# PCR PROTOCOLS

## A GUIDE TO METHODS AND APPLICATIONS

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**ACADEMIC PRESS, INC.**
*Harcourt Brace Jovanovich, Publishers*
San Diego New York Berkeley Boston London Sydney Tokyo Toronto

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# 40

# DETECTION OF HUMAN IMMUNODEFICIENCY VIRUS

David E. Kellogg and Shirley Kwok

Serological assays provide a rapid and sensitive procedure to screen blood and blood products for the presence of antibodies to human immunodeficiency virus type 1 (HIV-1). Samples that are repeatedly reactive are retested for HIV antibodies with supplemental tests such as Western blot, immunofluorescent assay (IFA), or radioimmunoprecipitation assay (RIPA). Although these tests are sensitive and specific for HIV, direct detection of the virus would be desirable for a number of reasons.

First, because of the latency associated with HIV, a direct assay may assist in the identification of individuals who are infected with the virus but have not seroconverted. Second, detection of HIV infection in newborns would be facilitated. Because maternal antibodies can persist up to 15 months, it is difficult to differentiate between maternal and infant antibodies. Third, direct viral detection should contribute significantly in clarifying the status of individuals with indeterminate Western blot patterns in the high- and low-risk communities. Fourth, a direct assay for the virus would provide a means to monitor both latent and actively replicating virus in patients on therapeutic drugs.

*PCR Protocols. A Guide to Methods and Applications*

Because of the low level of circulating free virus, direct detection of HIV in patient samples is difficult without *in vitro* propagation. Even with co-cultivation, the successful recovery of HIV varies from 10 to 75%, although some laboratories have reported close to 100% virus recovery from infected individuals (Jackson, Kwok, Sninsky, Sannerud, Rhame, Henry, Simpson, and Balfour, manuscript submitted). Virus culturing is time-consuming and expensive, and the procedures used to monitor viral growth are laborious. Often, the results from virus culturing are inconclusive. Reverse transcriptase assays, for example, are not specific for HIV. Many of the tests performed on cultured cells, such as electron microscopy, immunofluorescence, and Southern blot analysis on extracted DNA, lack sensitivity.

The heterogencity of HIV-1 has been extensively documented (Shaw *et al.* 1984; Saag *et al.* 1988; Goodenow *et al.* 1989; Meyerhans *et al.*, in press). To ensure efficient detection of all HIV-1 variants, only highly conserved regions of the viral genomes were targeted for amplification. (Nucleic acid alignments were provided by Los Alamos National Laboratory.) Two primer pairs have been extensively used for the analysis of HIV-1-infected samples. One primer pair, designated SK38 and SK39 (Ou *et al.* 1988; Kwok *et al.* 1989) amplifies a 115-bp region of *gag* of HIV-1. The second primer pair, SK145 and SK101, amplifies a 130-bp region of *gag* that is conserved among the HIV-1 isolates and HIV-2 (isolate ROD). It should be noted that SK101 has a single-base mismatch (9 nucleotides from the 5′ end) with the consensus sequence. Although this primer when coupled with SK145 amplified efficiently, we have since replaced it with SK150 (see Table 1). The probes for the SK38–39 and SK145–101 products are SK19 and SK102, respectively. The sequences of the primers and probes are presented in Table 1.

Because of the exquisite sensitivity of PCR, it is crucial that special care be taken to avoid cross-contamination of samples or carryover of amplified products that can result in false-positive results. Because of the generally small number of HIV molecules in a clinical specimen (potentially as few as 1 : 10,000 or less in peripheral blood), the amplification protocols and detection methods described here have been optimized to provide maximum sensitivity. The demand placed on PCR to detect small numbers of molecules necessitates the avoidance of even minute quantities of contaminating target molecules.

The guidelines for avoiding false positives with PCR have been described (Kwok and Higuchi 1989; Chapter 17) but merit reiteration.

**Table 1**
Primers and Probes for the Amplification and Detection of HIVs

| Oligonucleotide designation (orientation) | Sequence (5′-3′) | Virus | Position[a] |
|---|---|---|---|
| SK38 (+) | ATAATCCACCTATCCCAGTAGGAGAAAT | HIV-1 | 1541-1578 |
| SK39 (−) | TTTGGTCCTTGTCTTATGTCCAGAATGC | HIV-1 | 1665-1638 |
| SK19 (+) | ATCCTCGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC | HIV-1 | 1595-1635 |
| SK145 (+) | AGTGGGGGACATCAAGCAGCCATGCAAAT | HIV-1 | 1366-1395 |
| SK101 (−) | CCTATGTCAGTTCCCCTTGGTTCTC | HIV-2 | 1150-1121 |
| | | HIV-1 | 1506-1482 |
| | | HIV-2 | 1258-1234 |
| SK150 (−) | TGCTATGTCACTTCCCCTTGGTTCTC | HIV-1 | 1507-1480 |
| | | HIV-2 | 1259-1232 |
| SK102 (+) | GAGACCATCAATGAGGAAGCTGCAGAATGGGAT | HIV-1 | 1403-1435 |
| | | HIV-2 | 1158-1190 |

[a]HIV-1 isolate SF2, GenBank Accession number K02007
HIV-2 isolate ROD, GenBank Accession number M15390

Perhaps most important is the use of a dedicated area that is free of PCR product for the preparation of amplification reactions. In our laboratories, biological hoods equipped with UV lights that are turned on between use serve as efficient containment areas. It is critical that amplified products are kept away from this area. Other precautions include the use of positive-displacement pipettes with disposable capillaries and pistons, dispensing of reagents into small aliquots, and the preparation of master mixes whenever possible to minimize handling. Equally important, an investigator must be cognizant of manipulations that can potentially lead to contamination. Precautions must be taken not only in the preparation of the amplification reactions but in all aspects of sample handling, from collection to DNA extraction to PCR amplification.

The protocols that follow describe the procedures used for extraction of DNA, the conditions used in the amplification of HIV, and the analysis of PCR product by oligomer hybridization (OH). Although analysis of PCR product from high-copy-number targets can be achieved by visualization on agarose gels after ethidium bromide staining, Southern blot analysis, or dot blot analysis, detection of low-copy targets is best achieved by the OH analysis. The procedure is at least 25 times more sensitive than Southern blot analysis. When PCR is coupled with OH, as few as 18 copies of HIV molecules (prior to amplification) in a background of 1 $\mu$g genomic DNA (the equivalent of 150,000 cells) can be detected by using 30 cycles of amplification (Kwok *et al.* 1989).

## Protocols

### Reagents

| | |
|---|---|
| Solution A | 100 m*M* KCl<br>10 m*M* Tris–HCl (pH 8.3)<br>2.5 m*M* MgC12 |
| Solution B | 10 m*M* Tris–HCl (pH 8.3)<br>2.5 m*M* $MgCl_2$<br>1% Tween 20<br>1% NP40 |

Add self-digested Proteinase K to 120 $\mu$g/ml to solution B just before use.



| | |
|---|---|
| 10× *Taq* buffer | 500 m*M* KCl<br>100 m*M* Tris–HCl (pH 8.3)<br>25 m*M* $MgCl_2$<br>0.1% gelatin |

### Sample Preparation

All samples, including clinical specimens, must be handled and disposed of as if they contain a transmissible infectious agent. Lab coats, masks, safety glasses, and gloves should be worn. Wash hands thoroughly when work is completed.

1. Collect 5 to 10 ml of whole blood by venipuncture in either an ACD (acid citrate dextrose) or heparin tube.
2. Pipette 5 ml of Ficoll-Hypaque into a conical centrifuge. Carefully layer an equal volume of whole blood on top of the Ficoll-Hypaque.
3. Centrifuge at room temperature for 30 minutes at 2000 rpm in a swinging bucket rotor.
4. Use a sterile transfer pipette and collect the lymphocyte/monocyte fraction (opaque band located at the gradient interface) into a 2-ml Sarstedt tube.
5. Add phosphate-buffered saline (PBS) to the sample to 2 ml. Mix and spin in a microcentrifuge at 2500 rpm for 10 minutes to pellet the cells.
6. Remove the PBS. Resuspend cells in 2 ml PBS. Mix and spin.
7. At this point, DNA can be extracted from the pelleted cells or the cells can be frozen in RPMI plus 50% fetal calf serum and 10% DMSO at −70°C or in liquid nitrogen.

### DNA Extraction

This procedure describes a method for the rapid extraction of DNA from peripheral blood mononuclear cells (Higuchi 1989; Jackson *et al.* 1989). The cells are lysed in *Taq* buffer containing Tween 20 and NP40 and digested with Proteinase K. The DNA extracted with this procedure can be amplified directly, thereby obviating the need for a phenol/chloroform extraction step.

1. If cells have been stored frozen, quick thaw at 37°C. Wash once with 1 to 2 ml cold RPMI plus 10% fetal calf serum. Pellet by centrifugation at 2500 rpm for 10 minutes. Wash twice more with PBS.
2. Resuspend the cells to a concentration of approximately $6 \times 10^6$ cells/ml in solution A.
3. Add an equal volume of solution B.
4. Incubate at 60°C for 1 hour.
5. Inactivate the Proteinase K by placing the tube in a 95°C water bath for 10 minutes.
6. Cool to room temperature and refrigerate.

## DNA Amplification

Each sample is initially analyzed with a primer pair that amplifies a region of HLA-DQ alpha to determine if the DNA is of sufficient quality for PCR amplification (see Chapter 32). Samples are then amplified in duplicate with each of the HIV primer pairs. The conditions for optimal amplification need to be determined for each primer pair. The conditions recommended below were optimized for SK38–39 and SK145–101. Each amplification is performed in a volume of 100 μl.

1. Add 50 μl of mineral oil to 0.5-ml microcentrifuge tubes.
2. Add 50 μl of a reaction mix that consists of:
   - 5 μl of 10× *Taq* buffer
   - 50 pmol upstream primer
   - 50 pmol downstream primer
   - 2 μl of 10 μ*M* dNTPs
   - 2 units of *Taq* polymerase (Perkin-Elmer Cetus)
   - glass-distilled water to 50 μl.

   The amount of 10× *Taq* buffer used will depend on the buffer in which the DNA is resuspended. The DNAs as prepared above are already in 1× *Taq* buffer.
3. Add 50 μl of sample DNA to each tube (should contain about 1 to 2 μg of DNA [the equivalent of 150,000 to 300,000 cells]). To minimize aerosolization, dispense the samples under the oil. Cap each sample after the addition of DNA before proceeding to the next.



4. Spin the tubes for a few seconds in a microcentrifuge to bring the liquid to the bottom.
5. Place the samples in a DNA Thermal Cycler (Perkin-Elmer Cetus). Cycle as follows:
   a. Denature at 95°C for 30 seconds
   b. Anneal at 55°C for 30 seconds
   c. Extend at 72°C for 1 minute

   Samples are amplified for 30 cycles. At the completion of the 30 cycles, the DNA is extended for an additional 10 minutes at 72°C.
6. Store samples at 4°C until ready to assay.

## Detection of Amplified Products by Oligomer Hybridization (OH)

OH is a procedure whereby a $^{32}$P-end-labeled probe hybridizes in solution to one strand of the amplified product. The probe-target duplex is separated from the unhybridized probe by gel electrophoresis and is then autoradiographed.

1. Add 50 μl of mineral oil to 0.5-ml microcentrifuge tubes.
2. Add 10 μl of a probe mix consisting of:
   - 4 μl 60 m*M* NaCl
   - 1 μl 40 m*M* EDTA (pH 8.0)
   - 0.2 pmol $^{32}$P-end-labeled probe
   - glass-distilled water to 10 μl

   The oligonucleotide probes are end labeled with [$^{32}$P]ATP (6000 mCi/mmol) to a specific activity of 1.5 to 3 μCi/pmol by using polynucleotide kinase (Maniatis *et al.* 1982). The end-labeled oligonucleotides are separated from the unincorporated ATP by spin column dialysis with G50 Sephadex (Maniatis *et al.* 1982).
3. Add 30 μl of amplified DNA.
4. Mix and centrifuge briefly to bring down the content.
5. Denature DNA in a 95°C bath for 5 minutes.
6. Anneal the probe and target sequences by incubating the tubes at 55°C for 15 minutes.
7. Add 10 μl bromophenol blue/xylene cyanol dye mix to each tube.

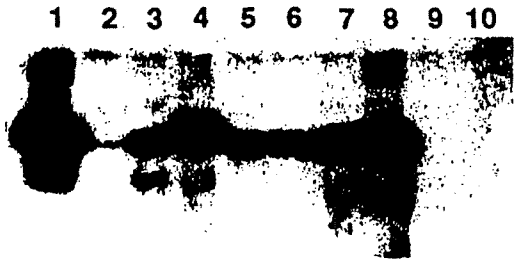


**Figure 1** Representative OH analysis of samples amplified with SK38/39 and probed with SK19. Lanes 1–5 and 7–8 represent DNAs from HIV-seropositive individuals; lane 6, positive control; lane 9, seronegative individual; and lane 10, a reagent control.

8. Extract the mineral oil with 100 μl chloroform. The dye and sample will float to the top upon the addition of chloroform.
9. Load 25 μl of the sample onto a 10% polyacrylamide minigel.
10. Run in 1× TBE buffer at 100 V until the bromophenol blue dye approaches the bottom of the gel.
11. Excise the unhybridized probe from the gel by slicing just below the xylene cyanol front. Dispose of the bottom gel strip in the radioactive waste.
12. Blot the gel with tissue. Wrap the gel in plastic and expose to XAR 50 film overnight with an intensifying screen.
13. Develop the autoradiograph. The presence of a probe-target duplex is indicative of HIV infection. See Fig. 1 for a representative OH analysis.

## Interpretation of Results

The HLA-DQ alpha amplifications serve as controls for these analyses. A sample can be interpreted as negative only if analysis of the DNA with the HLA primers indicates the DNA is suitable for amplification. The amount of HLA PCR product also provides a crude approximation of the number of cells being analyzed. The absence of an HLA product visible by ethidium bromide staining indicates either a presence of an inhibitor of PCR or an insufficient amount of



cellular DNA. HIV-amplified products have been detected in the absence of ethidium bromide-stainable HLA amplification products, suggesting that although the amount of cellular DNA was small, the HIV molecules were at a level sufficient for detection by OH analysis. However, the absence of evidence for chromosomal DNA prevents the ability to exclude PCR carry-over as a source of HIV sequence detected.

| Primer Pair | | |
|---|---|---|
| SK38–39 | SK145/150 | Interpretation |
| +/+ | +/+ | Positive |
| −/− | −/− | Negative |
| +/+ | +/− | |
| −/+ | +/+ | |
| +/+ | −/− | Repeat, if pattern repeats: |
| −/− | +/+ | positive |
| +/− | +/− | |
| −/− | −/+ | Repeat, if pattern repeats: |
| +/− | −/− | indeterminate |

## Applications

Primer pairs SK38–39 and/or SK145–101 have been successfully used in the detection of HIV in greater than 96% antibody-positive individuals (Ou *et al.* 1988; Jackson *et al.*, submitted; Lifson *et al.*, in press). In addition, the identification of HIV by PCR has contributed to other areas of AIDS diagnostics and research. First, the procedure has been successfully used to test infants born to seropositive mothers (Laure *et al.* 1988; Rogers *et al.* 1989; Chadwick *et al.*, in press). Since maternal antibodies can persist in the infants for up to 15 months, diagnosis of HIV infection in babies by serology is hampered. In several studies to date, detection of HIV by PCR in babies has correlated well with disease progression. Second, the procedure has been used to resolve the infection status of individuals with indeterminate serology (Jackson, MacDonald, Cadwell, Sullivan, Hanson, Sannerud, Stramer, Fildes, Kwok, Sninsky, Bowman, Polesky, Balfour, and Osterholm, submitted). Studies on low-risk individuals with indeterminate patterns suggest these individuals are not infected. Third, the procedure has detected HIV viral sequences

in seropositive symptomatic individuals who were negative by other direct detection assays, including virus culture (Ou *et al.* 1988; Jackson *et al.* 1988). Fourth, the procedure has identified infection in a small number of high-risk individuals prior to seroconversion (Imagawa *et al.* 1989). Fifth, when PCR was used to screen seronegative sexual partners of HIV-1-infected hemophiliacs, none (0/22) was found to harbor HIV sequences, suggesting that the frequency of HIV-1 infection in antibody-negative sexual partners is probably very low. Sixth, PCR was used to confirm the first case of HIV-2 in a West African undergoing treatment in the United States (CDC 1988). In addition, the first documented case of a West African who was co-infected with HIV-1 and −2 was confirmed by PCR (Rayfield *et al.* 1988). Finally, PCR has been used to evaluate viral heterogeneity in HIV-1 isolates (Goodenow *et al.* 1989; Meyerhans *et al.*, in press).

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