

## Isolation, Propagation, and Titration of Human Immunodeficiency Virus Type 1 From Peripheral Blood of Infected Individuals

Hanneke Schuitemaker and Neeltje A. Kootstra

### Summary

HIV-1 can be isolated from peripheral blood mononuclear cells and is easily propagated on primary cells in vitro. Here we describe the method for bulk isolation of the HIV-1 quasispecies and a limiting dilution virus isolation protocol by which single coexisting clones can be obtained. In addition, methods for propagation and titration of HIV-1 are provided.

**Key Words:** HIV isolation; bulk isolate; HIV variant; TCID<sub>50</sub>; stock preparation.

### 1. Introduction

To study biological properties of the human immunodeficiency virus type 1 (HIV-1) in relation to the clinical course of infection, the in vitro preservation of phenotypical characteristics of the virus that may be relevant in vivo is essential. Even in the early days of HIV-1 research, it was clearly recognized that passage through immortalized T-cell lines was successful with only some of the viruses (1). In addition, adaptation to T-cell lines changed the viral phenotype (2). Thus, primary HIV-1 isolates should be isolated and propagated on primary peripheral blood mononuclear cells (PBMC), as this provides the most optimal preservation of the biological phenotype of the virus. This chapter describes the isolation, propagation, and titration of HIV-1 bulk isolates and biologically cloned HIV variants.

### 2. Materials

1. Iscove's modified Dulbecco's medium (IMDM; BioWhittaker).
2. Heparin.

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3. Trisodium citrate dihydrate (TNC, Merck).
4. Phosphate-buffered saline (PBS).
5. Fetal calf serum (FCS; Hyclone).
6. Recombinant interleukin-2 (rIL-2; Chiron Benelux).
7. Penicillin (Pen), 100 U/mL (GibcoBRL).
8. Streptomycin (Strep), 100 U/mL (GibcoBRL).
9. Phytohaemagglutinin (PHA; Wellcome).
10. Heparinized peripheral blood or buffy coat.
11. Ficoll-Isopaque (Pharmacia).
12. Sterile 10-mL pipets.
13. Sterile 25-mL pipets.
14. Sterile 50-mL plastic tubes.
15. Pasteur pipets.
16. PFH medium: PBS supplemented with 5% FCS and 10 U/mL heparin.
17. PFT medium: PBS supplemented with 5% FCS, 10% TNC.
18. IF medium: IMDM supplemented with 5% FCS.
19. PHA medium: IMDM supplemented with 10% FCS, 1  $\mu$ g/mL PHA, Pen/Strep.
20. Interleukin (IL)-2 medium: IMDM supplemented with 10% FCS, Pen/Strep, 10 U/mL rIL-2.
21. 0.2% Triton X-100 solution: PBS supplemented with 0.2% Triton X-100.
22. 96-well flat-bottom microtiter plates.
23. Multichannel pipet.
24. Sterile pipet tips.

### 3. Methods

The methods described below outline (1) the isolation of peripheral blood mononuclear cells from patient blood and buffy coat, (2) the virus isolation from patient PBMC in bulk, (3) HIV isolation under limiting dilution conditions to obtain biological virus clones, (4) the propagation of primary HIV isolates and variants, and (5) the titration of virus stocks (*see Note 1*).

#### 3.1. Isolation of PBMC From Patient Blood and Buffy Coats

Transfer heparinized venous patient blood to plastic 50-mL tubes (maximum of 25 mL patient blood per tube) and dilute with an equal volume of PFH medium. When citrate has been used as an anticoagulant, add an equal volume of PFT medium. When PBMC are isolated from a buffy coat, transfer the buffy coat to a 250-mL flask and add PFT medium to a final volume of 150 mL. Gently load 25 mL of diluted blood on top of 12.5 mL of a Ficoll Isopaque solution with a density of 1.077 g/mL in a 50-mL tube. Centrifuge at 760g for 20 min at room temperature. Collect the cell band on top of the Ficoll layer. Wash the harvested cells twice by adding IMDM to a total volume of 50 mL and centrifuge at 425g for 10 min. PBMC can now either be cryopreserved in

IMDM containing 10% FCS and 10% dimethyl sulfoxide (DMSO) or directly used (*see* **Note 2**).

### **3.2. Isolation of Human Immunodeficiency Virus on Donor PBMC**

#### *3.2.1. Selection of Donor PBMC*

PBMC may vary in their susceptibility to infection with HIV. To optimize the efficiency of the isolation procedure, it may therefore be useful to select optimal susceptible donor PBMC.

#### *3.2.2. Exclusion of PBMC From Donors With a 32-Basepair Deletion in CCR5 Gene*

HIV-1 susceptibility is most obviously determined by the CCR5 genotype and associated  $\beta$ -chemokine production levels. The CCR5 gene encodes for the coreceptor for R5 HIV variants. To achieve optimal susceptible target cells during virus isolation, it is recommended to exclude buffy coats from donors who are homozygous or heterozygous for the 32-basepair (bp) deletion in CCR5 that results in a premature stop codon and the absence of functional CCR5 on the cell membrane. The presence of this 32-bp deletion is rapidly determined with a polymerase chain reaction (PCR) (3). Genomic DNA can be most conveniently isolated from donor PBMC using the Qiagen blood kit (Qiagen, Hilden, Germany). Subsequently, 100 ng of DNA can be used for PCR analysis with primers (sense, position 612 to 635 in CCR5, 5'-GATAGGTACCTGGCTGTCGTCCAT-3'; antisense, position 829 to 850 in CCR5, 5'-AGATAGTCATCTTGGGGCTGGT-3') flanking the described 32-nucleotide deletion in the CCR5 gene. Samples are amplified with 1 U of Taq polymerase (Promega, Madison, WI) in the buffer provided by the manufacturer, with a final  $MgCl_2$  concentration of 3 mmol/L. Conditions for PCR reaction are: 5 min of denaturation at 95°C; 30 cycles of 1 min at 95°C, 1 min at 56°C, and 2 min at 72°C; and 5 min of elongation at 72°C in a Perkin-Elmer Cetus DNA thermal cycler 480 (Perkin-Elmer, Foster City, CA). The PCR product lengths are 238 bp for the wild-type allele and 202 bp for the mutant allele. DNA samples that show both bands are obviously from donors with a CCR5 D32 heterozygous genotype. PCR products can be analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

#### *3.2.3. Measurement of HIV Susceptibility of PBMC*

To determine relative HIV susceptibility of donor PBMC, a cell-free virus stock of a R5 HIV isolate is titrated in quadruplicate on PBMC from different donors in microtiter plates. PBMC from donors in which the highest TCID<sub>50</sub> value is achieved are selected for further use. For a detailed description of the TCID<sub>50</sub> assay, *see* **Subheading 3.5**.

### 3.3. Isolation of Primary HIV-1 on PBMC

Primary HIV-1 can be isolated either in bulk or under limiting dilution conditions to obtain biological clonal virus variants. For both methods, PBMC from a healthy blood donor volunteer are used as target cells. These donor PBMC are stimulated with PHA medium for 2 to 3 d, starting at a cell density of  $5 \times 10^6/\text{mL}$ . PHA-stimulated cells are then centrifuged at 425g and resuspended in IL-2 medium at a cell density of  $1 \times 10^6/\text{mL}$ .

#### 3.3.1. Isolation of Bulk HIV (the "Quasi-Species")

Mix 5 mL of the PHA-stimulated donor PBMC suspension with 1 to 3 mL of isolated patient PBMC at a cell density of  $1 \times 10^6$  in IL-2 medium in a 15-mL tube (*see Note 3*). Spin the cells down and incubate the cell pellet in a 37°C water bath for 1 h prior to culture in a 25-cm<sup>2</sup> flask. Alternatively, put the cells directly in the 25-cm<sup>2</sup> flask and culture the cells overnight in the 37°C incubator standing under a 45° angle, allowing the cells to settle and be in close contact in a corner of the flask. Afterwards, place the flask in an upright position and further incubate the cells. Four days after the start of the co-cultivation, cells are collected, centrifuged, and resuspended in the original volume of fresh IL-2 medium. Collected supernatant is tested for the presence of p24 gag antigen reflecting virus production (*see Note 4*). On d 7, cells are centrifuged and the supernatant is again tested for the presence of p24 antigen. To half of the cell pellet,  $5 \times 10^6$  fresh 2–3 d PHA-stimulated donor PBMC are added to a total volume of 8 mL IL-2 medium and cultures are maintained. The other half of the pellet can be cryopreserved in IMDM with 10% DMSO. Medium changes are repeated on d 11, 18, and 25. The addition of fresh PHA-stimulated cells is repeated on d 14, 21, and 28. In general, cultures are continued for 4 to 5 wk. Supernatant is stored in small aliquots (1–1.5 mL/ampule) at –80°C.

#### 3.3.2. Isolation of Biological HIV Clones

Molecular studies have demonstrated that in HIV-1-infected individuals at each moment a large number of related but different virus variants may co-exist. Virus isolation as described in **Subheading 3.2.1.** will result in the rapid outgrowth of one or few of the infectious HIV-1 variants present in the patient's PBMC with the most fit phenotype under these isolation conditions. To obtain a more complete picture of the diversity of the HIV quasiespecies present in an individual in vivo, a virus isolation protocol was developed that allows for the isolation of multiple HIV-1 variants from a single PBMC sample, avoiding overgrowth and loss of slowly replicating variants (**Fig. 1**) (**4**).

Biological virus clones can be obtained by cocultivation of patient PBMC with PHA-stimulated healthy blood donor PBMC (donor PHA-PBMC) under

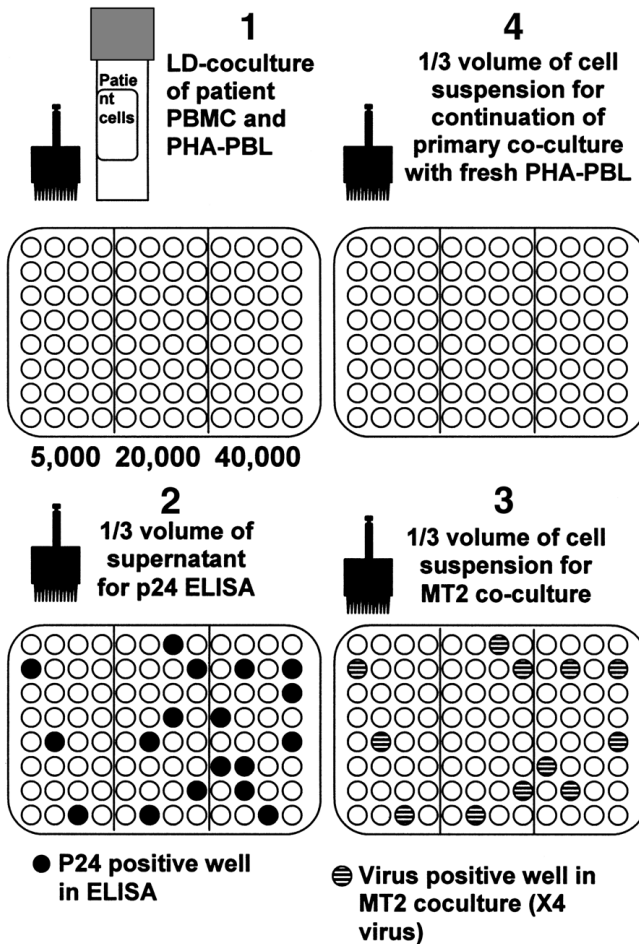


Fig. 1. Isolation of biological HIV-1 clones is achieved by co-culture of limiting dilutions of HIV-1-infected patient peripheral blood mononuclear cells (suggested cell numbers in figure are 5000, 20,000, and 40,000 patient cells per well; 32 wells per patient cell number) with phytohemagglutinin (PHA)-stimulated healthy donor peripheral blood lymphocytes (PBL). Step 1 is the initiation of the co-culture, step 2 is the harvesting of 50  $\mu$ L supernatant for analysis of virus production in a p24 enzyme-linked immunosorbent assay to be performed weekly, step 3 is the transfer of 50  $\mu$ L of the cell suspension to a MT2 co-culture to monitor the presence of X4 viruses that will induce large syncytia in MT2 cells, step 4 is the transfer of 50  $\mu$ L of the cell suspension to fresh PHA-stimulated healthy-donor PBL for continuation of the primary culture. From this plate, steps 2, 3, and 4 are repeated at d 14. At d 21, steps 2, 3, and 4 are repeated from the primary co-culture plate that was established at d 14, and so forth. See text for further details.

limiting dilution conditions (*see Note 4*). Patient PBMC are isolated by Ficoll density gradient centrifugation as described above and diluted in medium to cell concentrations of  $5 \times 10^4$ ,  $10 \times 10^4$ ,  $20 \times 10^4$ , and  $40 \times 10^4$  cells/mL IL-2 medium. These cell suspensions are then seeded in 96-well microtiter plates, 100  $\mu$ L/well and 32 to 48 wells per cell concentration (*see Note 5*). Subsequently,  $10^5$  PHA-stimulated healthy donor PBMC in 50 mL IL-2 medium are added to each well and cultures are incubated in an incubator at 37°C, with a humidified atmosphere and 5% CO<sub>2</sub>. Every week, 50  $\mu$ L culture supernatant is transferred from all wells, row by row, to wells of a new 96-well plate, using a multichannel pipet. With the same pipet tips, the remaining cells in that row of the microtiter plate are resuspended and 50  $\mu$ L is transferred from each well of that row to the corresponding row in a new 96-well microtiter plate. Subsequently,  $10^5$  freshly prepared PHA-stimulated healthy donor PBMC in 100  $\mu$ L IL-2 medium are added to propagate the culture. Microcultures are continued in an incubator as described above. To each well of the original 96-well plate, add 50  $\mu$ L of the initial PBMC co-culture and 100  $\mu$ L of  $10^5$  MT2 cells in log phase. These cultures will be microscopically studied with a  $\times 40$  magnification at d 5 after start of the MT2 co-culture for the presence of syncytia, which indicates that the HIV variant present in the co-culture has the capacity to use HIV coreceptor CXCR4.

To reveal virus production in the original PBMC co-cultures, the presence of p24 antigen in the supernatants is tested using a p24 specific antigen capture enzyme-linked immunosorbent assay (ELISA) (*see Note 3*). Prior to performing the ELISA, infectious HIV that may be present in the supernatant should be inactivated by adding 50  $\mu$ L of 0.2% Triton X-100 solution.

The whole procedure is repeated at d 14, 21, and 28. The frequency of infected PBMC is estimated using the formula for Poisson distribution ( $u = -\ln F_0$ , in which  $F_0$  is the fraction of negative cultures). Isolates can be considered clonal if 37% or less of the cultures become positive, which corresponds to  $\leq 0.5$  infected cells/well.

### 3.4. Propagation of Primary HIV

Once virus replication is detected by p24 ELISA (*see Note 3*), the virus should be stored as quickly as possible to minimize the number of passages, as this may lead to adaptation of the virus to in vitro conditions. Virus can be stored as cell-free supernatant at  $-80^\circ\text{C}$ , or as infected viable frozen PBMC at  $-80^\circ\text{C}$  or in liquid nitrogen.

#### 3.4.1. Expansion of Primary HIV From Cell-Free Supernatant

To expand HIV from cell-free supernatant, PHA-stimulated PBMC are prepared as described above (*see Subheadings 3.1. and 3.2.*). For each inocula-

tion, a cell suspension with an absolute cell number of  $8 \times 10^6$  cells is transferred to a 15-mL tube. Cells are centrifuged (425g, 10 min at room temperature) and the supernatant is decanted. The cell pellet is then resuspended in 1–1.5 mL HIV-positive supernatant from the thawed ampule and incubated for 2 h in a 37°C shaking water bath. Subsequently, 7 mL IL-2 medium is added and the cell suspension is transferred to a 25-cm<sup>2</sup> tissue culture flask and cultured at 37°C. Every 3–4 d, 100  $\mu$ L is sampled from the supernatant to monitor for virus production in a p24 antigen capture ELISA. At d 4, 11, 18, and 25, half of the medium is replaced with fresh IL-2 medium. At d 7, 14, and 21, half of the cell suspension is removed and  $4 \times 10^6$  fresh PHA-stimulated PBMC are added. When the optical density (OD) of the sample in the p24 ELISA is four times the OD of the negative control, the supernatant is aliquoted in 1–1.5-mL samples and stored at –80°C until further use.

#### 3.4.2. Expansion of Primary HIV From Viable Frozen HIV-Infected PBMC

The propagation of HIV from HIV-infected viable frozen PBMC is identical to the procedure described for virus isolation from HIV-infected patient PBMC (see **Subheading 3.3.1.**).

### 3.5. Determination of Infectious Titer of HIV in Supernatant

To establish the titer of a virus stock, serial 1:5 dilutions of the stock are made in IL-2 medium. For each virus, six 15-mL tubes with 2 mL IL-2 medium each are prepared. Then 0.5 mL from the original virus stock is transferred to the first 15-mL tube with 2 mL IL-2 medium. After thorough mixing, 0.5 mL is transferred from this tube to the next tube with 2 mL IL-2 medium. This is repeated until six dilutions have been made.

From 2-d PHA-stimulated PBMC, a cell suspension of  $10^6$ /mL IL-2 medium is prepared. This suspension is then seeded in a 96-well plate, 100  $\mu$ L per well, 4 columns (32 wells) per virus titration. Subsequently, undiluted virus stock is added to 4 wells in row A of the plate, 50  $\mu$ L/well. The 1:5 dilution of the virus stock is added to 4 wells in row B, the 1:25 dilution of the virus stock is added to 4 wells in row C, and so on. There is room for three virus titrations on one 96-well plate. Cultures are incubated at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. On d 7 and 10, 75  $\mu$ L supernatant is harvested using a multichannel pipet and transferred to a new plate to be inactivated with 75  $\mu$ L/well of a 0.2% Triton X-100 solution. On d 7, subsequently 75  $\mu$ L fresh IL-2 medium is added to each well, to propagate the cultures. Cultures are terminated after supernatant sampling on d 10.

The 50% tissue culture infectious dose (TCID<sub>50</sub>) in the supernatant is calculated as follows:

$-\log \text{TCID}_{50} \text{ endpoint} = -\log a - [(b - 0.5) \times \log c]$ , in which  $a$  = the lowest virus dilution in the assay (e.g.,  $10^{-1}$ );  $b$  = sum of the percent p24 positive cultures at each dilution; and  $c$  = the dilution factor.

#### 4. Notes

1. Working with replication-competent HIV-1 requires a laboratory at biological safety level 2 (BSL-2).
2. Working with primary cells from blood donor and HIV-infected patients may result in the frequent introduction of mycoplasma. Not only should precautions be taken to prevent infection of permanent cell lines that are being used in the laboratory, but it is also recommended to add cyproxin to the cultures, to inhibit replication of mycoplasma.
3. An inhibitory effect of CD8 cells on HIV replication has been reported. When an attempt is made to isolate virus from an HIV-infected individual with low virus load, it is recommended to deplete CD8 cells from patient PBMC and from PHA-stimulated PBMC. The latter should be performed after PHA stimulation, as the absence of CD8 cells seems to interfere with PHA stimulation.
4. As an alternative for the p24 antigen capture ELISA, a reverse transcriptase (RT) assay can be performed to detect virus production.
5. It is possible to isolate HIV-1 variants from specific peripheral-blood T-cell subsets. For this purpose, the cells of choice should be sorted on a Fluorescence Activated Cell Sorter (FACS) first and then used for virus isolation according to one of the protocols described earlier.

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