8
Direct Detection of HIV Infection Using Nucleic Amplification Techniques

GERALD SCHOCHETMAN and JOHN J. SNINSKY

Although infectious viral particles of human immunodeficiency virus (HIV) encapsidate single-stranded RNA (ssRNA) as the genetic information, the viral life cycle includes a compulsory conversion to double-stranded DNA (dsDNA), termed the provirus, which becomes integrated into the host cells' chromosomes (see Chap. 2 for more details of the virus life cycle). The integrated provirus remains associated with the cellular chromosomal DNA for the life of the infected cell. Furthermore, the integrated provirus can either actively transcribe the genes for the structural proteins of the virus, which results in the assembly and release of infectious virions, or by selective transcription of only the complex array of viral regulatory genes remain transcriptionally constrained and thereby not release viral particles. The latter condition is frequently referred to as the "latent state." Because proviral DNA is present regardless of the transcriptional state of the cell, early efforts targeted to direct detection of the virus used proviral DNA as a template.

Because of the low frequency of HIV-1-infected peripheral blood mononuclear cells (PBMCs) in a seropositive person,[1] conventional molecular biology techniques[2] were not sensitive enough to routinely detect and characterize HIV proviral DNA directly from patients' lymphocytes. Therefore HIV proviral DNA must first be amplified to detectable levels using the polymerase chain reaction (PCR). Prior to PCR, successful direct detection of HIV-1 required culturing the virus. The ability of PCR to amplify HIV sequences several orders of magnitude in vitro has obviated the need to propagate the virus for direct detection.

For the study of HIV infection and acquired immunodeficiency syndrome (AIDS), PCR has demonstrated both clinical and research utility for: (1) direct detection and quantitation of HIV DNA and RNA from cells of infected persons; (2) detecting infected persons during the window period (i.e., prior to the generation of HIV-specific antibodies); (3)

G. Schochetman et al. (eds.), *AIDS Testing*

resolving the infection status of individuals with an indeterminate Western blot assay; (4) screening neonates for HIV infection; (5) distinguishing HIV-1 from HIV-2 infections; and (6) defining the patterns of transmission and evolution of the virus throughout the population.

PCR Methodology

The PCR process was originally developed as a technique for the in vitro amplification of targeted DNA sequences.[3–6] For PCR, sample preparation has employed separation of mononuclear from polymorphonuclear cells of the blood using a Ficoll-Hypaque gradient. After preparation of the PBMCs the cells are incubated with nonionic detergents and finally treated with proteinase K. Heat treatment (95°C for 15 minutes) is used to inactivate the proteinase K. The resulting DNA preparation is ready for PCR. Usually 1 μg of DNA (equivalent to about 150,000 mononuclear cells) (Table 8.1) is used per PCR reaction. PCR is a repetitive process consisting of three distinct steps (Fig. 8.1): (1) denaturation of dsDNA; (2) annealing of specific primers; and (3) extension of annealed primers. Because of the complementary and antiparallel nature of DNA, ssDNA can also serve as a template for amplification. When amplification of a specific RNA sequence is required, a DNA copy of the RNA sequence is produced using the enzyme reverse transcriptase prior to PCR amplification of the resulting DNA.[7,8] After the PCR process, a variety of techniques can be used to detect the amplified DNA sequences. Although the amplified DNA is of a defined size and can sometimes be visualized after gel electrophoresis, this method cannot provide definitive identification of the product. The confirmation of am-

TABLE 8.1. Numbers to consider for HIV infection.

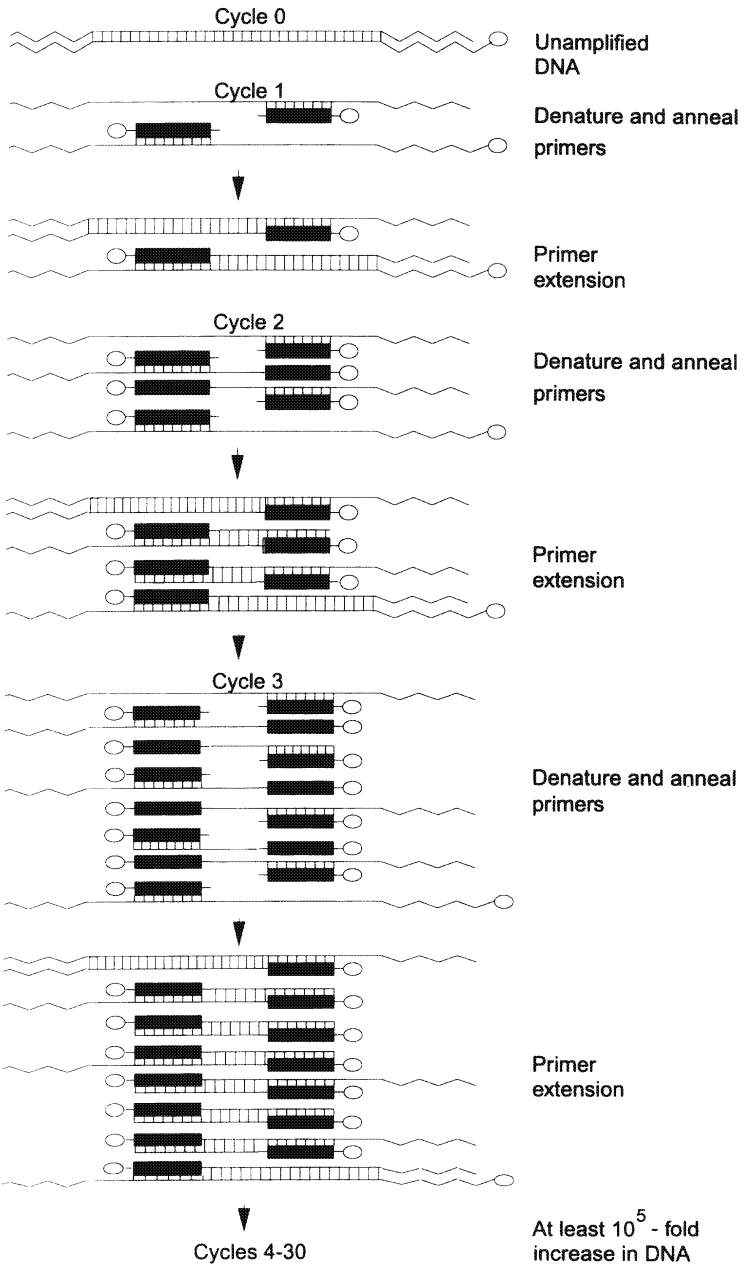
General
Blood volume: 5.6 liters in a 70 kg person
White blood cells: 500,000/65 μl whole blood
White blood cells:
Polymorphonuclear cells (70%), 350,000
Mononuclear cells (30%), 150,000
Mononuclear cells
Monocytes (10%), 15,000
Large granular lymphocytes (LGLs) (10%), 15,000
B cells (10%), 15,000
T cells (70%), 105,000
T cells
T8 (suppressor) cells (35%), 37,000
T4 (helper) cells (65%), 68,000

plification of HIV DNA includes hybridization of a portion of the amplified DNA to a synthetic DNA probe that is complementary to a portion of the amplified DNA sequence. The probe can be labeled by a variety of means, isotopic (radioactive) or nonisotopic (colorimetric or chemiluminescent) (Figs. 8.2, 8.3, 8.4).

PCR Test System

A PCR system for qualitatively detecting HIV-1 has been developed as a simplified diagnostic test kit[9] that provides the inherent sensitivity and specificity of any research PCR assay in a user-friendly system format. The kit consists of three components: a specimen collection and preparation kit, an amplification kit, and a detection kit. All components are premixed for easy and reliable amplification and detection using the well known microwell plate colorimetric format. The test specimen for this kit is whole blood, and ambient temperature storage allows convenient shipping of clinical specimens. The whole blood lysis procedure is a simple method that requires only 0.5 ml of blood. A modified procedure using 0.1 ml of blood has also been developed for neonates, from whom virus culture requires too large a volume of blood. For the whole-blood lysis procedure red blood cells are selectively lysed while the leukocytes remain intact. After the leukocytes are pelleted and then washed several times, DNA is extracted from the final cell pellet. The entire procedure takes less than 2 hours. A 50 µl aliquot (approximately 200,000 cells) is used to amplify proviral HIV-1 DNA. Biotinylated primers used to amplify the HIV-1 DNA represent a 142 basepair sequence of the *gag* gene region. These primers have reportedly been tested on thousands of specimens and have been shown to amplify all samples efficiently, detecting virtually 100% of HIV-1 isolates worldwide.

During the early days of PCR, the potential to amplify minute amounts of contaminating material from previous reactions resulted in problems of false-positivity. Extremely small amounts of amplified target DNA or amplicon (10^{-8} µl) from a previous amplification contaminating a reaction tube may result in a false-positive reaction. To solve this problem, deoxyuridine 5′-triphosphate (dUTP) was incorporated into the amplification reaction, making it possible to distinguish amplicon DNA from native T-containing DNA found in specimen cellular DNA (Fig. 8.5). The active enzyme uracil-*N*-glycosylase (UNG) selectively recognizes and destroys the U-containing amplicons while leaving target DNA unaffected. Because all amplified products are synthesized with dUTP rather than deoxythymidine 5′-triphosphate (dTTP), they are susceptible to UNG activity in subsequent reactions. The development of this procedure has been critical to the successful transfer of PCR from the research laboratory to the clinical laboratory.


Cycle 0
Unamplified DNA
Cycle 1
Denature and anneal primers
Primer extension
Cycle 2
Denature and anneal primers
Primer extension
Cycle 3
Denature and anneal primers
Primer extension
Cycles 4-30
At least 10^5 - fold increase in DNA

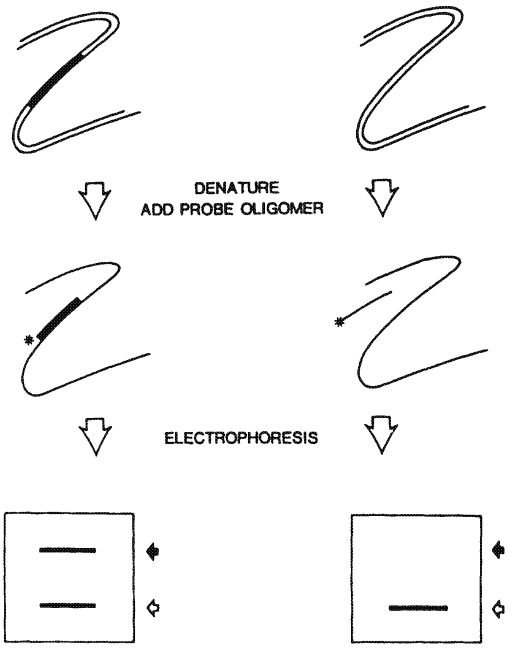


FIGURE 8.2. Detection of the amplified DNA involves hybridization of a portion of the amplified DNA product to a radioactive ^{32}P-labeled synthetic probe complementary to a portion of the amplified sequences followed by gel analysis and autoradiography.

All reagents necessary for amplification are provided in the kit; it is only necessary to add UNG to the premixed master mix vial. An aliquot of premixed master mix is then added to the appropriate number of amplification tubes. Subsequently, an aliquot of prepared specimen is added to the appropriate tube, the tube is capped, and the rack of reaction tubes is placed in the thermal cycler. At the end of the amplification cycle, the reaction tubes should be held at 72°C until the dena-

FIGURE 8.1. Polymerase chain reaction (PCR), which is a repetitive process that includes denaturation of double-stranded DNA (dsDNA), annealing of primers, and extension of bound primers. One PCR cycle usually takes about 3 minutes, and the cycle is repeated many times (usually 25–35 times). The dsDNA is first heated to 95° to 100°C to separate the strands of the duplex. During the subsequent annealing phase, oligonucleotide primers hybridize to the dissociated HIV DNA. Each primer is complementary to one of the original DNA strands, either the 5′ or the 3′ side of the sequence of interest. After annealing, a thermostable DNA polymerase from *T. aquaticus* (*Taq*) is used to catalyze the synthesis of new strands of DNA that are complementary to the intervening sequences primed by the opposing oligonucleotide primers.

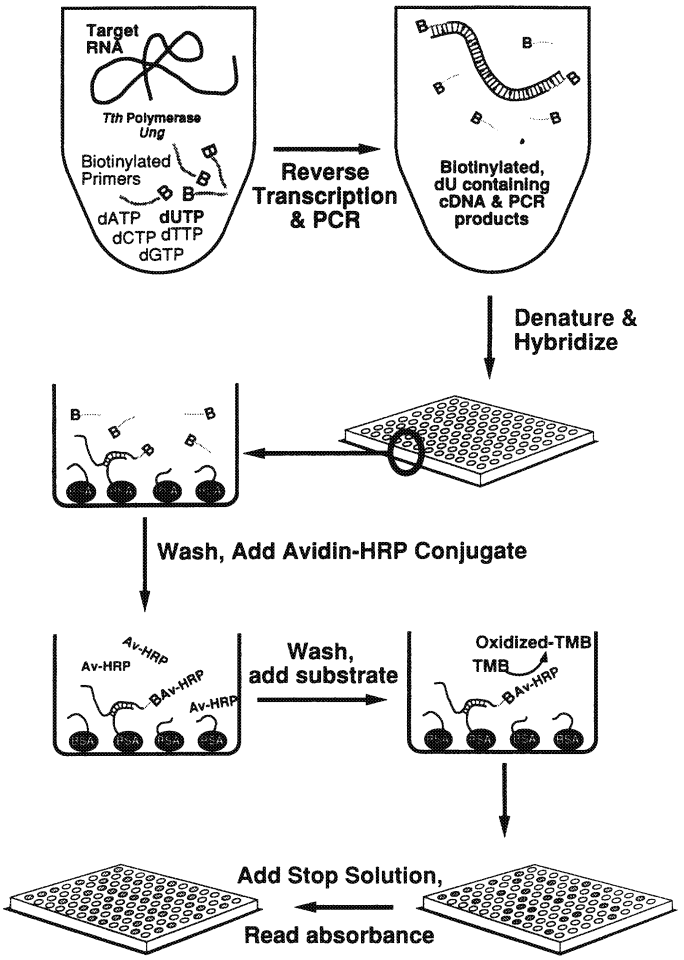


FIGURE 8.3. Detection of the amplified DNA involves the use of biotinylated primers to generate a tagged amplified DNA product, which is then hybridized to an immobilized probe complementary to a portion of the amplified sequences. This step is followed by incubation with avidin conjugated to horseradish peroxidase. This standard EIA format in a microplate yields a colorimetric readout for positive samples (C. Silver, M. Sulzinski, E. Dragon, and M. Longiaru, personal communication).

turation solution is added. Detection is based on the enzyme immunoassay (EIA)-like colorimetric microwell plate format (Fig. 8.3). Amplicons are captured by bovine serum albumin (BSA)-conjugated DNA probes specific for HIV that have been coated on the bottom of the well of a microwell plate. The specificity of the detection derives from the

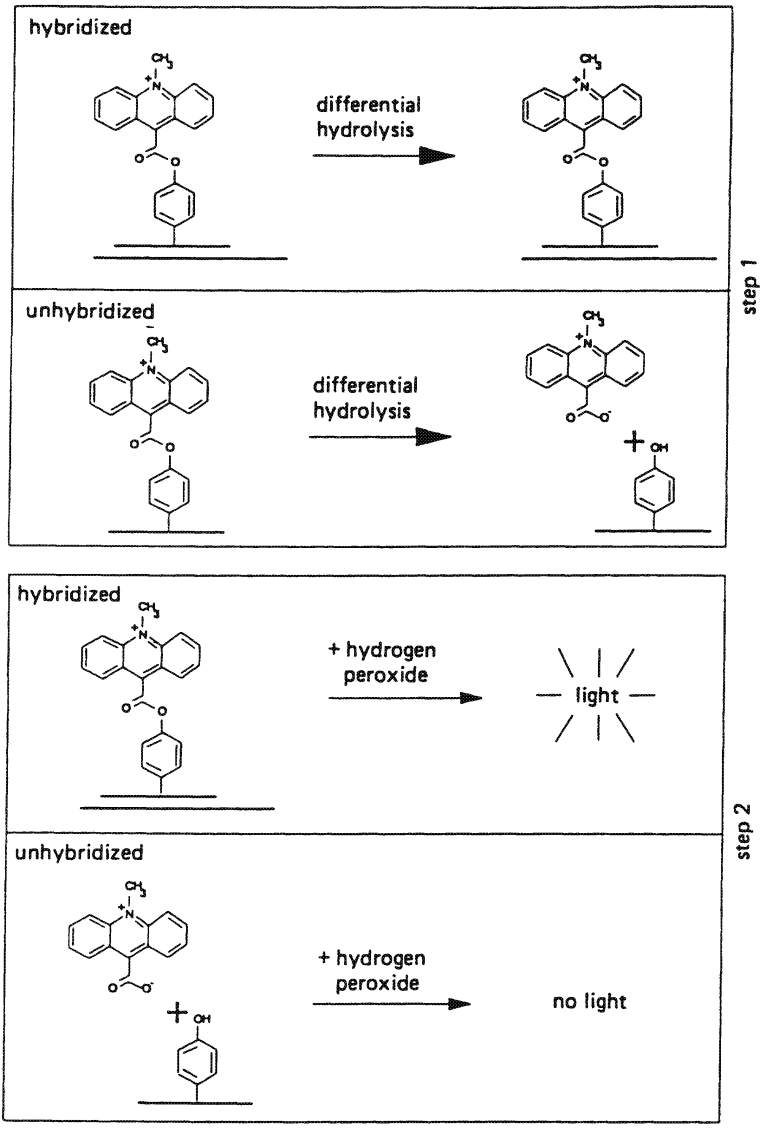


FIGURE 8.4. Detection of the amplified DNA involves hybridization of a portion of the amplified DNA product to an acridinium-labeled synthetic probe complementary to a portion of the amplified sequences followed by differential alkaline hydrolysis. The acridinium attached to the hybridized probe is relatively resistant to alkaline degradation and can chemiluminesce after oxidation by the addition of hydrogen peroxide. In contrast, the acridinium attached to the unhybridized probe is highly sensitive to alkaline degradation and loses its ability to chemiluminesce almost immediately.

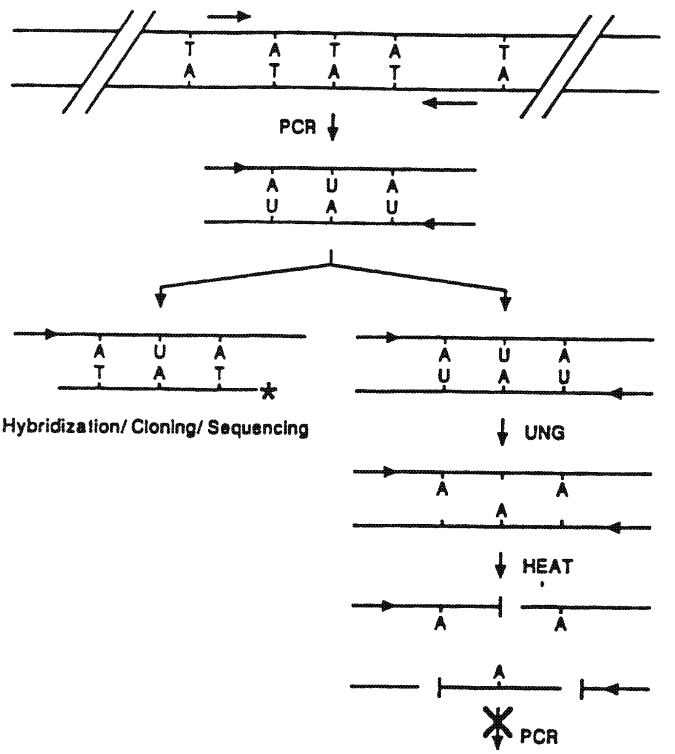


FIGURE 8.5. Use of dUTP and uracil DNA glycosylase to eliminate errant PCR products as templates for subsequent amplifications leading to false-positive results (see text for details).

BSA-conjugated DNA capture probe. Denatured amplification reaction is pipetted into the wells that contain the hybridization solution. After being covered and incubated for 1 hour at 37°C, the plate is washed, and avidin–horseradish peroxidase (HRP) conjugate is added to the wells and incubated for 15 minutes at 37°C. The plate is then washed again, and the substrate/chromogen is added. Color development is allowed to proceed for 10 minutes at room temperature in the dark. Acidic stop solution is added to the wells, and the resulting absorbances are read at 450 nm. The test kit should be rapid (results can be obtained within one working day), easy to perform, and amenable for use in the clinical laboratory. This system, which should provide a means of standardizing testing among laboratories, is being investigated at a number of designated clinical trial sites in the United States for Food and Drug Administration (FDA) licensure.

Direct Detection of HIV Proviral Sequences

The PCR has proved to be a powerful technique for diagnosing HIV infections. It has been used successfully for direct detection of HIV-1 proviral DNA sequences in PBMCs of seropositive persons[10–13] and to detect HIV-1 DNA in cells from seropositive subjects who were negative by virus co-culture.[10] Studies have also demonstrated that PCR has simplified the ability of researchers to directly clone and sequence HIV-1 DNA[14–16] and HIV-1 cellular RNA.[7] HIV sequences obtained by this method more accurately reflect the repertoire of viral sequences actually present in a patient.[15] It is possible because PCR does not require virus isolation, which leads to the selection of only a subset of the HIV strains that are present in any particular patient.[15,16]

The PCR has been used to establish that virtually all antibody-positive individuals are infected with HIV, lending further proof that HIV is the causative agent of AIDS.[17] This large-scale study involved testing PBMCs obtained from 409 individuals who were HIV antibody-positive. The group consisted of 56 individuals who had AIDS, 88 who had AIDS-related complex (ARC), and 265 who were asymptomatic. In addition, blood samples from 131 persons who were HIV antibody-negative were examined. All samples from the 56 AIDS patients, 87 of the 88 ARC patients (99%), and 259 of the 265 asymptomatic but HIV antibody-positive persons (98%) tested positive for virus by culture, PCR, or both analyses; in contrast, none of the 131 HIV-1 antibody-negative persons was positive for virus by culture or by PCR.

Quantitation of HIV Proviral Sequences

Because the amount of the original target DNA can be quantitated by PCR, the technique can be used to measure the number of infected cells[18–20] or to quantitate the amount of cell-free virus in the patient's plasma (viremia). A quantitative assay for proviral HIV-1 would be important for the evaluation of new drugs and vaccines or for monitoring disease progression. Quantitation of viral load in infected persons has been performed with varying success by limiting dilution culture methods in which culture supernatants were tested for the presence of HIV-1 *gag* protein (p24) or the presence of viral reverse transcriptase activity (or both).[21,22] Virus culture is expensive and time-consuming, requires handling large volumes of infectious material, and has not always been reliable. By amplifying a dilution series of known amounts of HIV DNA (e.g., a plasmid containing a full-length copy of HIV-1 DNA or DNA from a cell line containing one integrated copy of HIV-1 per cell), it is possible to quantify the virus burden in a person by determining the number of HIV proviral copies per given number of cells in a

patient.[18–20,23–27] To ensure the biosafe use of a full-length proviral DNA standard, a replication-deficient HIV-1 proviral DNA has been developed.[28] A cell line, ACH2, which contains one integrated copy of HIV-1 and produces a noninfectious HIV, has also been developed.[29] Methods have also been developed for the quantitation of specific RNAs by the PCR technique.[30–32]

A rapid, quantitative detection procedure has been developed using a nonisotopic chemiluminescent DNA probe.[19] The total time for PCR amplification and DNA probing using this technique requires about 4 hours. Thus detection and quantitation of HIV DNA can be achieved within 1.0 to 1.5 days from the time of receipt of the blood sample.

The PCR has been used by a number of investigators to determine the relation between infected cell burden and immunologic status in persons with asymptomatic and symptomatic HIV infection.[20,25–27] In those studies, lysates of patient PBMCs were serially diluted, amplified, and detected with a radiolabeled probe. The signal intensity from each amplification was compared with the PCR performed on serial dilutions of the plasmid containing the HIV-1 genome or the ACH2 cell line that contains one integrated copy of HIV-1 per cell. The results of those studies demonstrated that there was a significant increase in viral burden per constant $CD4^+$ cells in patients as they progressed to clinical disease. There was also a concomitant quantitative depletion of $CD4^+$ cells. This finding contrasted with the stable viral burden and the maintenance of a relatively constant level of $CD4^+$ cells in patients who were clinically stable.[25] The number of HIV-infected cells can be calculated from the number of proviral copies, as it has been estimated that there is approximately one proviral copy per cell.[26] These results can be compared to the number of cells producing virus in asymptomatic versus symptomatic persons as determined by limiting dilution cultures.[21,22] From this type of analysis it can be estimated that approximately 10% of the infected cells in the blood of asymptomatic persons are actively expressing virus compared to about 100% of the infected cells in symptomatic persons. These results indicate that not only do the number of infected cells increase substantially, but the proportion of infected cells actively expressing HIV increases substantially as patients move from an asymptomatic to a symptomatic state. These results are consistent with a direct and probable causal relation between an increase in viral burden and immunosuppression and disease—presumably due to the increase in HIV expression leading to cell destruction.

Latent HIV-1 infection can be differentiated from active viral transcription[7,8,33] because HIV-specific RNA sequences can be detected in cells of infected persons by amplifying cDNA copies of reverse-transcribed cellular RNA. This assay was capable of detecting HIV RNA in one infected cell among 10^6 uninfected cells.[7] Direct comparison of the presence of detectable HIV serum antigen with HIV RNA expression in

the same patients[7] demonstrated that RNA PCR was more sensitive than serum antigen detection (i.e., all patients who were antigen-positive were HIV RNA-positive, but all patients who were HIV RNA-positive were not antigen-positive).

Virus Infection and Seroconversion

Various reports have indicated that in some individuals HIV can be detected by virus isolation or by antigen detection prior to seroconversion.[34–38] Other studies have reported that HIV-1 proviral DNA can be detected in PBMCs before seroconversion.[39–42] Preliminary replication of one of these studies[42] has not confirmed the original results (unpublished data) and merits additional investigation. Those who have been followed prospectively after exposure to HIV-1 have generally seroconverted within 6 months.[43] However, cases of positive antigen reactions for as long as 14 months without detectable antibody have also been reported.[36,40] To define the length of time from infection to the development of detectable levels of HIV antibodies, a before and after seroconversion study was undertaken of 26 homosexual men and 11 men with hemophilia.[43] PBMCs from these men were analyzed for HIV-1 DNA by PCR using primers from two distinct regions of the viral genome. Using a Markov statistical model, the median time from infection with HIV-1 to seroconversion was estimated to be 3 months and that 95% of all persons who become infected would seroconvert within 6 months. Similar results were obtained studying infection in high risk seronegative prostitutes in Nairobi, Kenya,[44] where it was estimated that the interval between infection and seroconversion was 3 to 4 months. These results indicated that prolonged periods of latent infection without detectable antibody probably are rare.

Resolving Cases with Indeterminate Western Blot Assays

The PCR has been used to determine whether apparently healthy persons who have had repeatedly reactive EIAs and an indeterminate Western blot test for HIV antibody are infected with HIV-1.[45] A total of 99 volunteer blood donors in a low risk area of the United States with such a serologic outcome were coded and tested for the presence of HIV by culture and by PCR. Of the 99 blood donors, 98 had no reported risk factors for HIV-1 infection; 1 donor had used intravenous drugs. After a median 14 months from the time of the initial serologic tests, 65 donors (66%) were still repeatedly reactive for HIV-1 on at least one immunoassay. For 91 donors (92%) the Western blot results were still

indeterminate. None of the 99 donors had evidence of either HIV infection as determined by culture or PCR. These results demonstrate that persons at low risk (e.g., volunteer blood donors) for HIV infection and who have persistent indeterminate HIV-1 Western blots are rarely infected with HIV-1.

HIV Typing

There is partial but significant serologic cross-reactivity between the *gag* (core) proteins of HIV-1 and HIV-2, whereas cross-reactive antibodies to the *env* (envelope) proteins are thought to be considerably less common.[46] There have been reports of individuals who possess antibodies reactive against the *gag*, *pol*, and *env* proteins of both HIV-1 and HIV-2.[46–50] Serologically, it has been difficult to determine whether this dual reactivity was due to a single HIV infection generating a broad immune response to determinants common to both viruses, an infection with a recombinant or third virus containing determinants of both HIV-1 and HIV-2, or a true mixed infection with both viruses in the same person. In regions where HIV-1 but not HIV-2 is highly endemic (e.g., the United States), it would be unlikely to find an individual infected with both viruses. However, in certain areas of West Africa where HIV-1 and HIV-2 are both prevalent, the probability of finding someone infected with both viruses is much higher.

The PCR has been used successfully as an adjunct to serologic testing to determine if a patient is infected with HIV-1 or HIV-2.[50] It has also been used to confirm the first case of HIV-2 infection in a person living in the United States[51] and to confirm the first case of a mixed HIV-1 and HIV-2 infection in the same individual.[50] That person was seroreactive by whole-virus EIAs, type-specific peptide EIAs, and Western blot assays for both viruses and contained proviral sequences of both HIV-1 and HIV-2 as determined by PCR.

HIV Infection in Newborns

The fact that about 13% to 40% of infants born to women with HIV-1 infection have acquired their infection from the mother together with the presence of maternal antibodies to HIV-1 in the newborn makes diagnosis of HIV-1 infection difficult. PCR has been used successfully to diagnose HIV-1 infection by detecting HIV-1 DNA during the neonatal period (first 28 days of life),[52,53] particularly in those infants born to HIV-seropositive mothers who develop a severe, rapid course of the disease.[53,54] The PCR has also detected virtually all HIV-infected children who are in the postneonatal period, usually a few months old.[53–55] Those HIV-infected infants born to seropositive mothers who develop a less severe, slow

course of the disease become PCR HIV-positive by 4 to 6 months. However, diagnosis of HIV-1 infection during the neonatal period and assessment of disease outcome in seropositive infants[53,54] are essential for identifying the infants who might benefit from early therapeutic intervention[54,56] (see Chap. 16 for a more complete description).

Use of PCR to Monitor for Drug-Resistant HIV

The reverse transcriptase inhibitor 3′-azido-3′-deoxythymidine (AZT, or zidovudine) has demonstrated clinical utility for the treatment of AIDS and ARC. Specifically, this therapeutic strategy has been shown to extend life expectancy and to lower the frequency and severity of opportunistic infections. The initially reported isolates with reduced sensitivity in vitro to this nucleoside analogue,[57] cultured from patients receiving zidovudine, were subsequently found to harbor specific mutations within the coding sequence for the HIV reverse transcriptase.[58] Whereas the most resistant isolates have four amino acid substitutions (e.g., positions 67, 70, 215, and 219), isolates that have a subset of these four mutations are less resistant to the drug. Larder and his colleagues have identified six AZT-resistant mutations, including two at the same position, codon 215.[59] A "nested" or "double" PCR procedure was developed[60] to detect the common mutations found in residue 215 (for example, conversion of the threonine codon to one for either tyrosine or phenylalanine requires a two base change). High-level (>50-fold) AZT resistance required two or more of the mutations in the laboratory constructs examined. The mutations at codon 215 are the most important of the AZT resistance mutations, being almost invariably present in highly resistant isolates. Codon 215 mutations have been monitored recently in several clinical studies and have proved to be a good marker for imminent clinical or immunologic decline in AZT-treated persons from various cohort studies.[61] Under modified conditions,[62] PCR can also be used to selectively amplify sequences varying in a single nucleotide, and therefore the other codon changes should be amenable to similar analysis. The role these mutations play in the declining efficacy of zidovudine after protracted periods of treatment remains unclear, but rapid diagnostic procedures for their detection should assist in resolving their contribution.

The increasing use of ddI and ddC of course raises the threat of ddI and ddC drug resistance. In fact, such resistance occurs clinically, although reports thus far concern small numbers of patients. HIV-1 resistant to ddI was isolated[63] from three patients after 6 to 12 months on ddI; the resistance was due to a leucine 74 to valine mutation, which conferred cross-resistance to ddC. In another report[64] a methionine 184 to valine mutation, which has similar effects, has been detected in five ddI-treated patients. A resistance mutation has been detected in two ddC-treated patients, threonine 69 to aspartate.[65]

Analytic Sensitivity and Specificity

The PCR has the highest analytic sensitivity of any procedure used in the diagnostic arena. Single molecule detection has been reported by numerous laboratories. This exquisite analytic sensitivity does not necessarily translate into diagnostic sensitivity with the ultimate clinical utility. The procedure does not have an intrinsic analytic or diagnostic sensitivity and specificity. The diagnostic sensitivity and specificity are inextricably linked to the laboratories performing the procedure. As a result, the confidence in the reported results is directly proportional to the experience and critical interpretive criteria used by the laboratories performing the assay. Multiple parameters have been shown to dramatically affect the overall analytic sensitivity and, correspondingly, its diagnostic sensitivity. Factors that have been demonstrated to affect overall amplification efficiency beyond the obvious contribution of the selected primers and probes for amplification and detection, respectively, include the concentration of the various reagents and the thermocycling profiles used for amplification (Table 8.2). As a result, as with other diagnostic assays, the use of well characterized controls to monitor inter- and intraassay variability is essential. Introduction of the use of PCR for detecting HIV has resulted in several controversial reports that run counter to the experience of the remainder of the diagnostic community carrying out this procedure. PCR data in the absence of patient follow-up and supporting results from more established procedures, such as the FDA-approved EIAs or virus culture by an experienced laboratory, should be viewed with caution.

Similar to other diagnostic assays, the application of PCR to the detection of HIV proviral DNA has resulted in false positives and false negatives. False positives have been demonstrated to result from (1) cross-contamination of a negative sample from a positive sample; (2) contamination of clinical samples or the reagents for amplification with recombinant plasmids or phage harboring the entire HIV proviral genome or portions of it; and (3) "carryover" of PCR products from previous positive reactions. The latter is usually the reason for false positive because of the number of copies generated by the PCR (e.g., 10^6–10^{12}). Higuchi and Kwok[66] have recommended specific precautions to follow to minimize this type of contamination (see Carryover, below). In addition, two laboratories[67,68] have described the use of dUTP instead of dTTP and the other three conventional deoxynucleoside triphosphates in PCR as well as pretreatment of all reactions with uracil DNA glycosylase to eliminate or "sterilize" errant PCR products as templates for the amplification (Fig. 8.5). Just as PCR harnesses the replication capacity of cells, this procedure exploits the restriction/modification and excision/repair systems of cells. Because PCR products containing dU hybridize as efficiently as dT-containing PCR products and can be cloned and se-

quenced, this procedural modification promises to increase the reliability of positive results from a large number of laboratories.

Amplification of low copy numbers by PCR is vulnerable to interference by the amplified extension of primer pairs annealed to nontarget nucleic acid sequences in the test sample (termed mispriming) and by the amplified extension of two primers across one another's sequence without significant intervening sequence (termed primer dimerization). Primer dimers may experience amplified oligomerization during PCR to create complex mixtures of primer artifacts. The quantity of these primer artifacts often varies inversely with the yield of specific PCR product in low copy number amplifications. The resulting nonspecificity has numerous negative consequences for detection and quantitation of low copy number blood-borne infectious agents usually in the presence of high copy number host nucleic acid. PCR detection of HIV is typical of such an analysis, where amplification for 20 µl of blood containing about 1.6×10^5 diploid human genomes in 1 µg of DNA often generates an uninterpretable ethidium-stained amplified DNA pattern. It has been shown that, using PCR amplification of HIV-1 targets, most of the observed mispriming and primer dimerization arises during the customary and poorly controlled time interval (minutes) when reactants are mixed at room temperature before starting an amplification. To prevent this problem, a method called "hot start PCR" is employed. With this method some of the additions are delayed until all the reactants have been heated to a temperature that prevents primer annealing to nontarget sequences. Hot start PCR has been shown to increase amplification efficiency, specificity, and yield of low copy numbers of target sequences as with three gene targets of HIV-1.[69] An added benefit of the dUTP-UNG procedure is increased specificity and sensitivity of amplifications. By preincubating the amplification reactions at 50°C for 2 minutes prior to thermal cycling, non-specific extension products that were formed at ambient temperature are cleaved. Since the temperature of subsequent cycles is kept at or above the annealing temperature, non-specific extensions are reduced and an enzymatic hot start is achieved.[69a]

False-negative results have been attributed to compromised analytic sensitivity because of insufficient specificity either because of less than

TABLE 8.2. Reaction parameters to be evaluated for efficient PCR.

Annealing temperature and time
Denaturation temperature and time
Taq DNA polymerase addition at elevated temperature
Enzyme concentration
$MgCl_2$ concentration
Primer concentration
Co-solvents

optimal amplification conditions or the selection of primers and probes that do not readily recognize different sequence variants.[62]

As with all diagnostic assays, replication is an important factor for being confident that the results are reproducible. Samples that have disparate results in duplicate, not unlike the discordant EIA assays, can be caused by signals at the cutoff point for positivity or by sample mixup. Stochastically, a sample must contain five copies of HIV template to have a 99% likelihood of being reproducibly positive. If there are fewer than an average of five copies in a sample, the reactions may appear irreproducible due to sampling bias.

Previously, a multicenter, blinded proficiency trial was conducted using 105 HIV-1-seronegative, culture-negative samples from low risk blood donors and 99 HIV-1-seropositive and culture-positive samples.[70] The five laboratories participating in the study had significant experience with PCR, but the procedure and interpretive criteria varied somewhat, and only one of the multiple primer pairs was used in common. The average sensitivity for the laboratories was 99.0% and the average specificity 94.7%. One laboratory achieved 100% sensitivity and specificity. The overall false-positive, false-negative, and indeterminate rates were 1.8%, 0.8%, and 1.9%, respectively. This study demonstrated that PCR is a highly sensitive, specific assay for HIV-1 proviral DNA but that rigorous procedural and critical testing algorithms are required. Furthermore, the two primer pair systems targeted to the *gag* gene showed 100% sensitivity and specificity. This observation suggests that the inability to detect all samples known to contain HIV-1 proviral DNA at the requisite level with different primer pairs may be due to a laboratory's experimental performance rather than a viral sequence variant incapable of amplification.

The lack of concordance between duplicate samples may be due to sample mix-up. Resolution of sample mix-up when using serologic assays is difficult. Often the sample is either rerun, or another sample is obtained for analysis. However, PCR assays for HIV, particularly if amplification of the histocompatibility region is used as a control for the number of cells examined and amplification integrity of the sample, allows for simple resolution. The pioneering PCR studies of Erlich and colleagues[71] on HLA genotyping were later exploited for HIV[72] to demonstrate that due to the polymorphic nature of the region between the HLA DQα DNA primers used, samples from different individuals could be discerned because of the differential hybridization of HLA sequence-specific probes[73] (Fig. 8.6).

Quantitation of Plasma Viremia

Accurate determination of virus load, both cell-free virus and infected cell burden, is required for understanding the natural history of HIV-1

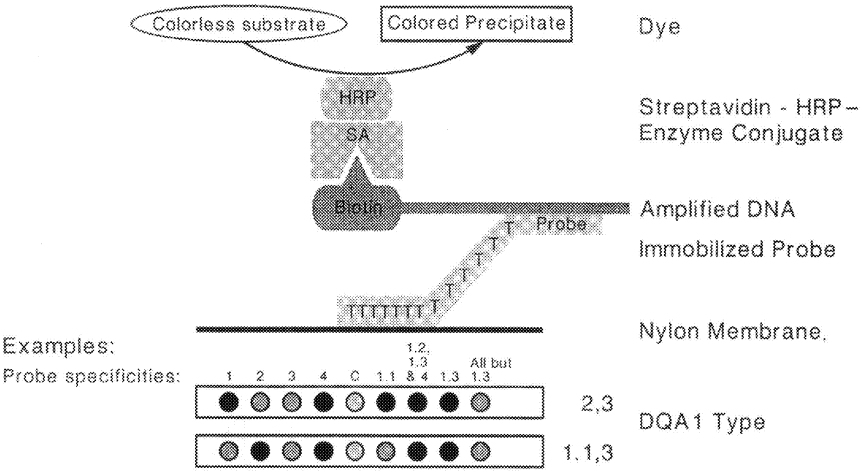


FIGURE 8.6. Immobilized oligonucleotide probe detection of amplified DNA. Reverse dot blot hybridization is used to rapidly and precisely type HLA DQα class II polymorphism. A sequence-specific probe is "tailed" with a dT homopolymer and immobilized on a solid support. The amplified PCR product, which has incorporated a biotinylated primer, hybridizes to the probe. After washing away unbound DNA and primers, the biotinylated, amplified DNA binds an avidin–horseradish peroxidase conjugate. The enzyme then converts a colorless dye to a colored precipitate. The format for detecting specific alleles in samples of amplified DQα DNA from heterozygous individuals is shown.

infection, predicting progression, and evaluating the efficacy of therapeutic drug regimens and vaccines. To date, CD4 T-helper lymphocytes together with elevated levels of β_2-microglobulin, neopterin, and interferon, delayed-type hypersensitivity, and clinical symptoms have served as surrogate markers for disease progression.[74,75] It is clear, however, that these markers represent indirect measures of the state of the viral infection and that virus load may provide better insight into the dynamics of the HIV-1 infection; moreover, virus load measurement is expected to complement the measurement of CD4 counts.

The ability to quantitate virus load in infected persons has been hampered by the low number of (1) circulating infected cells in the blood, (2) viral particles, and (3) expressed viral gene products. Viral cultures employing endpoint dilution have been used to quantify HIV-1 particles in plasma and in infected peripheral blood mononuclear cells[22,23] (see Chap. 7). This procedure is laborious, time-consuming, and expensive; and it requires handling large quantities of infectious virus. Direct detection of the HIV-1 p24 antigen in patient serum has been used, but the assay lacks sensitivity owing to its complexing with antigen-specific antibodies and to the low levels of the protein in the peripheral blood (see Chap. 6 for a detailed discussion). Acid dissociation of the immune

complexes has been used to increase the sensitivity of p24 detection and has had some limited success.

The PCR technique for quantitation of RNA has been extensively reported and reviewed by Ferr[76] and Clementi et al.[77] The quantification of plasma HIV-1 RNA by PCR may prove to be a more important measure of infection status than culturable virus. RNA from both infectious and noninfectious particles can be detected by PCR; and given the fact that physical particles may be at a 10^4- to 10^7-fold excess over infectious particles,[78] PCR should be more sensitive that virus culture. The amount of viral RNA released into the plasma is an indirect measure of the transcriptional status of those infected cells that shed virus. The transcriptional activity of the virus is in turn associated with the expression of viral and cellular proteins that participate in viral pathogenesis. Most symptomatic patients with low CD4 counts have been shown to have higher viral titers than asymptomatic persons with high CD4 counts, although a wide range of viral titers have been reported at all stages of the infection cycle.[22,23] The variability of plasma virus titers among infected persons suggests that only by following plasma viremia in a single patient over time can the results assist in evaluating therapeutic efficacy. To quantitate HIV-1 RNA in patients encompassing the full spectrum of CD4 counts and disease states, an assay with the ability to detect as few as 200 copies/ml is required to avoid the need for excessively large volumes of plasma to monitor viremia.

The typical method of RNA PCR requires first the reverse transcription of the RNA (viral RNA in the case of HIV-1) to convert it to a DNA form, followed by standard PCR amplification. An assay has been described that has certain advantages over conventional reverse transcription coupled to PCR (RT-PCR) assays.[79] The sample preparation procedure requires only a single guanidium isothiocyanate (GuSCN) treatment of the plasma followed by an alcohol precipitation step. Instead of using commercially available retrovirus reverse transcriptases combined with *Taq* DNA polymerase, the new procedure uses a thermostable DNA polymerase that contains efficient RT and DNA polymerase activities.[80] The enzyme rTth DNA polymerase simplifies the two-enzyme systems and has been used successfully to detect hepatitis C virus.[81] The sensitivity of the amplification requires fewer cycles than are normally required. This assay incorporates an RNA quantitation standard that amplifies HIV-1 RNA without compromising amplification of the target. This point is critical for monitoring reaction variability. The new assay system utilizes the microwell detection assay, which provides a quantitative, colorimetric readout over a four-log dynamic range. Furthermore, the sensitivity of the amplification and detection system, in conjunction with the quantitation standard, provides quantification from a single amplification using only 50 µl of the sample being tested. This method contrasts with competitive PCR amplification systems, which require multiple amplifi-

cations.[82–84] The reported analytic sensitivity of this assay is 10 copies/50 µl, or 200 copies/ml of plasma. The effects of differential collection, storage, and processing of the patient's specimen have been studied. Studies have indicated that optimally sera or plasma should be separated from cells within 3 hours of collection and stored at −70°C.

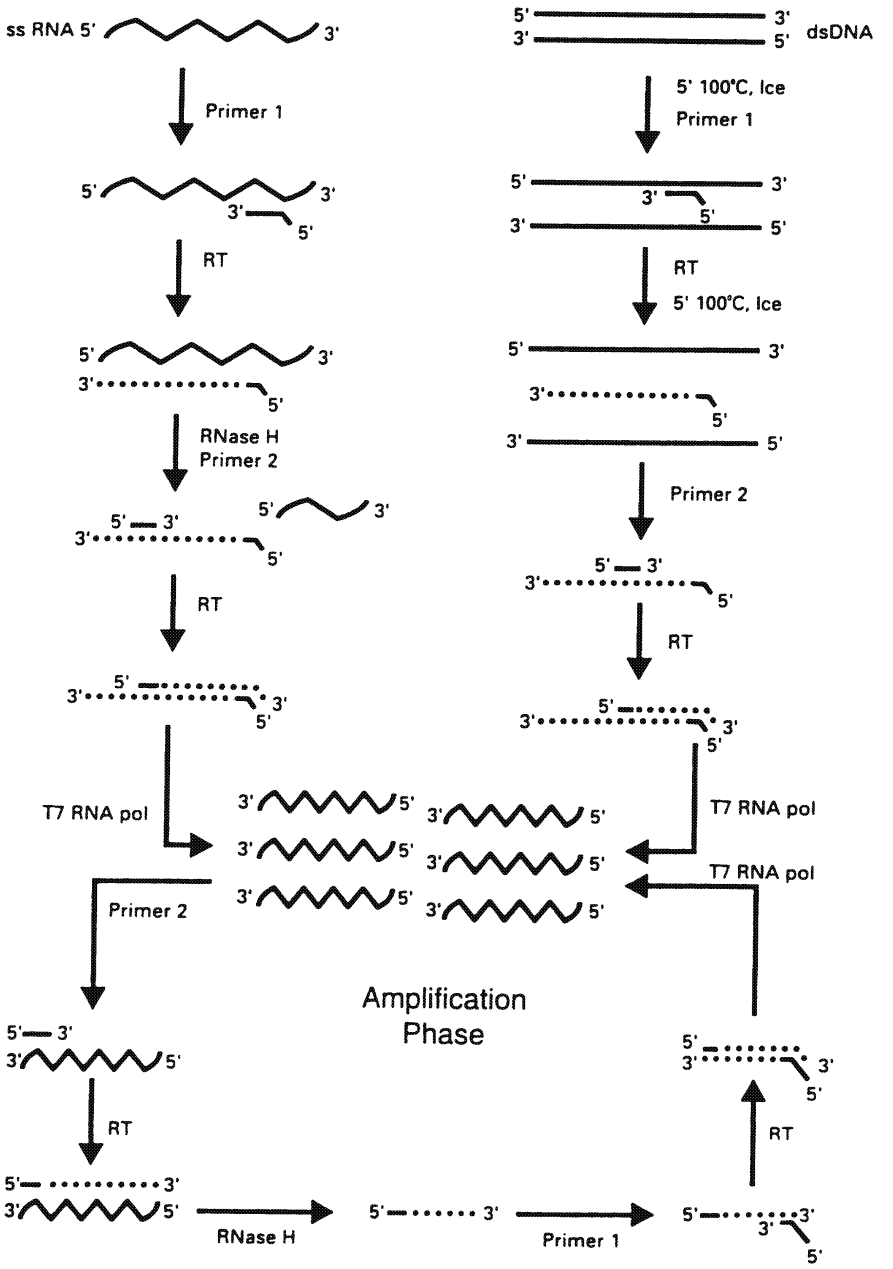
Amplification procedures other than PCR have been developed, as well,[85–87] one of which is an RNA-based amplification system. It is an isothermal amplification technology capable of selective amplification of RNA in a large DNA background. The amplification is based on the simultaneous enzymatic activity of RNase H, AMV-reverse transcriptase (RT), and T7 RNA polymerase at 41°C. The isothermal reaction results in the accumulation of the target HIV sequence in single-stranded RNA, which allows direct, specific detection (Fig. 8.7). The method is based on the extension of primer 1 (which contains at T7 promoter) by AMV-RT on a (+) single-stranded HIV RNA, strand separation through RNA degradation by RNase H, synthesis of double-stranded DNA by AMV-RT, and large-scale (−) strand RNA synthesis by T7 RNA polymerase. The (−) strand RNA synthesis is the first step into the cyclic phase. The reported analytic sensitivity of detection is 10 to 100 copies of HIV-1 RNA. This technology has the capability of being adapted for quantitation of HIV RNA.[88]

Carryover

As mentioned above, considerable care must be taken to avoid carryover of DNA from one tube to another in order to prevent false positives.[66,89,90] Because amplified sequences are present in large numbers, carryover of minute quantities of amplified DNA can lead to significant false-positive problems. The following are procedures that should minimize carryover.

1. *Physical separation of pre- and post-PCR reactions.* To prevent carryover, a separate room or containment unit, such as a biosafety cabinet, should be used for setting up amplification reactions. A separate set of supplies and pipettes should be kept in this area and should be used only for setting up PCR reactions. Care must be taken to ensure that amplified DNA is not brought into this area. Reagents, devices, and supplies should never be taken and returned from an area where PCR analyses are being performed.

2. *Aliquot reagents.* Reagents should be aliquoted to minimize the number of repeated samplings. All reagents used in the PCR process must be prepared, aliquoted, and stored in an area that is free of PCR-amplified product. Similarly, oligonucleotides used for amplification should be synthesized and purified in an environment free of PCR product.


ss RNA 5'
3'
Primer 1
RT
RNase H
Primer 2
RT
T7 RNA pol
Primer 2
RT
RNase H
dsDNA
5' 100°C, Ice
Primer 1
RT
5' 100°C, Ice
Primer 2
RT
T7 RNA pol
T7 RNA pol
Amplification
Phase
RT
Primer 1

3. *Positive displacement pipettes*. Contamination of pipetting devices can result in cross-contamination of samples. To eliminate cross-contamination of samples by pipetting devices, positive displacement pipettes with disposable tips or disposable tips with filters are recommended.

4. *Careful laboratory technique*. The following precautions should be taken during all aspects of PCR, from sample collection to PCR: (1) change gloves frequently; (2) uncap tubes carefully to prevent aerosols; (3) minimize sample handling; and (4) add nonsample components (mineral oil, dNTPs, primers, buffer, and enzyme) to the reaction mixture before adding the sample DNA. Cap each tube after the addition of DNA before proceeding to the next sample.

5. *Selection of controls*. For a positive control, select a sample that amplifies weakly but consistently. The use of a strong positive control results in the unnecessary generation of large amounts of amplified DNA sequences. Well characterized negative controls should also be used. The extreme sensitivity of the PCR process has the potential to amplify a nucleic acid sequence in a sample that is negative by all other criteria. Finally, multiple reagent controls should be included with each amplification because the presence of a small number of molecules of PCR product in the reagents may lead to sporadic positive results. The reagent controls should contain all the necessary components for the PCR process minus the template DNA.

In Situ PCR

The PCR has been successfully utilized to amplify DNA from formalin-fixed and even wax-embedded tissues. However, for the histopathologist, a limitation of PCR has been the inability to localize amplified DNA in

←

FIGURE 8.7. Isothermal amplification technology procedure applied to both single-stranded RNA (ssRNA) and double-stranded DNA (dsDNA). For RNA (top left) the technique is based on extension of primer 1 (which contains a T7 TNA polymerase promoter) by AMV-reverse transcriptase (RT) on the ssRNA template. RNase H degrades the RNA strand (or heat denaturation for the dsDNA) in the RNA:DNA hybrid that results from the AMV-RT enzyme activity. Subsequently, primer 2 can anneal to the resulting single-stranded cDNA, and the second DNA strand is synthesized by the DNA-dependent DNA polymerase activity of the AMV-RT, yielding a dsDNA molecule including a T7 RNA polymerase promoter sequence. The T7 RNA polymerase gives a 100- to 1000-fold increase in specific RNA. When DNA is used as input, two heat-denaturing steps (top right) are compulsory to obtain single-stranded DNA intermediates available for primer annealing. During the cyclic phase the events are the same as for RNA imput, but primer 1 and primer 2 are incorporated in reverse order.

cells or tissue sections. This limitation has been overcome, however, with the reports of studies employing a combination of PCR with in situ hybridzation, termed in situ PCR. This technique allows localization of specific amplified DNA segments within isolated cells and tissue sections.

In situ PCR was first described by Haase and his colleagues,[91] who amplified a 1200 base pair *gag* gene segment of visna virus DNA from infected sheep choroid plexus cells followed by detection of the amplified DNA using in situ hybridization with a 150 basepair probe. The sensitivity of this in situ PCR was at least severalfold greater than in situ hybridization alone. The technique has subsequently been modified for the identification of different types of human papillomavirus in formalin-fixed, wax-embedded tissue sample.[92,93] In situ PCR has been used to detect low proviral copy HIV-1 in PBMCs when in situ hybridization alone could not detect HIV infection.[94,95] The usefulness of in situ PCR has also been demonstrated numerous times, including the identification of single-copy immunoglobulin gene arrangements in human B lymphocytes and the identification of various HLA DQ haplotypes in human PBMCs. The technique has also been successfully used to demonstrate latent and permissive HIV infection in routinely fixed and paraffin-embedded tissue.[96]

When compared with PCR performed in solution, DNA amplification from tissue sections or isolated cells is less efficient. It may be due in part to the relatively poor access of DNA primers, DNA polymerase, and other components to the target DNA. The most consistent in situ PCR results apparently were achieved using the "hot start" method of PCR in which the oligonucleotide primers and DNA polymerase are added only at high temperature and appeared to improve the DNA amplification efficiency.

Successful in situ PCR depends on the amplified DNA remaining localized. Why the amplified DNA does not diffuse during the procedure is unclear, but it may result from the network formation of the amplified DNA, which leads to the development of an insoluble high-molecular-weight DNA complex. Studies of in situ PCR have demonstrated that the length of the DNA fragment to be amplified did not alter the final localization of DNA after in situ hybridization.

After PCR, in situ hybridization is performed using standard methodology, including use of an oligonucleotide probe complementary to a portion of the amplified DNA sequences with the hybridization time kept to a minimum. Amplified DNA can be detected directly without performing in situ hybridization by the incorporation of labeled nucleotides or oligonucleotides in the PCR, which results in more rapid detection of the amplified DNA fragment.

To ensure that in situ PCR is amplifying and detecting a specific DNA sequence (e.g., from HIV proviral sequences) various controls must be included in the assay. The DNA amplification reaction must be shown to be enzyme-mediated, primer-dependent, and (if possible) of the ap-

propriate molecular weight. Finally, omitting PCR should result in a reduced or absent in situ hybridization signal compared with in situ PCR.

Conclusion

As the AIDS epidemic continues to grow and spread, there is an increasing need for sensitive, quantitative assays for HIV. Quantitative tests for monitoring the infected cell load and cell-free particulate virus in infected persons are needed to monitor the *clinical* status of the patient and to evaluate the efficacy of new antiviral agents and potential vaccines.

The application of PCR technology to AIDS research opens up exciting possibilities for the sensitive, specific, direct detection and quantitation of HIV. PCR in its short existence has proved valuable for (1) detecting infection in seronegative persons, (2) quantifying the virus burden in a patient, (3) typing HIV infections, (4) measuring virus expression, (5) diagnosing perinatal transmission of HIV at an early stage, and (6) resolving indeterminate Western blottest results. As a research tool, the PCR technique is also proving useful for studying variant HIVs, distinguishing the important human retroviruses HTLV-I and HTLV-II, and discovering new pathogenic human retroviruses. Further simplification of PCR technology with the addition of sensitive nonisotopic detection systems requiring less than 1 hour for a quantitative readout should guarantee PCR a significant role in the diagnosis of HIV infection and AIDS.

References

1. Harper MH, Marselle LM, Gallo RC, Wong-Staal F. Proc Natl Acad Sci USA 1986;83:772–776
2. Shaw GM, Hahn BH, Arya SK, et al. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science 1984;226:1165–1171
3. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 1987;155:335–350
4. Saiki PK, Scharf S, Faloona F, et al. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for the diagnosis of sickle cell anemia. Science 1985;230:1350–1354
5. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 1988;239: 487–491
6. Schochetman G, Ou C-Y, Jones W. Polymerase chain reaction. 1988;158: 1154–1157
7. Hart C, Spira T, Moore J, et al. Direct detection of HIV RNA expression in seropositive subjects. Lancet 1988;2:596–599
8. Byrne BC, Li JJ, Sninsky J, Poiesz BJ. Detection of HIV-1 RNA sequences by in vitro DNA amplification. Nucleic Acids Res 1988;16:4165

9. Butcher A, Spadoro J. Using PCR for detection of HIV-1 infection. Clin Immunol Newslett 1992;12:73–76
10. Ou C-Y, Kwok S, Mitchell SW, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 1988;239: 295–297
11. Kwok S, Mack DH, Mullis KB, et al. Identification of human immunodeficiency virus sequences by using in vitro enzymatic amplification and oligomer cleavage detection. J Virol 1987;61:1690–1694
12. Kwok S, Mack DH, Sninsky JJ, et al. Diagnosis of human immunodeficiency virus in seropositive individuals: viral sequences in peripheral blood mononuclear cells. In Luciw PA, Steimen KS (eds): HIV Detection by Genetic Engineering Methods. New York Marcel Dekker, 1989:243–255
13. Kellogg DE, Kwok S. Detection of human immunodeficiency virus. In Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds): PCR Protocols. Orlando, FL: Academic Press, 1989:337–347
14. Ou C-Y, Schochetman G. Polymerase chain reaction in AIDS research. In Erlich HA, Gibbs R, Kazazian HH Jr (eds): Current Communications in Molecular Biology, Polymerase Chain Reaction. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989:165–170
15. Meyerhans A, Cheynier R, Albert J, et al. Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell 1989;58:901–910
16. Goodenow M, Huet T, Saurin W, et al. HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions. J Acquir Immune Defic Synd 1989;2:344–352
17. Jackson JB, Kwok SY, Sninsky JJ, et al. Human immunodeficiency virus type 1 detected in all seropositive symptomatic and asymptomatic individuals. J Clin Microbiol 1990;28:16–19
18. Kellogg DE, Sninsky JJ, Kwok S. Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. Anal Biochem 1990;189:202–208
19. Ou C-Y, McDonough SH, Cabanas D, et al. Rapid and quantitative detection of enzymatically amplified HIV-1 DNA using chemiluminescent oligonucleotide probes. AIDS Res Hum Retrovir 1990;6:1323–1329
20. Schnittman SM, Psallidopoulos MS, Lane HC, et al. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science 1990;245:305–308
21. Ho DD, Moudgil T, Alam M. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N Engl J Med 1989;321:1626–1625
22. Coombs RW, Collier AC, Allain J-P, et al. Plasma viremia in human immunodeficiency virus infection. N Engl J Med 1989;321:1626–1631
23. Ratner L. Measurement of human immunodeficiency virus load and its relation to disease progression. AIDS Res Hum Retrovir 1989;5:115–119
24. Lion T, Nighet R, Hutchinson MA, Golomb HM, Brownstein BH. Rapid dot blot quantitation for viral DNA and amplified genes in less than 1000 cells. DNA 1989;8:361–367
25. Schnittman SM, Greenhouse JJ, Miltiades BS, et al. Increasing viral burden in $CD4^+$ T cells from patients with human immunodeficiency virus (HIV)

infection reflects rapidly progressive immunosuppression and clinical disease. Ann Intern Med 1990;113:438–443
26. Simmonds P, Balfe P, Peutherer JF, et al. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cell and at low copy numbers. J Virol 1990;64:864–872
27. Spear GT, Ou C-Y, Kessler HA, et al. Analysis of lymphocytes, monocytes, and neutrophils from human immunodeficiency virus (HIV) infected persons for HIV DNA. J Infect Dis 1990;162:1239–1244
28. Hart C, Chang S-Y, Kwok S, et al. A replication-deficient HIV-1 DNA used for quantitation of the polymerase reaction (PCR). Nucleic Acids Res 1990; 18:4029–4030
29. Clouse KA, Powell D, Washington I, et al. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J Immunol 1989;142:431–438
30. Wang AM, Doyle MV, Mark DF. Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci USA 1989;86:9717–9721
31. Becker-Andre M, Hahlbrock K. Absolute mRNA quantification using the polymerase chain reaction (PCR): a novel approach by a PCR acided transcript titration assay (PATTY). Nucleic Acids Res 1989;17:9437–9446
32. Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci USA 1990;87:2725–2729
33. Murakawa GJ, Zaia JA, Spallone PA, et al. Direct detection of HIV-1 RNA from AIDS and ARC patient samples. DNA 1988;7:287–295
34. Goudsmit J, Paul DA, Lange J, et al. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. Lancet 1986;2:177–180
35. Kessler H, Blaauw B, Spear J, et al. Diagnosis of human immunodeficiency virus infection in seronegative homosexuals presenting with an acute viral syndrome. JAMA 1987;258:1196–1199
36. Ranki A, Valle S, Krohn M. Long latency precedes overt seroconversion in sexually transmitted human immunodeficiency virus infection. Lancet 1987; 2:589–593
37. Simmonds P, Lainson FAL, Cuthbert R, et al. HIV infection and antibody detection: variable responses to infection in the Edinburgh haemophilic cohort. BMJ 1987;296:593–598
38. Ward JW, Schable C, Dickinson GM, et al. Acute human immunodeficiency virus (HIV) infection: antigen detection and seroconversion in immunosuppressed patients. Transplantation 1989;47:722–724
39. Loche M, Mach B. Identification of HIV-infected seronegative individuals by a direct diagnostic test based on hybridization to amplified viral DNA. Lancet 1988;2:418–421
40. Wolinsky SM, Rinaldo CR, Kwok S, et al. Human immunodeficiency virus type 1 (HIV-1) infection a median of 18 months before a diagnostic Western blot: evidence from a cohort of homosexual men. Ann Intern Med 1989; 111:961–972
41. Hewlett IK, Gregg RA, Mayner RE, et al. Detection of HIV proviral DNA and p24 antigen in plasma prior to seroconversion. IVth Int Conf AIDS 1988;1:137

42. Imagawa DT, Lee MH, Wolinsky SM, et al. Human immunodeficiency virus type 1 infection in homosexual men who remain seronegative for prolonged periods. N Engl J Med 1989;320:1458–1462
43. Horsburgh CR Jr, Ou C-Y, Jason J, et al. Duration of human immunodeficiency virus infection before detection of antibody. Lancet 1989;2:637–640
44. Farzadegan H, Vlahov D, Solomon L, et al. Detection of human immunodeficiency virus type 1 infection by polymerase chain reaction in a cohort of seronegative intravenous drug users. J Infect Dis 1993;168:327–331
45. Jackson JB, MacDonald KL, Cadwell J, et al. Absence of HIV infection in blood donors with indeterminate Western blot tests for antibody to HIV-1. N Engl J Med 1990;322:217–222
46. George JR, Rayfield MA, Phillips S, et al. Efficacies of U.S. Food and Drug Administration-licensed HIV-1 screening enzyme immunoassays for detecting antibodies to HIV-2. AIDS 1989;4:321–326
47. Rey F, Salaun D, Lesbordes JL, et al. HIV-1 and HIV-2 double infection in Central African Republic. Lancet 1986;2:1391–1392
48. Rey MA, Girard PM, Harzic M, et al. HIV-1 and HIV-2 double infection in French homosexual male with AIDS related complex. Lancet 1987;1:388–389
49. Foucault C, Lopez O, Jourdan G, et al. Double HIV-1 and HIV-2 seropositivity among blood donors. Lancet 1987;1:165–166
50. Rayfield M, DeCock K, Heyward WL, et al. Mixed human immunodeficiency virus (HIV) infection of an individual: demonstration of both HIV type 1 and HIV type 2 proviral sequences by polymerase chain reaction. J Infect Dis 1988;158:170–176
51. Centers for Disease Control. AIDS due to HIV-2 infection—New Jersey. MMWR 1988;37:33–35
52. Krivine A, Firtion G, Cao L, et al. HIV replication during the first weeks of life. Lancet 1992;339:1187–1189
53. Rogers MF, Ou C-Y, Rayfield M, et al. Use of the polymerase chain reaction for early detection of the proviral sequences of human immunodeficiency virus in infants born to seropositive mothers. N Engl J Med 1989;320: 1649–1654
54. Rogers MF, Ou C-Y, Kilbourne B, Schochetman G. Advances and problems in the diagnosis of HIV infection in infants. In Pizzo PA, Wilfert CM (eds): Pediatric AIDS, The Challenge of HIV Infection in Infants, Children and Adolescents. Baltimore: Williams & Wilkins, 1990:159–174
55. Chadwick EG, Yogev R, Kwok S, et al. Enzymatic amplification of the human immunodeficiency virus in peripheral blood mononuclear cells from pediatric patients. J Infect Dis 1989;160:954–959
56. Pizzo PA, Eddy J, Falloon J, et al. Effects of continuous intravenous infusion of zidovudine (AZT) in children with symptomatic HIV infection. N Engl J Med 1988;319:889–896
57. Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 1988;246: 1731–1734
58. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science 1989;246: 1155–1158
59. Kellam P, Boucher CAB, Larder BA. Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development

of high-level resistance to zidovudine. Proc Natl Acad Sci USA 1992;89: 1934–1938
60. Boucher CAB, Tersmette M, Lange JMA, et al. Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy. Lancet 1990;336:585–590
61. Kozal MJ, Shafer RW, Winters MA, Katzenstein DA, Merigan TC. A mutation in human immunodeficiency virus reverse transcriptase and decline in CD4 lymphocyte numbers in long-term zidovudine recipients. J Infect Dis 1993;167:526–532
62. Kwok S, Kellogg DE, McKinney N, et al. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nuclieic Acids Res 1990;18:999–1005
63. St Clair MH, Martin JL, Tudor-Williams G, et al. Resistance to ddI and sensitivity to AZT induced bya mutation in HIV-1 reverse transcriptase. Science 1991;235:1557–1559
64. Wainberg MA, Gu Z, Gao Q, et al. Clinical correlates and molecular basis of HIV drug resistance. J Acquir Immune Defic Syndr 1993;6(suppl 1): S536–546
65. Fitzgibbon JE, Howell RM, Haverzettl C, et al. Human immunodeficiency virus type 1 pol mutations which cause decreased susceptibility to 2′,3′-dideoxycytidine. Antimicrobial Agents Chemother 1992;36:153–157
66. Higuchi R, Kwok S. Avoiding false positives with PCR. Nature 1989;339: 237–238
67. Longo MC, Berninger MS, Hartley JL. Use of uracil DNA glycosylase to control carryover contamination in polymerase chain reactions. Gene 1990; 93:125–128
68. Sninsky JJ, Gates C, McKinney N, et al. dUTP and uracil-N-glycolase in the polymerase chain reaction: a resolution to carryover. In preparation
69. Chou Q, Russell M, Birch DE, Raymond J, Bloch W. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res 1992;20:1717–1723
69a. Persing DH, Cimino GD. Amplification product inactivation methods in diagnostic molecular microbiology: principles and application. In Persing DH, Smith TF, Tenover FC, White TJH (eds): ASM, Washington, D.C. 1983; pp 1050–121.
70. Sheppard HA, Ascher MS, Busch MP, et al. A multicenter proficiency trial of gene amplification (PCR) for the detection of HIV-1. J Acquir Immune Defic Syndr 1991;4:277–283
71. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA. Analysis of enzymatically amplified beta-globin and HLA DQ alpha with allele-specific oligonucleotide probes. Nature 1986;324:163–165
72. Farzadeagan H, Polis MA, Wolinsky SM, et al. Loss of human immunodeficiency virus type 1 (HIV-1) antibodies with evidence of viral infection in asymptomatic men. Ann Intern Med 1988;108:785–790
73. Saiki R, Walsh R, Levenson CH, Erlich HA. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotidę probes. Proc Natl Acad Sci USA 1989;86:6230–6234
74. Anderson RE, Lang W, Shiboski S, et al. Use of β_2-microglobulin level and CD4 lymphocyte count to predict development of acquried immunodeficiency virus infection. Arch Intern Med 1990;15:73–77

75. Fahey JL, Taylor JMG, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. N Engl J Med 1990;322:166–172
76. Ferre F. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl 1992;2:1–9
77. Clementi M, Menzo S, Bagnarelli P, et al. Quantitative PCR and RT-PCR in virology. PCR Methods Appl 1993;2:191–196
78. Layne SP, Merges MJ, Dembo M, et al. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology 1992;189:695–714
79. Mulder J, McKinney N, Christopherson C, et al. A rapid and simple PCR assay for quantitation of HIV-1 RNA in plasma: application to acute retroviral infection. J Clin Microbiol 1994;32:292–300
80. Myers TW, Gelfand DH. Reverse transcription and DNA amplification by a Thermus thermophilis DNA polymerase. Biochemistry 1991;30:7661–7666
81. Young KKY, Resnick RM, Myers TW. Detection of hepatitis C virus RNA by a combined reverse transcription-polymerase chain reaction assay. J Clin Microbiol 1993;31:882–886
82. Becker-Andre M, Hahlbrock K. Absolute mRNA quantification using the polymerase chain reaction (PCR): a novel approach by a PCR aided transcript titration assay (PATTY). Nucleic Acids Res 1989;17:9437–9446
83. Gilliland G, Perrin S, Banchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci USA 1990;87:2725–2729
84. Platak M, Saag MS, Yang LC, et al. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science 1993;259: 1749–1754
85. Wu DY, Wallace RB. The ligation amplification reaction (LAR)—amplification of specific DNA sequences using sequential rounds of template-dependent ligation. Genomics 1989;4:560–569
86. Kwoh DY, Davis GR, Whitfield KM, et al. Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc Natl Acad Sci USA 1989; 86:1173–1177
87. Kievits T, van Gemen B, van Strijp D, et al. NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J Virol Methods 1991;35:273–286
88. Van Gemen B, Kievits T, Schukkink R, et al. Quantification of HIV-1 RNA in plasma using NASBA during HIV-1 primary infection. J Virol Methods 1993;43:177–188
89. Dragon EA. Handling reagents in the PCR laboratory. PCR Methods Appl 1993;3:S8–S9
90. Hartley JL, Rashtchian A. Dealing with contamination: enzymatic control of carryover contamination in PCR. PCR Methods Appl 1993;3:S10–S14
91. Haase AT, Retzel EF, Staskus KA. Amplification and detection of lentiviral DNA inside cells. Proc Natl Acad Sci USA 1990;87:4971–4975
92. Nuovo GJ, MacConnell P, Forde A, Delvenne P. Detection of human papillomavirus DNA in formalin-fixed tissues by in situ hybridization after amplification by polymerase chain reaction. Am J Pathol 1991;139:847–854

93. Nuovo GJ, Becker J, Margiotta M, et al. Histological distribution of polymerase chain reaction-amplified human papillomavirus 6 and 11 DNA in penile lesions. Am J Surg Pathol 1992;16:269–275
94. Nuovo GJ, Margiotta M, Mac Connell P, Becker J. Rapid in situ detection of PCR-amplified HIV-1 DNA. Diagn Mol Pathol 1992;1:98–102
95. Bagasra O, Hauptman SP, Lischner HW, Sachs M, Pomerantz RJ. Detection of human immunodeficiency virus type-1 provirus in mononuclear cells by in situ polymerase chain reaction. N Engl J Med 1992;326:1385–1391
96. Embretson J, Zupancic M, Beneke J, et al. Analysis of human immunodeficiency virus-infected tissues by amplification and in situ hybridization reveals latent and permissive infections at single-cell resolution. Proc Natl Acad Sci USA 1993;90:357–361