Detection of HIV DNA and RNA Using PCR

The polymerase chain reaction (PCR) and reverse transcriptase (RT) PCR can be used to amplify and detect HIV DNA and RNA, respectively, with high sensitivity and specificity. These two techniques are useful tools to monitor changes in viral burden and viral replication during the course of HIV infection. The techniques presented in this unit are specific variations of protocols presented elsewhere (UNITS 10.20-10.23).

The protocols in this unit present methods for detecting HIV DNA and RNA sequences in HIV-infected cells. In HIV-infected individuals, these samples can originate from any body compartment suspected of harboring HIV. Alternatively, these protocols can also be applied to cells infected with HIV in vitro. In DNA-PCR, HIV DNA sequences are amplified by PCR and detected by hybridization to an HIV-specific radiolabeled oligonucleotide probe (see Basic Protocol 1). In reverse transcription–PCR (RT-PCR), RNA is extracted from infected cells and reverse-transcribed into cDNA prior to the DNA-PCR steps (see Basic Protocol 2).

**CAUTION:** Handling of HIV-infected cells for nucleic acid preparations is to be carried out in a biosafety level 3 facility. After virus inactivation, the samples should be transferred to a work area designed to minimize cross-contamination. All protocols should be carried out using aerosol barrier pipet tips as well as sterilized DNase/RNase-free plastic tubes and water.

**AMPLIFICATION AND DETECTION OF HIV DNA USING PCR (DNA-PCR)**

In this protocol, DNA isolated from HIV-infected cells is subjected to HIV DNA-PCR. PCR products are analyzed by gel electrophoresis followed by Southern hybridization using a $^{32}$P-labeled probe, and visualized by phosphor imaging analysis. The T cell line, ACH-2, carrying one copy of the integrated form of HIV DNA, is used as a positive control. The quantity and integrity of the DNA used in this assay is monitored by parallel PCR amplification of a reference DNA sequence, typically β-globin. The parameters in this section have been optimized for the amplifications shown in Figure 12.6.1 with the primer/probe sets given in Table 12.6.1. The authors recommend optimization of PCR reactions for each primer/probe set.

**Materials**

- HIV-infected cells to be tested for the presence of HIV DNA
- HIV-negative cells
- ACH-2 T cell line (AIDS Research and Reference Reagent Program, # 349)
- DNA isolation kit (Puregene, Gentra Systems)
- Isopropanol
- 70% ethanol
- Phosphate-buffered saline (PBS; APPENDIX 2A)
- 20 mg/ml proteinase K
- Cell lysis buffer (used only for step 4b; see recipe)
- HIV-specific PCR primers (Table 12.6.1)
- 10 mM dNTP mix (Perkin-Elmer; UNIT 10.20)
- 10× PCR buffer (see recipe)
- 50 mM MgCl$_2$
- 2.5 U Platinum Taq DNA polymerase (Life Technologies)
- 40 pmol oligonucleotide probe (Table 12.6.1)
- 5× T4 polynucleotide kinase forward reaction buffer (provided with T4 polynucleotide kinase)
Detection of HIV DNA and RNA Using PCR

10 U/µl T4 polynucleotide kinase (Life Technologies)
10 µCi/µl [γ-32P]ATP
1.8% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide
0.5x, 1x, and 2x SSC containing 0.1% (w/v) SDS
Hybridization solution (see recipe)
10 mg/ml salmon sperm DNA (Life Technologies)

56° and 94°C water baths
8-tube strip MicroAmp optical tubes (PE Biosystems)
8-cap strip MicroAmp optical caps (PE Biosystems)
Thermal cycler (e.g., GeneAmp PCR System 9700, Perkin-Elmer)
0.5- and 1.5-ml microcentrifuge tubes
Elutip-D columns (Schleicher & Schuell)
Nylon membrane (Zeta Probe GT, Bio-Rad)
Chromatography paper (Whatman)
UV cross-linker (Stratagene)
Glass hybridization tube (Stovall)
Hybridization oven (Stovall)
Plastic dish with tight cover
Phosphor imager system, including screen, image eraser and Storm 860
(Molecular Dynamics)
Image Quant software (Molecular Dynamics)

Additional reagents and equipment for culture of T cells (UNIT 12.1), counting cells (APPENDIX 3A), isolation of mononuclear cells (UNIT 12.2), concentration and purification of DNA with Elutip-D columns (UNIT 10.5), Southern blot hybridization (UNIT 10.7), autoradiography (APPENDIX 3J), and agarose gel electrophoresis (UNIT 10.4)
Prepare sample

To prepare genomic DNA using a Puregene DNA isolation kit

1a. Pellet 1 × 10^6 cells in a 15-ml conical tube by centrifuging 10 min at ∼500 × g. Remove the supernatant leaving a small amount in the tube (i.e., <20 µl) and resuspend pellet by vortexing.

2a. Add 143 µl cell lysis solution (from Puregene kit). Lyse the cells by pipetting up and down with a large bore pipet tip. If cell clumps are visible, incubate at 37°C until the solution is homogeneous. At this point, samples can be stored at −80°C, for up to 1 year.

3a. Transfer cell lysate into a clean 1.5-ml microcentrifuge tube. Add 2 µl of RNase A solution (from Puregene kit) invert tube 25 times, and incubate at 37°C for 10 to 30 min.

4a. Cool samples to room temperature, then add 48 µl of protein precipitation solution (from Puregene kit), vortex vigorously for 20 sec, and incubate on ice for 5 min.

5a. Microcentrifuge 5 min at maximum speed, room temperature. Transfer the supernatant into a 1.5-ml microcentrifuge tube and add 143 µl of isopropanol. Gently invert the tubes until the DNA aggregates become visible.

6a. Microcentrifuge 5 min at maximum speed, discard supernatant, and wash the DNA pellet with 1 ml 70% cold ethanol. Microcentrifuge 5 min at maximum speed, aspirate ethanol, and air dry pellet.

7a. Dissolve DNA pellet in 20 to 40 µl H2O and allow DNA to rehydrate overnight at 4°C. Determine the amount of DNA in each sample by absorption spectroscopy (APPENDIX 3L). An A260/A280 ratio of 1.8 is optimal. Adjust volumes to standardize concentrations of DNA in each sample; typically they should be ∼0.10 µg/µl. This will translate into PCR reactions being carried out on ∼1.5 × 10^5 cell equivalents of DNA.

To prepare cell lysates for direct PCR

1b. Wash cells that are to be assessed for HIV twice in PBS (UNIT 12.1).
2b. Count cells and pipet aliquots containing $1 \times 10^6$ cells each into 1.5-ml microcentrifuge tubes.

*PCR is performed on an amount of DNA corresponding to $1 \times 10^5$ cells (50 µl of the lysate are tested in the PCR, corresponding to 1/10 of the original lysate). An amount of lysate corresponding to fewer cells (e.g., $10^4$ or $10^3$) can be used, but in this case the sample lysates are first layered onto a cushion lysate corresponding to $1 \times 10^5$ Jurkat cells to obtain similar amounts of total DNA in all samples tested.*

3b. Microcentrifuge cells 5 min at maximum speed and discard supernatant.

*Cells may be used immediately or stored at −20°C. For indefinite storage, cell pellets should be placed at −70°C.*

4b. Prepare fresh lysis solution (PK/LB) by mixing 15 µl of 20 mg/ml proteinase K and 500 µl cell lysis buffer per sample.

5b. Add 500 µl of fresh PK/LB solution to each pellet and resuspend cell pellet completely by vortexing the tube. Allow lysis to proceed 1 hr in a 56°C water bath.

*If cell pellets are frozen, the PK/LB solution should be added directly to the frozen pellet.*

6b. Inactivate proteinase K (PK/LB solution) by incubating the tubes 15 min in a 94°C water bath.

*Lysates may be used immediately for amplification or stored at −20°C. For long-term storage, lysates should be placed at −70°C.*

7b. Proceed to step 8.

**Carry out PCR amplification**

8. Label one series of tubes in an 8-tube strip of thin-walled PCR reaction tubes with test sample identification. Label a second parallel set for β-globin PCR.

*Include an HIV-negative control such as normal donor peripheral blood mononuclear cells (PBMC), and positive controls (10-fold serial dilutions of ACH-2 DNA equivalent to 5000 to 5 cells) for each primer pair, including primers for β-globin.*

9. In a separate tube, prepare the master mix (multiply the following volumes by the total sample number plus 1 or 2 to allow for pipetting losses):

- 5 µl 5 µM 5’ primer
- 5 µl 5 µM 3’ primer
- 2 µl 10 mM dNTP mix
- 5 µl 10× PCR buffer
- 2 µl 50 mM MgCl2
- 0.5 µl 2.5 U Platinum Taq DNA polymerase
- 20.5 µl sterile H2O.

Vortex the master mix and pipet 40 µl into each labeled tube.

10. Add 10 µl of DNA samples (step 7 a or b) to each tube containing the reaction mix and cap the tubes with an 8-cap strip.

11. Carry out the amplifications in a thermal cycler with the following parameters:

- 1 cycle: 2 min 94°C
- 26 to 32 cycles: 30 sec 94°C (denaturation)
- 30 sec 58°C (annealing)
- 45 sec 72°C (extension)

- 1 cycle: 10 min 72°C (final product extension).
12. Maintain samples at 4°C after cycling. 

*Samples may be stored at −20°C until analyzed.*

**Label hybridization probe used for detection**

13. Mix the following reagents in a 0.5-ml microcentrifuge tube:

- 4 µl 40 pmol oligonucleotide probe
- 6 µl molecular grade H₂O
- 4 µl 5× T4 polynucleotide kinase forward reaction buffer
- 1 µl 10 U/µl T4 polynucleotide kinase
- 5 µl 10 µCi/µl [γ-³²P]ATP.

Microcentrifuge briefly and incubate 60 min at 37°C followed by 5 min at 95°C in the thermal cycler. Microcentrifuge briefly and chill on ice.

14. Purify the labeled probe using Elutip-D columns according to manufacturer’s instructions (see also UNIT 10.5).

**Detect amplified product by gel electrophoresis and Southern hybridization**

(see also UNITS 10.4 & 10.6 for detailed protocols)

15. Resolve PCR-amplified DNA fragments on a standard 1.8% agarose gel containing 0.5 µg/ml ethidium bromide, and photograph gel (UNIT 10.4).

16. Transfer DNA to nylon membrane overnight (UNIT 10.7).

17. Wash the membrane twice with 2× SSC containing 0.1% SDS, each time for 5 min, and transfer the membrane to a piece of chromatography paper to remove excess buffer on the membrane.

18. Immediately cross-link the DNA using a UV cross-linker at the automatic setting.

19. Transfer the membrane into a glass hybridization tube containing 10 to 15 ml hybridization solution and 100 µl of 10 mg/ml denatured salmon sperm DNA. Prehybridize the membrane at least 30 min at 50°C (UNIT 10.7).

20. Remove prehybridization solution and add 10 to 15 ml of hybridization solution containing 100 µl denatured salmon sperm DNA and 1 × 10⁷ cpm labeled probe. Hybridize the membrane 6 hr to overnight at 50°C (UNIT 10.7).

21. Wash the membrane using the following sequence of solutions in a plastic dish with a tight cover:

- 2× SSC containing 0.1% SDS (2 min at room temperature) 
- 2× SSC containing 0.1% SDS (2 min at room temperature) 
- 2× SSC containing 0.1% SDS (30 min at 50°C) 
- 1× SSC containing 0.1% SDS (30 min at 50°C) 
- 0.5× SSC containing 0.1% SDS (30 min at 50°C).

22. Expose the membrane to a phosphor imager screen 1 to 24 hr and quantify the radioactive signals using Image Quant software.

**DETECTION OF HIV RNA BY REVERSE TRANSCRIPTASE-PCR (RT-PCR)**

In this protocol, RNA isolated from HIV-infected cells is subjected to HIV RT-PCR to detect unspliced messages. Cellular RNA is extracted from HIV-infected cells and reverse transcribed into cDNA. The rest of the protocol is very similar to Basic Protocol 1. In order to control for false positives, each reaction is performed in duplicate with one tube containing reverse transcriptase and the other without that enzyme. The quantity and integrity of the RNA, as well as efficiency of the reverse transcription reaction, is monitored by parallel amplification of a reference gene, typically β-actin. To obtain standards for determination of
the absolute copy numbers of HIV mRNA in each sample, another set of parallel reactions is carried out on in vitro–transcribed HIV RNA. The source of pure HIV transcripts is a 647-base insert in the plasmid SP64AH2 (Saksela et al., 1993).

**Materials**

- SP6 in vitro transcription kit (e.g., MEGAscript, Ambion)
- Control plasmid (SP64AH2) to transcribe unspliced form of HIV RNA
- DEPC-treated H₂O (*UNIT 12.8*)
- Lithium chloride precipitation solution (see recipe)
- 70% and 75% ethanol
- HIV-infected cells
- TRIzol reagent (Life Technologies)
- Chloroform
- Isopropanol
- 500 mM EDTA, pH 8.0 (*APPENDIX 2A*)
- 10 mM 4dNTP mix (Life Technologies)
- 50 ng/µl random hexamers (Life Technologies)
- 10× RT-PCR buffer (see recipe)
- 25 mM MgCl₂
- 1.5 M DTT (*UNIT 12.8*)
- 40 U/µl recombinant ribonuclease inhibitor (e.g., RNaseOUT, Life Technologies)
- 50 U/µl Superscript II reverse transcriptase (Life Technologies)
- 2 U/µl RNase H (Life Technologies)
- 5 µM 5′ primer
- 5 µM 3′ primer
- 2.5 U Platinum Taq DNA polymerase
- 0.5- and 1.5 ml microcentrifuge tubes
- 25°C, 37°C, 65°C, and 95°C water baths
- 8-tube strip MicroAmp optical tubes (PE Biosystems)
- 8-cap strip MicroAmp optical caps (PE Biosystems)
- Thermal cycler (e.g., GeneAmp PCR System 9700, Perkin-Elmer)

**NOTE:** Precautions must be taken to avoid the risk of RNA degradation by ribonuclease contamination. These include the use of RNase-free sterile plasticware and solutions, the addition of an RNase inhibitor during the RT reaction, and the use of DEPC-treated water in the preparation of RNA transcripts and cDNA templates. To avoid DNA contamination during the PCR, the RNA transcripts are pre-treated with DNase.

**Prepare unspliced form of HIV RNA by in vitro transcription of control plasmid**

1. Add the following reagents (provided in the in vitro transcription kit) to a sterile 0.5-ml microcentrifuge tube:
   - 2 µl 10× reaction buffer
   - 2 µl 50 mM ATP
   - 2 µl 50 mM CTP
   - 2 µl 50 mM GTP
   - 2 µl 50 mM UTP
   - 2 µl enzyme mix
   - 1 µg linearized plasmid SP64AH2
   - DEPC-treated H₂O to 20 µl.

   **Linearize the control plasmid by digestion with a restriction endonuclease that cleaves at the 3′ end of the insert. The DNA should then be purified by phenol extraction and ethanol precipitation (*UNIT 10.1*).
2. Incubate tube 2 to 6 hr at 37°C.

3. Add 1 µl of 2 U/µl RNase-free DNase I to the reaction tube, mix by flicking, microcentrifuge briefly, and incubate 15 min at 37°C.

4. Precipitate RNA with 30 µl DEPC-treated water and 25 µl of lithium chloride precipitation solution. Incubate 30 min at −20°C.

5. Microcentrifuge 15 min at maximum speed, 4°C, to pellet RNA. Aspirate the supernatant and wash pellet once with cold 70% ethanol.

6. Microcentrifuge 10 min at maximum speed, 4°C. Allow RNA pellet to air dry and resuspend in 100 µl DEPC-treated water.

The RNA may be stored at −80°C for up to 1 year.

Determine RNA concentration by absorption spectroscopy (APPENDIX 3L). Estimate the copy number at this concentration and make ten-fold dilutions to give rise to 5 × 10⁴, 5 × 10⁵, 5 × 10⁶, 5 × 10⁷ RNA copies/µl. In order to increase efficiency of cDNA preparation, the authors recommend adding carrier RNA in the form of tRNA or RNA from HIV-negative cells.

IMPORTANT NOTE: All following steps apply to HIV-infected samples and HIV-negative control PBMC.

Prepare RNA from HIV-infected cells

7. Pellet 5 × 10⁶ cells in a 15-ml conical tube by centrifuging 10 min at ~500 × g. Remove the supernatant, leaving a small amount in the tube (<20 µl). Resuspend the pellet by vortexing and transfer to a 1.5-ml microcentrifuge tube.

8. Add 1 ml TRIzol reagent, lyse cells by repetitive pipetting, and incubate 5 min at room temperature.

9. Add 0.2 ml of chloroform, shake vigorously 15 sec, and incubate 2 to 3 min at room temperature.

10. Microcentrifuge 10 min at maximum speed, 4°C. Transfer the aqueous layer to a clean tube.

11. Add 0.5 ml isopropanol, vortex, and incubate 10 min at room temperature.

12. Microcentrifuge 10 min at maximum speed, 4°C. Discard the supernatant, add 1 ml 75% ethanol, and vortex.

13. Microcentrifuge 10 min at maximum speed, 4°C. Discard the supernatant, allow pellet to air-dry, and resuspend in ~20 µl DEPC-treated water.

14. Determine the concentration of RNA as described in step 6.

Treat samples with DNase

15. Add 1 µl of 10× DNase buffer and 2 U RNase-free DNase I to 2 µg of RNA and bring the volume to 10 µl with DEPC-treated water. Incubate 5 min at 37°C.

16. Terminate reaction by adding 1 µl of 25 mM EDTA, pH 8.0, and heating 10 min at 65°C.

17. Reprecipitate RNA with lithium chloride as described in steps 4 to 6.
Prepare cDNA
18. Prepare two sets of the following RNA/primer mixture, labeled RT⁺ and RT⁻, in 0.5-ml microcentrifuge tubes and incubate 5 min at 65°C then place on ice at least 1 min:

- 2 μg sample RNA
- 1 μl 10 mM 4dNTP mix
- 1 μl 50 ng/μl random hexamers
- DEPC-treated H₂O to 10 μl.

19. Prepare the following master mix by adding each component in the indicated order. Multiply the following volumes by the total number of samples, adding 1 or 2 to allow for pipetting losses:

- 2 μl 10× RT-PCR buffer
- 4 μl 25 mM MgCl₂
- 2 μl 0.1 M DTT
- 1 μl 40 U/μl RNase inhibitor.

20. Add 9 μl master mix to each RNA/primer mixture from step 18, mix gently, microcentrifuge briefly, and incubate 2 min at 25°C.

21. Add 1 μl of 50 U/μl Superscript II RT to each tube of the RT⁺ mixes, mix, and incubate 10 min at 25°C, followed by 50 min at 42°C and 15 min at 70°C to terminate the reaction. Chill tubes on ice and collect reactions by brief microcentrifugation.

22. Add 1 μl of 2 U/μl RNase H to each tube and incubate 20 min at 37°C.

Carry out PCR amplification
23. Prepare the following master mix. Multiply the following volumes by the total number of sample adding 1 or 2 to allow for pipetting losses:

- 5 μl 5 μM 5′ primer
- 5 μl 5 μM 3′ primer
- 2 μl 10 mM dNTP mix
- 5 μl 10× PCR buffer
- 2 μl 50 mM MgCl₂
- 0.5 μl 2.5 U Platinum Taq DNA polymerase
- 28.5 μl sterile H₂O.

Vortex the reaction mix and dispense 48 μl into each labeled 8-tube strip MicroAmp optical tube.

24. Add 2 μl of cDNA samples from step 22 to each tube containing the reaction mix and cap tubes with a strip of MicroAmp caps.

25. Carry out the amplifications in a thermal cycler with the following program:

- 1 cycle: 2 min 94°C (denaturation)
- 26 to 32 cycles: 30 sec 94°C (denaturation) 30 sec 60°C (annealing) 45 sec 72°C (extension)
- 1 cycle: 10 min 72°C (final product extension).

26. Maintain samples at 4°C after cycling.

*Samples may be stored at ~20°C until analyzed by the methods described in Basic Protocol 1, steps 15 to 22.*
REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.

Cell lysis buffer
- 0.001% (v/v) Triton X-100
- 0.0001% (w/v) SDS
- 10 mM Tris-Cl, pH 8.0 (APPENDIX 2A)
- 1 mM EDTA (APPENDIX 2A)

Store indefinitely in aliquots at 4°C

Hybridization solution
- 1× SSC
- 1× Denhardt solution (APPENDIX 2A)
- 1% (w/v) SDS

Store at −20°C, up to 6 months

Lithium chloride precipitation solution
- 7.5 M lithium chloride
- 50 mM EDTA

Store indefinitely at 4°C

PCR buffer and RT-PCR buffer, 10×
- 200 mM Tris-Cl, pH 8.4 (APPENDIX 2A)
- 500 mM KCl

Store indefinitely at −20°C

COMMENTARY

Background Information
The analysis of HIV DNA and RNA by PCR amplification represents one of the most widely used methods of detecting and quantifying the virus present in susceptible cell populations. The assays described herein and their variations can be used to evaluate numerous aspects of HIV infection, including the dynamics of HIV replication (Ho et al., 1995; Wei et al., 1995), the kinetics of reverse transcription (Zack et al., 1990), the presence of latent reservoirs (Chun et al., 1997), and the distribution of quasispecies (Delwart et al., 1993). The various applications can give a wealth of information; however, success relies on judicious decisions, such as the source of infected cells, the choice of primer/probe combinations, and which type of PCR amplification to perform.

The use of PCR technology to monitor the viral burden during HIV infection provides a level of sensitivity and accuracy that can greatly surpass other viral markers, such as the level of p24 antigen in plasma or culture supernatant (UNIT 12.5) and RNA in situ hybridization on tissue sections (UNIT 12.8). Two basic protocols for determining levels of HIV DNA and RNA extracted from infected cells are described in this unit. There are numerous protocols for evaluating HIV RNA levels in the plasma that have not been addressed in this unit but are available on line (see Internet Resources). Recently, there have also been tremendous developments in the area of automated PCR technology, such as real-time PCR and the use of novel methods of detection such as molecular beacons (Lewin et al., 1999). However, since these developments are relatively new, difficult to adapt to the heterogeneity and complexity of HIV, and presently very expensive, they are also beyond the scope of this unit.

There are limitations to the application of PCR technology in HIV research; the most important are the limits on interpretations drawn from the amplification data. It is well accepted that levels of proviral DNA are of limited value in trying to assess ongoing viral replication. The information that HIV RNA amplification yields is complicated by the variety of HIV RNA species that can be present in an infected cell. These include full-length genomic RNA to be packaged into virions, unspliced (US) and singly spliced mRNA of the structural genes, and multiply-spliced (MS) mRNA of the regulatory genes. While the authors have restricted Basic Protocol 2 to the quantitative analysis of full-length and
unspliced messages, other protocols and materials are available for the analysis of MS HIV mRNA (Saksela et al., 1993).

**Critical Parameters and Troubleshooting**

Successful quantitative analyses of HIV DNA and RNA in test specimen rely on quality of the template preparations, choice of primers/probe, and use of adequate standards. While it is true that DNA-PCR can be performed directly on cell lysates (see Basic Protocol 1, alternate b steps), a preparation of quality genomic DNA ensures better quantification and reproducibility, and minimizes the amplification of nonspecific background. Selection of appropriate primer pairs and probe is also critical for high-specificity amplification; however, there is an additional concern in HIV PCR, namely choosing primers from conserved regions of the HIV genome. The primer/probe sets described in Table 12.6.1 have proven to be suitable for amplifying HIV DNA and RNA from diverse sources. The AIDS Research and Reference Reagent Program (see Internet Resources) also offers conserved HIV-1 primer pairs that can be used to amplify regions in the LTR as well as gag and env genes.

For DNA-PCR, standardization is achieved by amplifying test DNA in parallel with serial dilutions of DNA derived from ACH-2, a T cell clone containing one integrated copy of HIV per cell. For RT-PCR, the choice of standards presents a more difficult decision in light of the intrinsic variability in cellular mRNA expression. An adequate standard for determining absolute copy numbers in test samples is to conduct parallel RT-PCR reactions with serial dilutions of pure HIV RNA transcripts. These can be generated by in vitro transcription of a reference plasmid, such as the one described in Basic Protocol 2, which contains a 647-bp insert of HIV. Alternatively, HIV-1 quantification standards may be obtained from the AIDS Research and Reference Reagent Program. Together with adequate phosphor imaging software, these analyses can give reproducible quantitative results.

Control for input DNA or RNA quantity and quality is achieved by parallel amplification of a reference cellular gene, such as β-globin or β-actin, shown in Figures 12.6.1 and 12.6.2, respectively. Additional precautions are incorporated into protocols to control for false-positive PCR results. For HIV-1 DNA-PCR, as shown in the last two lanes of Figure 12.6.1, negative controls include extraction of genomic DNA from HIV-negative cells and a reaction tube where DNA is replaced with water. For HIV-1 RT-PCR, shown in Figure 12.6.2, negative controls include extraction of RNA from HIV-negative cells and omission of reverse transcriptase during cDNA preparation for each test sample.

*Figure 12.6.2* PCR amplification of in vitro transcribed and cell-associated HIV RNA. Negative controls include omission of reverse transcriptase (RT) during cDNA preparation and RT-PCR on RNA extracted from HIV-negative cells (HIV−).
The protocols described in this unit have been optimized for the particular primer/probe sets listed in Table 12.6.1. To adapt protocols to other primer/probe sets it may be necessary to modify certain parameters, such as concentration of MgCl₂, annealing temperature, and number of cycles during the amplification phase. Purity of preparations can also affect results, especially the integrity of RNA in the RT-PCR protocol.

The authors recommend Southern hybridization for the detection of PCR products because it provides a greater degree of specificity and clarity of band resolution compared to alternative methods such as direct liquid hybridization or labeling one of the primers.

**Anticipated Results**

The frequency of infected cells in HIV-infected individuals ranges from undetectable to 1/10, depending on the stage of disease, source of cells, and anti-retroviral regimen. Therefore, an amount of cellular DNA corresponding to >10⁵ cells must be analyzed when performing DNA-PCR on samples ex vivo. Preparations derived from cells that have been infected in vitro usually contain considerably more proviral DNA; however, the amount of input DNA to use will depend on the multiplicity of infection used and the type of cells infected (UNIT 12.2).

For US RT-PCR, frequencies of HIV RNA can vary. Therefore, reactions are typically carried out on 0.1 to 1 µg of RNA. It is very difficult to approximate how many cells these quantities of RNA represent because the amount of RNA per cell will vary tremendously depending on the level of activation at the time of extraction. For detection of MS HIV-1 mRNA, nested PCR strategies are generally recommended (Vesanen et al., 1997).

**Time Considerations**

DNA-PCR involves three steps: preparation of genomic DNA, amplification of DNA, and Southern hybridization. Genomic DNA should be prepared the day before the DNA-PCR step to allow overnight rehydration of the pellet. The DNA-PCR step takes several hours, running the gel can be carried out in an afternoon, and transfer of DNA to nylon membrane is an overnight step. The hybridization steps and exposure of the membrane can be completed within the next 24 hr. Altogether, including the overnight incubations, Basic Protocol 1 takes 2 to 3 days to perform. For RT-PCR, the preparation of RNA and cDNA adds another half day of work. The preparation of RNA transcripts by in vitro transcription can take up to 8 hr; however, stock preparations can be prepared in large quantities and aliquots may be stored at −70°C for several months.

**Literature Cited**


**Internet Resources**

http://www.niaid.nih.gov/daids/vir_manual

*Provides many detailed techniques for those who wish to use commercial kits to determine HIV RNA levels in plasma samples and HIV proviral DNA levels in cells.*

http://www.aidsreagent.org

*Offers a wealth of HIV-related reagents to the scientific community.*

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