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Standard Guide for Detection of Nucleic Acid Sequences of the Human Immunodeficiency Virus HIV-1 by the Polymerase Chain Reaction Technique[1]

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INTRODUCTION

This guide covers the amplification and detection of nucleic acids [ribonuclcic acid (RNA) and deoxyribonucleic acid (DNA)] of the human immunodeficiency virus type 1 (HIV-1) by the polymerase chain reaction (PCR) technique. PCR is used in clinical laboratories to aid in the diagnoses of genetic and infectious diseases; it is also used as a general tool in molecular biology and biotechnology laboratories. PCR involves the replication, or amplification, of a fragment of DNA by as many as several million times in only several hours. The amplified DNA fragments, which are identical or nearly identical, can then be detected, identified and quantified by classical procedures of molecular biology and biochemistry. An advantage of PCR is that as few as several molecules of target RNA or DNA in a biological test specimen can be rapidly and accurately amplified and subsequently identified. The value of using PCR to detect HIV is the rapidity of the procedure (~1 day for amplification and detection). Rapid clinical intervention can delay disease progression and prevent opportunistic infections.

Acquired immune deficiency syndrome (AIDS) is the end-stage outcome of infection with HIV. It is one of the leading causes of human death in the world. As of 1998, about 12 million people have died from AIDS. In the United States, until 1996, AIDS was the leading cause of death among persons 25 to 44 years of age **(1)**[2]. In 1996 the total number of HIV-infected adults and children worldwide was over 20 million **(1)**.

At present two serotypes of HIV are recognized: HIV-1 and HIV-2. HIV-1 is the primary serotype found in the United States; two principal genetic groups have been identified: M (main) and O (outlying). Subtype M is further classified into 10 envelope subtypes, A through J. HIV-2 is primarily restricted to West Africa. At present, no generally accepted primers exist for the detection of HIV-2 nucleic acids.

This guide was developed by Subcommittee E48.02 on Characterization and Identification of Biological Systems in collaboration with DIN (German Institute for Standardization) Committee E9 on Serodiagnosis of Infectious Diseases and Diseases of the Immune System of DIN's Department for Medical Standards (Normenausschuss Medizin). It is recommended that this HIV-specific PCR guide be used in conjunction with ASTM's general PCR guide E 1873 (Standard Guide for Detection of Nucleic Acid Sequences by the Polymerase Chain Reaction Technique). The combination of the two guides provides guidelines, recommendations, basic considerations, criteria, and principles that should be employed when developing, utilizing or assessing PCR protocols for the detection of the RNA or DNA form of HIV nucleic acid.

This guide assumes a basic knowledge of virology and molecular biology. It assumes the availability of, and the ability to search the literature for, HIV target-specific PCR protocols.

1. Scope

1.1 This guide covers considerations, criteria, principles and recommendations that should be helpful when developing,

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utilizing, or assessing PCR protocols to amplify and detect DNA (by PCR) or RNA [by reverse transcriptase PCR (RT-PCR)] from HIV. This guide is not a specific protocol for the detection of HIV. It is intended to provide information that will assist the user in obtaining quality and reliable data. The guide is closely related to and should be used concurrently with the general PCR guideline E 1873.

1.2 This guide has been developed for use in any molecular biology or biotechnology laboratory. This includes but is not limited to clinical and diagnostic laboratories involved with HIV detection.

1.3 This guide does not cover details of the various methods such as gel electrophoresis, that can be utilized to identify PCR-amplified HIV nucleic acid sequences, nor does it cover details of instrument (thermocycler) calibration.

1.4 This guide does not cover specific variations of the basic PCR or RT-PCR technology (e.g., quantitative PCR, multiplex PCR and in situ PCR).

1.5 This guide does not address the additional considerations necessary for the performance and validation of a quantitative PCR test for HIV.

NOTE 1—**Warning:** Laboratory work involving certain clinical specimens and microorganisms can be hazardous to personnel.

NOTE 2—**Precaution:** Biosafety Level 2 facilities are recommended for potentially hazardous work **(2)**, and many laboratories use Biosafety Level 3 facilities when working with HIV. Safety guidelines should be adhered to according to NCCLS M29–A and other recommendations **(2)**.

2. Referenced Documents

2.1 *ASTM Standards:*

E 1873 Guide for Detection of Nucleic Acid Sequences by the Polymerase Chain Reaction Technique[3]

2.2 *NCCLS Standards:*[4]

M29–A Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).

C24-A Internal Quality Control Testing: Principles and Definitions; Approved Guideline (1991)

MM3-A Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (1995)

3. Terminology

3.1 Basic PCR definitions apply according to the general PCR guideline E 1873 (Section 3).

4. Significance and Use

4.1 This guide is intended for use in any laboratory utilizing PCR to amplify DNA sequences or RT-PCR to amplify RNA sequences of HIV from cells, tissues, or body fluids such as whole blood, sera and plasma.

4.2 The criteria used for the identification and evaluation of the amplification reactions should be administered by an individual trained in the use of molecular biological and microbiological techniques associated with PCR and HIV.

5. Additional Background Information about HIV and detection of HIV by PCR

5.1 HIV-1 is a complex retrovirus belonging to the lentivirus family. It is generally accepted to be the causative agent of AIDS. Each of the two strains of the virus, HIV-1 and HIV-2, contains two identical RNA strands. HIV-1 enters the cell by binding to the transmembrane glycoprotein receptor $CD4^+$ and a chemokine coreceptor **(1)**. Following reverse transcription from RNA to double-stranded DNA, the HIV DNA is covalently integrated into the host genome (the proviral state).

5.2 The HIV provirus is approximately 9.8 kilobases in length and contains open reading frames that encode both structural and regulatory genes located between flanking long terminal repeat (LTR) sequences **(1)**. The structural genes encode the polyproteins gag, pol and env, all of which are common to retroviruses. The proteins tat, rev, vif, vpr, nef and vpu provide regulatory functions as well as assist in assembly of the viral particle. As is typical for RNA containing viruses, HIV has no single, stable RNA sequence in vivo. The HIV RNA sequence is constantly changing (evolving) within host cells through the production of mutations. However, the RNA sequences that code for certain genes, such as the pol (polymerase) gene, env (envelope) gene and gag gene (includes the capsid structural protein), are genetically more stable than other HIV genes. Consequently, regions of the HIV genome that are located within these genes can be successfully amplified with certainty. The PCR primers presented in Table 1 are

[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] *Annual Book of ASTM Standards*, Vol 11.05.

[4] Available from the NCCL Standards 940 West Valley Road, Suite 1400, Wayne PA 19087.

TABLE 1 Primers and DNA Probes for PCR Amplification of HIV-1 DNA

Target Gene	Description	Position	Sequence	Reference
HIV-1 gag	JA4 Primer	1319-1338	5'GAAGGCTTTCAGCCCAGAAG3'	**(4)**
HIV-1 gag	JA5 Primer	1446-1465	5'ACCATCAATGAGGAAGCTGC3'	
HIV-1 gag	JA6 Primer	1577-1558	5'TATTTGTTCCTGAAGGGTAC3'	
HIV-1 gag	JA7 Primer	1615-1596	5'TCTCCTACTGGGATAGGTGG3'	
HIV-1 gag	SK 38 Primer	1551-1578	5'ATAATCCACCTATCCCAGTAGGAGAAAT3'	**(5)**
HIV-1 gag	SK 39 Primer	1665-1638	5'TTTGGTCCTTGTCTTATGTCCAGAATGC3'	**(6)**
HIV-1 gag	SK 19 DNA Probe	1595-1635	5'ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC3'	
HIV-1 gag	SK 145 Primer	1366-1395	5'AGTGGGGGGACATCAAGCAGCCATGCAAAT3'	**(6)**
HIV-1 gag	SK CC1B Primer	1493-1520	5'TACTAGTAGTTCCTGCTATGTCACTTCC3'	**(7)**
HIV-1 gag	SK 102 DNA Probe	1403-1435	5'GAGACCATCAATGAGGAAGCTGCAGAATGGGAT3'	**(6)**
HIV-1 pol	P3 Primer	2356-2381	5'TGGGAAGTTCAATTAGGAATACCAC3'	**(8)**
HIV-1 pol	P4 Primer	2663-2637	5'CCTACATACAAATCATCCATGTATTC3'	
HIV-1 pol	Lauré DNA Probe	2508-2545	5'ATGAGACACCAGGGATTAGATATCAGTACAATGTGCT3'	

sufficient to amplify HIV-1 DNA with a high degree of certainty.

5.3 PCR has proven to be a useful procedure for the detection of HIV-1. PCR can be used to detect the AIDS virus in HIV-infected people, including individuals with latent infections, in the newborns of HIV-infected mothers, in cells of human origin, in cell culture, and in body fluids such as whole blood, serum, and plasma. Either the RNA form (using RT-PCR) or the DNA form of the virus can be detected by PCR. The PCR methodologies developed for detecting HIV-1 are probably more sensitive, uniform, and specific than for any other organisms due to the large amount of effort expended on this task (**3**).

5.4 For reference, PCR-based kits are commercially available for HIV amplification.

6. Principle of the Method

6.1 See E 1873 (Section 5).

6.2 In addition to the above, the amplification of target sequences takes place *in vitro*.

7. Target Material

7.1 For general information see E 1873 (Sections 6 and 9.6).

7.2 In addition to the above, it is highly important to avoid contamination with HIV target sequences. Blood derived from the umbilical cord shall not be used for the PCR detection of HIV.

7.3 Typical biological specimens for the detection of HIV nucleic acid sequences include cells in culture, tissues or body fluids such as whole blood, sera or plasma.

7.4 The method to be used for isolating the target sequence depends on the test material (e.g. lymphocytes or sera) and upon the type of HIV nucleic acid to be detected (DNA or RNA). The target sequence shall be made accessible and/or concentrated from the test material efficiently and in a reproducible manner. Methods currently used for extraction of the target sequence(s) from HIV-containing biological preparations include treatment with detergent and proteinase K, treatment with alkali or guanidium isothiocyanate, phenol/chloroform extraction and alcohol precipitation or chromatographic procedures.

7.5 Substances that inhibit PCR such as heparin, hemoglobin, etc., should be considerably reduced in amount through the extraction procedures used. Usual methods for the reduction in amount of inhibitors are solid phase adsorption of the nucleic acids or inhibitors, phenolchloroform extraction, etc. Purification of the extracted nucleic acids by solid phase adsorption, e.g. to silicates or resins, can lead to increased purity; however it can also lead to considerable losses of nucleic acids.

8. PCR Laboratory Design

8.1 According to E 1873 (Section 7).

9. Special Equipment/Supplies

9.1 *General*—See the recommendations of E 1873 (Section 8).

9.2 In addition to the above, equipment that ensures safe disruption of HIV-infected cells and/or tissues, such as programmed incubators, enclosed sonicators, or the equivalent.

9.3 Nucleic acids from HIV-containing biological specimens should be isolated in Biosafety Level 2 (BSL2) or BSL3 laboratories.

10. Chemicals and Reagents

10.1 *General*—See the recommendations of E 1873 (Section 9).

10.2 *HIV-Specific PCR Primers*—See the recommendations of E 1873 (Section 9.5). Table 1 lists several primers and probes that can be used for the amplification and detection of HIV-1 DNA.

10.3 *Target Sequences for the Detection of HIV by PCR*—The use of several primer pairs for amplification of different target sequences of the HIV genome may be necessary for an increase in reliability of detection. If two or more target sequences are used, then non-coding (e.g., LTR) as well as coding nucleic acid sequences (e.g., gag, pol, env) can be used. Amplified DNA products should have expected sequences as verified by: (*1*) hybridization analysis with oligonucleotide probes, or (*2*) restriction fragment analysis, or (*3*) DNA sequence determination.

11. Conditions Preliminary to the Performance of PCR

11.1 *General*—See recommendations of E 1873 (Section 10).

12. Performance of PCR

12.1 *General*—See recommendations of E 1873 (Section 11).

12.2 The positive control(s) used should be added to the biological test material at a sufficiently early stage in order to control the course of the entire process from extraction of nucleic acids to detection of the amplified products.

12.3 See NCCLS MM3-A for establishing sensitivity and specificity for diagnostic or confirmatory tests in clinical laboratories.

13. Evaluation and Interpretation of Results

13.1 *General*—See E 1873 (Section 12).

13.2 In addition to the above, for clinical, *in vitro* diagnostic PCR testing, the nucleic acid sequence detection of HIV is to be evaluated as:

13.2.1 *Positive*—A signal obtained by hybridization was above the cut-off value taken as baseline for the procedure in comparison with the accompanying controls, or a specific amplified product was obtained and characterized by restriction fragment analysis or DNA sequence determination.

13.2.2 *Negative*—In the case of hybridization, a cut-off value taken as baseline for the procedure was not exceeded, or a specific amplified product was not obtained when consideration was given to the result of the accompanying controls.

13.2.3 *Indeterminate*—The control reactions did not work correctly, or the amplification was negative, and/or an inhibition of the sample could be demonstrated. Under these conditions the test is to be repeated with consideration given to the problem that occurred.

13.2.4 *Inconclusive*—In the case of hybridization, a test result was obtained that lies in a narrow region close to the threshold value, or a specific amplified product could not be

generated in sufficient amount. Inconclusive results are to be repeated in the same laboratory and an attempt made to obtain an interpretable result.

13.3 Evaluation and documentation of the amplified product should be carried out.

13.4 Interpretation of results of the PCR reaction depends on: what results were obtained with the controls; which primers were used for the amplification; which target sequences were used; which test procedure was used for obtaining the amplified product; whether DNA or RNA was used as starting material; and from which test specimens the virus was obtained and concentrated.

14. Report

14.1 General requirements for the report are given in E 1873 (Section 13).

14.2 For a report of *in vitro* diagnostic PCR test results, in order to assure a correct diagnosis:

14.2.1 A report of positive findings should include a recommendation that a repeat of the test should be made using fresh test material when a patient has a positive nucleic acid detection for the first time. The recommendation should take into consideration the results of any other test procedures that were performed such as an ELISA or Western Blot.

14.2.2 A report of negative findings is generated in the case of a validated, negative test result. A negative nucleic acid detection report should always contain an addendum that states even in the case of a negative result, an infection with HIV is not completely excluded. When HIV infection continues to be suspected, a recommendation is to be included in the report concerning a suitable time for a test repeat and the type of test specimens.

14.2.3 A report of questionable findings should be made only in the case that after repeated, unclear results have been obtained, the existence or absence of HIV infection cannot be eliminated or verified. The performance of further tests with newly obtained test specimens should be recommended.

14.2.4 In all the above cases, the report should comment upon the validity of all associated diagnostic methods utilized. The report should also take into consideration any other test procedures used at that time.

15. Quality Assurance

15.1 See the general requirements given in E 1873 (Section 14).

15.2 Basic concepts for quality assurance in the laboratory should be followed, according to NCCLS C24-A.

15.3 In addition to the above, the user should keep in mind the fact that the detection of the nucleic acid forms of HIV by PCR is a rapidly developing area, and the user needs to take into consideration current innovations in the field and to integrate them into the test methodology and report interpretation.

16. Troubleshooting

16.1 See E 1873 (Section 15).

17. Keywords

17.1 AIDS; amplification; DNA primer; DNA probe; HIV; human immunodeficiency virus; PCR; polymerase chain reaction

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