have been pelleted, quickly thaw the virus inoculum (2 vials) using a 37°C water bath, removing the vials before the virus stock has completely melted.

   Decant the supernatant from the cells.

   Pool the virus supernatants and remove a 200 µl sample, placing it into a labeled cluster tube. Record the volume of the viral supernatant and use it to gently resuspend the cell pellet, making a final volume of 1–1.5 ml.

   Incubate at 37°C ± 2°C, 5% CO2 , and 95% humidity for ~ 2 hours, mixing by gently swirling ever 20-30 minutes.

   After the incubation bring the volume up to 30 ml with Wash Medium (page 3) and centrifuge for 10 minutes at 1000 RPM.

3. Virus Adsorption Cycle #2

During the centrifugation, thaw the second cycle of virus inocculm using a 37°C water bath, removing the vials before the virus stock has completely melted.

Repeat Step 2, *Virus Adsorption Cycle #1* (use 2 vials of virus again).

4. Gently resuspend the cell pellet in 10 ml of Co-culture Medium (page 2) and then divide the volume equally into two T-75 flasks labeled with specimen ID # and date. Add an additional 25 ml Co-culture Medium (page 2) per flask (the final volume in each flask is 30 ml).

5. Incubate at 37°C ± 2°C, 5% CO2, and 95% humidity.

Culture Monitoring

- Beginning on day 3, the cultures are monitored for p24. From this point on each culture is treated individually based on the antigen capture data, cell density, and the pH of the culture. Here are some general guidelines of what should be done at various time points.

Day 3

AM: Monitor p24 by removing ~0.1 ml from each flask and pooling this into a labeled cluster tube. Prepare ten-fold dilutions to get an endpoint (usually 1:10 and 1:100 will work for day 3).

PM: If the p24 data shows the culture to have a reading of 100 ng/ml or more, the culture is split. To split the culture, remove ~20 ml supernatant from each flask and collect into a labeled 50 ml conical tube. Store this pooled batch of virus at -70°C and discard after successful virus expansion. Gently resuspend cells in the remaining supernatant (~10–15 ml) and divide this equally between the original flask and a new flask with the appropriate label. (Be sure to indicate which flask is new by writing S/C on it.) There are now a total of 4 flasks per isolate. Bring the volume up to 30 ml with fresh Co-culture Medium (page 2) and incubate until day 4.

Day 4

AM: Monitor p24 as described for day 3. Split any culture that was not split on day 4 by following the procedure above.

PM: Review data. For any culture split on day 3, observe the flasks and add only enough fresh Co-culture Medium (page 2) to make the pH just neutral. (Medium does not necessarily need to be added).

Day 5, 6 and 7

AM: Monitor p24 as described above.

PM: Review p24 data. Add medium to maintain a optimal pH and cell density or harvest as appropriate.

- When p24 value reaches between 50 and 100 ng/ml or higher, the expansion cultures may be ready to harvest. Follow the harvest procedure below. If p24 does not reach the harvestable range by day 7, harvest day 7 supernatant and refeed the cells with fresh Co-culture Medium (page 2) and add 4 x 10^6 PHA-stimulated polybrene cells per flask.

Day 10

AM: Monitor p24 as described above and harvest day 10 virus stock as necessary.

Harvest of Virus Supernatant

1. Aspirate 25-30 ml cell-free virus stock from each T-75, leaving 10-15 ml of cell suspension in each flask. Collect a 300µl sample into a cluster tube for antigen capture assay.

2. Clarify by centrifugation twice, initially at 1400 rpm (370 x g), 10 minutes at 4°C, then transfer virus stock into another 250 ml centrifuge tube (do not transfer cells). A second centrifugation is done at 2400 rpm (1100 x g), 10 minutes at 4°C.

3. During the clarification label the appropriate number of cryotubes (use cryotubes with an internal threads and a silicone gasket such Nunc or Sarstedt brands).

4. Aliquot the "cell-free" virus stock at 1.0 ml/vial.

Termination of Cultures

1. Cryopreserve the infected PBMC at a concentration of 6 x 10^6 cells/ml or more in Freeze Medium (page 2).

2. Dispose of unneeded culture material using proper biosafety handling methods.

REFERENCES

Division of AIDS, National Institute of Allergy and Infectious Diseases. 1997. DAIDS Virology Manual for HIV laboratories. Publication NIH-97-3838. US Department of Health and Human Services, Washington, DC. The manual is available in PDF format at http://www.niaid.nih.gov/daids/vir_manual/full_vir_manual.pdf