Designation: E 1873 – 04

Standard Guide for Detection of Nucleic Acid Sequences by the Polymerase Chain Reaction Technique[1]

This standard is issued under the fixed designation E 1873; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

INTRODUCTION

This guide applies to the detection of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences by the polymerase chain reaction (PCR) technique. The PCR is used as a tool in many molecular biology laboratory settings and for diverse reasons, for example, for amplification and detection of nucleic acid sequences and for DNA sequencing. There is an abundance of publications addressing laboratory procedures and specific protocols for various applications. The field of PCR is advancing so rapidly that it is necessary to frequently modify and update these procedures and specific protocols. This guide consists of guidelines, recommendations, basic considerations, criteria, and principles that should be employed when developing, utilizing, or assessing PCR procedures and specific protocols for the amplification and detection of nucleic acid sequences.

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, Department for Medical Standards (NAMed).

This guide assumes a basic knowledge of molecular biology. It assumes the availability of basic references in PCR for general procedures (see Refs **1-7**)[2] and the ability to search the literature for target-specific protocols.

1. Scope

1.1 This guide covers guidelines, recommendations, basic considerations, criteria, and principles to be employed when developing, utilizing, or assessing PCR procedures and specific protocols for the amplification and detection of nucleic acid sequences. This guide is not intended to be a standard procedure with a list of requirements for PCR detection of nucleic acids. This guide is intended to provide information that will assist the user in obtaining quality and reliable data.

1.2 Nucleic acid sequences that can be amplified by PCR include DNA, as well as RNA sequences; RNA sequences are suitable targets for PCR following reverse transcription of the RNA to complementary DNA (cDNA). This type of amplification technique is often called reverse transcription-PCR (RT-PCR).

1.3 This guide has been developed for use in any molecular biology/biotechnology laboratory. This includes, but is not limited to, laboratories that specialize in the diagnosis of human, animal, plant, or bacterial diseases.

1.4 This guide conveys the general procedural terminology of PCR technology used for the detection of nucleic acids.

1.5 This guide is a general one; it does not cover the additional guidance that would be needed for specific applications, for example, for the PCR detection of nucleic acid sequences of specific microorganisms.

1.6 This guide does not cover details of the various methods that can be utilized to identify PCR-amplified DNA sequences.

1.7 This guide does not cover specific variations of the basic PCR or RT-PCR technology (for example, quantitative PCR, real-time PCR, multiplex PCR, and in situ PCR), and it does not cover details of instrument calibration.

1.8 **Warning**—Laboratory work involving certain clinical specimens and microorganisms can be hazardous to personnel. **Warning**—Biosafety level 2 (or higher) facilities are recommended for biohazard work **(8)**. Safety guidelines should be adhered to in accordance with NCCLS M29-A2 and other recommendations **(8)**.

[1] This guide is under the jurisdiction of ASTM Committee E48 on Biotechnology and is the direct responsibility of Subcommittee E48.02 on Characterization and Identification of Biological Systems.

Current edition approved July 1, 2004. Published July 2004. Originally approved in 1997. Last previous edition approved in 2002 as E 1873 – 97 (2002).

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

2. Referenced Documents

2.1 *NCCLS Standards:*

C24-A2 Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline—Second Edition (1999)[3]

M29-A2 Protection of Laboratory Workers from Occupationally Acquired Infections—Second Edition; Approved Guideline (2001)[3]

GP5-A2 Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002)[3]

MM3-A Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (1995)[3]

3. Terminology

3.1 Basic concepts for quality assurance in the laboratory should be followed, in accordance with NCCLS C24-A2.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *annealing*, *v*—in PCR, the binding or hybridization of two complementary strands of DNA or of DNA and RNA, as in the hybridization of oligonucleotide primers with DNA or RNA.

3.2.2 *carryover contamination*, *n*—in PCR, the adulteration of a PCR with amplified material (PCR products) from a previously amplified sample or with nucleic acids from another source. This potentially can cause a false positive result.

3.2.3 *chaotrope*, *n*—a chemical agent used to lyse cells.

3.2.4 *denaturation*, *n*—in PCR, separation of double-stranded DNA into single-stranded DNA. This can be accomplished with heat, extremes of pH, or exposure to certain chemicals such as chaotropic agents, plus heat, usually.

3.2.5 *DNA polymerase*, *n*—for PCR, usually a thermostable enzyme used in PCR that catalyzes the repeated synthesis of DNA under the selected reaction conditions of the method. The PCR, however, also can be performed with DNA polymerases that are not thermostable, if fresh enzyme is added before each cycle. It should be noted that other enzymes with DNA polymerase activity exist.

3.2.6 *DNA probe*, *n*—for PCR or RT-PCR, an oligonucleotide that is complementary to a portion of the amplified DNA product, contains a sufficient number of deoxynucleotides, generally 20 to 35, to facilitate specific hybridization with the amplified DNA product, and may be labeled with a detection molecule. It is common to use probes that can bind to the amplified DNA product at positions located between annealing sites of PCR primers. In some procedures, however, the probe can be used as the PCR primer.

3.2.7 *extension (of primer)*, *n*—in PCR, the synthesis of a new strand of DNA that is complementary to the target DNA, used as a template, by the addition of deoxynucleotide triphosphates to the oligonucleotide primer annealed to the target DNA. Extension is catalyzed by DNA polymerase.

3.2.8 *hot-start PCR*, *n*—a variation of PCR designed to minimize the formation of non-specific amplification products, often exhibited as smearing on electrophoretic gels, that may occur during the reaction setup, thereby enhancing the specificity, sensitivity, and precision of the amplification reaction. There are several ways to achieve hot-start PCR. All methods involve withholding a critical component (for example, polymerase or Mg^{++}) during the reaction setup at room temperature. Reaction tubes are then heated to a temperature exceeding 60°C, at which point the critical component is either added to the tube, comes in contact with the reaction mixture (for example, a hot start wax), or is activated (for example, a DNA polymerase used for hot start PCR).

3.2.9 *hybridization*, *n*—the specific annealing of a complementary DNA strand (for example, an oligonucleotide DNA primer) to the target DNA or RNA or amplified DNA segment in a medium containing an appropriate buffer composition, pH value, and temperature range.

3.2.10 *nested PCR*, *n*—a modification of PCR that uses nested sets of PCR primers to enhance the sensitivity and specificity of the reaction. A nested PCR protocol consists of two rounds of PCR amplification and utilizes two sets of primer pairs. In the first round of amplification, the outermost primer pair is used to generate an amplification product that is then subjected to a second round of amplification in the presence of the internal primer pair.

3.2.11 *polymerase chain reaction, PCR*, *n*—an in vitro laboratory method for the enzymatic amplification of nucleic acid sequences **(1-7)**. Two DNA oligonucleotide primers anneal with their complementary DNA strands and flank (that is, border) the segment to be amplified. The increase in amount (amplification) of the DNA segments occurs during repeated cycles consisting of three steps: heat denaturation of the double-stranded DNA, cooling to effect annealing of the primers to their complementary DNA strands, and enzymatic extension of the annealed primers by DNA polymerase at an optimal temperature. The amplification results in a near exponential increase in the amount of the nucleic acid target defined by the primers.

3.2.12 *PCR buffer*, *n*—a liquid medium that provides the appropriate salts, cofactors, and pH value required for amplification of DNA by PCR.

3.2.13 *PCR product*, *n*—the amplified DNA synthesized by DNA polymerase in a PCR. This term is sometimes called *amplicon*.

3.2.14 *primer*, *n*—for PCR, an oligonucleotide of defined length, generally 20 to 30 bases, complementary to one strand of DNA from the target sequence of interest. A primer pair defines the segment of the target DNA to be amplified. Primers can be labeled for detection. For RT-PCR, a primer can be an oligonucleotide of defined length complementary to an RNA target sequence of interest, a random hexamer or polydeoxythymidine.

3.2.15 *primer-dimer*, *n*—in PCR, the extension and amplification of primer pairs (independent of target DNA), usually caused when there is some complementarity between the two primers at the 3′ ends. These form products that are usually the length of the total of the two primers minus the overlap. Since they are relatively small and are complementary to the free primers, they can amplify efficiently and compete with and hinder amplification of the target DNA in PCR.

[3] Available from NCCLS, 940 West Valley Road, Suite 1400, Wayne, PA 19087.

3.2.16 *reaction tubes*, *n*—for PCR, tubes that can be heated to 100°C and cooled to 4°C without any damage resulting in leakage of contents. They should allow a rapid and homogeneous transfer of heat from the instrument heating block to the reaction mix to occur during the PCR procedure. They should not be made with substances known to be inhibitory to PCR **(9,10)**.

3.2.17 *reverse transcription–PCR, RT-PCR*, *n*—an in vitro laboratory method for using RNA as the target molecule for amplification **(11)**. A cDNA copy of the RNA target is made by annealing a complementary DNA oligonucleotide to the RNA target followed by the enzymatic extension of the annealed primer with reverse transcriptase. The cDNA:RNA hybrid is then heat denatured and a complementary primer anneals to the cDNA; a second DNA strand is synthesized by DNA polymerase. The increase in amount (amplification) of the cDNA segments occurs during repeated cycles of heat denaturation of the double-stranded DNA, annealing of the primers to their complementary DNA strands, and enzymatic extension of the annealed primers by DNA polymerase (see 3.2.11). The amplification (of DNA) results in a near exponential increase in the amount of the original RNA segment defined by the complementary primer.

3.2.18 *reverse transcription/polymerase enzyme*, *n*—for RT-PCR, a single enzyme that mediates reverse transcription of RNA to cDNA as well as amplification of cDNA by PCR. The enzyme rTth is an example of a thermostable DNA polymerase that can be used to reverse transcribe RNA efficiently in the presence of Mn^{++} at elevated temperature, and then subsequently act as a DNA polymerase to amplify the cDNA target in a single tube. Alternatively, two enzymes may be used, one a reverse transcriptase which transcribes the RNA to cDNA [such as M-MLV (Moloney murine leukemia virus) or AMV (avian myeloblastosis virus)], and the other, a DNA polymerase that amplifies the cDNA by PCR.

3.2.19 *target sequence*, *n*—the DNA or RNA (RT-PCR) that is selected for amplification and the portion of nucleic acid that is extracted or liberated from its source to allow PCR to occur.

3.2.20 *thermal cycler*, *n*—an instrument used to amplify DNA sequences by PCR or RNA sequences by RT-PCR. This instrument should be capable of changing temperature rapidly, automatically and in a reproducible fashion and have little well-to-well temperature variation.

3.2.21 *thermostable DNA polymerase*, *n*—for PCR, a DNA polymerase that preserves activity at the high DNA denaturation temperatures of PCR. A number of commercial sources are available. Taq polymerase is a thermostable enzyme commonly used in PCR.

3.2.22 T_m *(melting temperature)*, *n*—the midpoint of the temperature range over which two complementary strands of DNA denature.

4. Significance and Use

4.1 This guide is intended for use in any laboratory utilizing PCR or RT-PCR to amplify and detect a specific nucleic acid sequence.

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

5. Principle of the Method

5.1 The PCR is an in vitro method that enables the amplification and subsequent detection of segments of nucleic acid sequences. The increase in amount (amplification) of target DNA occurs by DNA polymerase in the presence of two, or more, oligonucleotide primers, deoxynucleoside triphosphates, Mg^{++} and a defined reaction buffer. Amplification of the DNA follows a reaction cycle that consists of the following.

5.1.1 Heating the reaction mixture to a temperature sufficient to *denature* the two strands of the target DNA.

5.1.2 Cooling the reaction mixture to a temperature that allows the primers to *anneal* with the target DNA strands.

5.1.3 Controlling at a temperature that allows *extension* of the annealed primers by DNA polymerase to occur on both single strands.

5.1.4 The preceding three steps (5.1.1-5.1.3) constitute a cycle. The cycle is repeated, (for example, 30 times), providing for repeat amplification of the target and any amplified material produced in the previous cycles. Some amplification protocols lack a discrete extension step, and extension occurs while the reaction mixture is being heated or cooled between the annealing temperature and the denaturation temperature.

5.2 The RT-PCR is an in vitro method that enables the amplification and subsequent detection of cDNA reverse-transcribed from segments of RNA. The replication of RNA segments occurs in two steps. A cDNA copy of the original RNA target is made with reverse transcriptase in the presence of a reverse transcription oligonucleotide primer, deoxynucleoside triphosphates, a divalent cation and a defined reaction buffer. In the second step the amount of cDNA is increased (amplified) by PCR. The increase in amount (amplification) of cDNA is catalyzed by DNA polymerase in the presence of two oligonucleotide primers, deoxynucleoside triphosphates, Mg^{++}, and a defined reaction buffer. Amplification of the RNA follows a reaction cycle that consists of the following.

5.2.1 Incubation at a temperature that allows the annealing of a reverse transcription primer(s) and extension of the annealed reverse transcription primer by reverse transcriptase, thus making a cDNA copy of the RNA target.

5.2.2 Heating the reaction mixture to a temperature sufficient to *denature* the strands of the cDNA:RNA hybrid.

5.2.3 Cooling the reaction mixture to a temperature that allows the amplification primers to *anneal* with the DNA strands.

5.2.4 Controlling the reaction mixture at a temperature that allows *extension* of the annealed amplification primers by DNA polymerase to occur on both single strands.

5.2.5 Steps 5.2.2-5.2.4 constitute a cycle. The cycle is repeated (for example, 30 times), providing for repeat amplification of the target and any amplified material produced in the previous cycles. Note that in cycles subsequent to the first, the denaturing step separates newly synthesized double strands of DNA. Some amplification protocols lack a discrete extension step, and extension occurs while the reaction mixture is being heated or cooled between the annealing temperature and the denaturation temperature.

5.3 The PCR product from PCR or RT-PCR is analyzed by sizing, radiolabeling or fluorescence labeling (see 8.5). There is

a constant evolution of nucleic acid detection techniques, and the selection of technique to use depends on the particular PCR application to be made. It is beyond the scope of this guide to provide recommendations and details about these various procedures. For additional information, see Refs **1-4** and NCCLS MM3-A.

6. Target Material

6.1 This represents nucleic acids isolated from biological sources including, but not limited to, cells, body fluids, and tissues. Collection of samples should be done carefully to minimize shearing of DNA and the introduction of potential PCR inhibitors, such as heparin, phenol, chloroform, and salts. There are many methods used routinely to prepare samples for PCR amplification. These methods include, but are not limited to, boiling in buffer or water, lysing in the presence of detergents, using chaotropic agents, and using phenol-chloroform extraction methods. Care should be taken to avoid contamination between specimens. This can be accomplished by changing the pipette tip between the handling of each specimen and frequent glove changes. Samples should be stored in such manner that isolated nucleic acids are preserved. Short-term storage (<5 days) generally can be done at 4°C. Longer-term storage should be done at − 20°C or less. Storage buffer will vary from experiment to experiment and application to application. A commonly used DNA storage buffer is TE (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

6.2 The objectives in preparing target nucleic acids include disrupting the cells, isolating the nucleic acids and removing or destroying any known PCR inhibitors, and performing the preceding in such manner that new PCR inhibitors are not introduced. Strategies include boiling, use of detergents, use of chelating agents, sonication or the use of one of many commercially available nucleic acid preparation kits. Since different research endeavors and applications target different samples and employ different enzymes, a discussion of all the possibilities is beyond the scope of this guide. When trying a method that provides nucleic acid of lesser purity than by standard phenol-chloroform extraction, it is recommended that studies comparing the results of that method to those obtained with phenol-chloroform extracted nucleic acid be performed.

7. PCR Laboratory Design

7.1 As a precaution against possible contamination with target DNA or amplified target DNA segments, two areas should be established in a laboratory. These two areas are:

7.1.1 *Pre-amplification Area*—Pre-amplification activities include reagent preparation and specimen preparation. The PCR reagent tubes should be sealed in this area and not reopened before PCR.

7.1.1.1 *Reagent Preparation Section*—For the preparation of PCR amplification reagents.

7.1.1.2 *Specimen Preparation Section*—For the isolation and liberation of nucleic acids from the target source.

7.1.2 *Post-Amplification Area*—For the amplification, analysis, and characterization of the amplified product.

7.1.3 Supplies and equipment should be dedicated to each activity and should not be moved between areas. There should be unidirectional traffic flow of reagents, tubes, and personnel from pre-amplification to post-amplification areas. Once working in a post-amplification area, one should not enter any pre-amplification work areas until changing of work clothing has occurred. The different stages of the PCR procedure should occur preferably in separated rooms or at least in separated areas within one room. When minimal reagent preparation is necessary, as with commercial kits, the reagent preparation and specimen preparation sections may be combined. One-way work flow and dedicated equipment for each area, however, should be strictly maintained. Reagent preparation and specimen preparation functions should not be performed at the same time. If only a single room is available, the use of table-top containment hoods to separate pre- and post-amplification areas is recommended. Additionally, attention should be paid to airflow within or across the work areas.

8. Special Equipment/Supplies

8.1 Instrument specifically designed to control the temperatures and times required for PCR. The temperature range should be at least 4 to 99°C. At a given temperature between 35 and 99°C, temperature uniformity should be ±0.5°C for 30 s. The temperature difference in the solution of individual reaction tubes should not be greater than ±1°C.

8.2 Positive displacement pipettes or pipette tips containing a filter that prevents contamination of the pipette or the withdrawn solution in the pipette tip.

8.2.1 For further prevention of contamination it is recommended that different sets of pipettes be used for preparing samples, setting up PCR reactions and manipulating PCR products. In addition, pipette shafts should be regularly cleaned (for example with 1M HCl overnight once or twice a month) to destroy DNA contamination.

8.3 The PCR sample tubes that can repeatedly withstand heating to 100°C and cooling to 4°C without leakage. It is recommended that the tubes be either sterile when purchased or autoclavable. It also is recommended that tubes from different vendors be screened for optimal PCR performance.

8.4 Some thermocyclers require reactioins with light mineral oil in the tubes to prevent evaporation of the reaction. The mineral oil should be purchased and sterile. Since it is inside the reaction tube, the oil should be asceptically aliquoted and stored as single assay reagents. In addition, with some thermal cyclers the use of mineral oil in the wells, outside the reaction tube, is recommended for improved heat transfer.

8.5 Examples of equipment or procedures that can be used to analyze the amplified DNA include, but are not limited to, gel or capillary electrophoresis with visualization or detection method, solid-phase detection formats such as Southern blots, in situ hybridization, microtiter wells or beads, solution hybridization formats such as exclusion-, hydroxyapatite-, or affinity capture-chromatography, homogeneous solution hybridization with labeled probes, HPLC (high-performance liquid chromatography) with detector, or an instrument for direct PCR product detection or detection assay system such as a colorimeter, fluorometer, or luminometer.

8.6 Equipment or containers for the removal of potentially hazardous substances, such as carcinogenic, infectious, environmentally hazardous, or radioactive material. Waste materials should be disposed of in accordance with NCCLS GP5-A2.