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

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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. 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. The 1997 edition of this guide was developed 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 targets for PCR include DNA, as well as RNA; RNA sequences are suitable targets for PCR following reverse transcription of the RNA to complementary DNA (cDNA). This type of amplification technique is known as 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 general; 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 CLSI M29-A2 and other recommendations **(8)**.

[1] This guide is under the jurisdiction of ASTM Committee E55 on Manufacture of Pharmaceutical Products and is the direct responsibility of Subcommittee E55.04 on General Biopharmaceutical Standards.

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

2. Referenced Documents

2.1 *CLSI Standards:*[3]

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

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

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

MM3-A2 Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—Second Edition (2006)

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *annealing*, *v*—in PCR, the binding or hybridization of two complementary strands of DNA or of DNA and RNA, as in the hybridization of two PCR primers to the complementary regions of the target DNAs or RNA at the annealing temperature.

3.1.2 *annealing temperature* (T_a), *n*—the temperature at which primers bind to a complementary sequence on the target. Annealing is usually performed at 5 °C below the melting temperature of the primer-template hybrid. If the T_a is too low, non-specific DNA sequences can be amplified. If the T_a is too high, the yield of the product can be reduced (**9**).

3.1.3 *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.

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

3.1.5 *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.

3.1.6 *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.

3.1.7 *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.

3.1.8 *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.

3.1.9 *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 complex of DNA polymerase and antibody which melts during the initial denaturation step.)

3.1.10 *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.

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

3.1.12 *nested PCR*, *n*—a modification of PCR that uses nested sets of PCR primers to enhance the sensitivity 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.

3.1.13 *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 its optimal temperature. The amplification results in a near exponential increase in the amount of the nucleic acid target defined by the primers.

3.1.14 *PCR buffer*, *n*—a liquid medium that supplies the appropriate salts plus the necessary ionic strength and pH value required for amplification of DNA by PCR. It provides the optimal conditions for functioning of DNA polymerase, enabling PCR amplification if the other cofactors and building blocks (dNTP's) are present.

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

3.1.16 *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. 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.

3.1.17 *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

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

primers at the 3′ ends or self-annealing sites. 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.

3.1.18 *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 (**10**,**11**).

3.1.19 *reverse transcription–PCR, RT-PCR*, *n*—an in vitro laboratory method for using RNA as the target molecule for amplification (**12**). A cDNA copy of the RNA target is made by annealing a complementary 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 3.1.13). The amplification (of DNA) results in a near exponential increase in the amount of the original RNA segment defined by the complementary primer.

3.1.20 *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.

3.1.21 *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.

3.1.22 *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.

3.1.23 *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.

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 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. When primers with high annealing temperature, that is, > 65°C, are being used, the annealing and extension steps can usually be combined.

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. When primers with high annealing temperature, that is, > 65°C, are being used, the annealing and extension steps can usually be combined.

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 **(1-4)** and CLSI MM3-A2.

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 *Reaction Preparation Section*—Area 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 and tubes 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 reaction preparation is necessary, as with commercial kits, the reaction preparation and specimen preparation sections may be combined. One-way work flow and dedicated equipment for each area, however, should be strictly maintained. Reaction 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.

8. Special Equipment/Supplies

8.1 An 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 When one uses a thermocycler without a heated lid or with a PCR reactions of small volumes (< 20 µl), a light mineral oil should be used to prevent altering the reaction volume due to evaporation during the thermal cycling. The mineral oil used should be sterile. 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 CLSI GP5-A2.

8.7 For additional information about PCR reagents, equipment, and detection systems, see catalogs from vendors that supply the biotechnology market. Also, see Section 7 for information on PCR laboratory design and 10.2.2 for information on the need for laboratory clothes, gloves, and hoods.

9. Chemicals and Reagents

9.1 *DNA Polymerase*—A thermostable polymerase that is capable of withstanding elevated temperatures and times of thermal cycling should be used for PCR. Either a purified native enzyme, or a purified, genetically engineered recombinant form of the enzyme can be used. The polymerase should have proven fidelity to ensure accurate replication of the target. The polymerase preparation should not contain substances known to interfere with the PCR. References for interfering substances are given in 3.1.4. With the diverse uses of PCR, different requirements for enzymes are needed, that is, for some systems, increased fidelity is necessary, and in others, relaxing fidelity will obtain the best results. Several vendors supply various polymerases. It is recommended a vendor be chosen that can provide quality assurance information with the enzyme, including the conditions under which the characteristics of the enzyme have been tested. It also is advised that new lots of enzyme or enzymes from new vendors be tested by running parallel tests with the resident enzyme and the new one. It is beyond the scope of this guide to recommend the types of enzyme characteristics needed for different situations.

9.2 *Enzyme(s) for RNA PCR (RT-PCR)*—An enzyme may be used that serves both as a thermostable reverse transcriptase and as a thermostable DNA polymerase. Alternatively, a reverse transcriptase, such as M-MLV or AMV may be coupled with a DNA polymerase. Either a purified native enzyme(s), or a purified, genetically engineered recombinant form of the enzyme(s), can be used. The reverse transcriptase or polymerase, or both, should have proven fidelity to ensure the accurate replication of the target. The reverse transcriptase or polymerase preparations should not contain substances known to interfere with the RT-PCR. Different temperature, fidelity, and processivity characteristics needed for the assay system may dictate the choice of enzyme(s). It is recommended a vendor be chosen that can provide this information, as well as quality assurance information with the enzyme, including the conditions under which the characteristics of the enzyme have been tested. It also is recommended that new lots of enzyme or enzymes from new vendors be tested by running parallel tests with the resident enzyme and the new one.

9.3 *Deoxynucleoside Triphosphates (dNTPs)*—Nucleoside triphosphates used in PCR for the synthesis of DNA include dATP (deoxyadenosine triphosphate), dCTP (deoxycytidine triphosphate), dGTP (deoxyguanosine triphosphate), and dTTP (deoxythymidine triphosphate). Nucleoside triphosphates such as dUTP can be used with many DNA polymerases. Nucleoside triphosphates should be purchased from vendors that have manufactured them for use in PCR. They can be purchased separately or as mixes and should be stored and used according to the vendor's recommendations. The performance of their incorporation into DNA by the DNA polymerase used for PCR should be verified. This can be accomplished by running parallel reactions with the new and the resident reagents. When developing a procedure, the concentration of dNTPs should be optimized. This can be done by varying the concentration of dNTPs over a range of reactions where the other components are kept at constant concentrations and examining each reaction for the best yield, that is, single band of product with expected length by gel electophoresis. A traditional starting concentration has been 200 µM of each dNTP in the PCR reaction.

9.4 *PCR Reaction Buffer*—Materials used for preparation of the PCR reaction buffer should be purchased from vendors which have manufactured them for use in PCR. They should be stable to storage and cycling conditions. The vendor's storage conditions should be noted and followed. In addition to buffer, the cofactor Mg^{++}(or Mn^{++} for rTth) is essential for enzyme activity. It is important to optimize these components and the pH to the target DNA and enzymes being used. Mg^{++}, for example, can be optimized by setting up several reactions, holding all components constant, and varying the Mg^{++} concentration in 0.5-mM increments, or less, over a range, that is, from 1 to 4 mM. This strategy also should be followed for dNTP concentrations that affect the available Mg^{++}.

9.5 *Primers*—In general, selection of oligonucleotide primers is determined by the sequence conservation, base composition, and size of the target nucleic acid sequence. Primers for PCR should have sufficient length, usually 20 to 30 bases long. Where possible, the G:C content should be in the range of 40-60 % unless otherwise needed for a specific purpose. Primer pairs having complementary 3′ ends that can result in primer-dimer formation, or primers that form hairpin structures may result in reduced PCR amplification efficacy. The calculated T_m values of both forward and reverse primers should be similar. Additionally, oligonucleotides with annealing temperatures between 55 to 65°C should be selected. The base composition at or near the 3′ end of the primer is critical for amplification efficiency. It has been shown that primers terminating in a 3′-thymidine residue provide more tolerance to mismatched bases (**13**). Therefore, generally it is recommended that primers end in a 3′- G or C. Several data bases (**4**) (see also National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health data bases) and computer programs (for example, on the Internet) are available to assist in primer design. If primers are synthesized in-house, they should be synthesized on dedicated columns, especially free of any prior reactions that contain the proposed target. Primers can be purified by any method shown through laboratory research to yield primers of adequate purity, such as HPLC or polyacrylamide gel electrophoresis. Such method should produce material with the purity needed for a specific reaction, for example, some reactions do not require pure primers, and some require a purity of 90 % or greater. For infectious disease

research and diagnostics, high-purity primers usually are required. When purchasing primers from vendors, select those that will supply a certificate of analysis (CA) with each shipment. The CA should contain the sequence synthesized, the molecular weight of the primer without any attachments, the purity and the method used to purify, as well as base composition analysis. Testing new primers in parallel reactions with proven lots and in several negative reactions (no target DNA added) is recommended.

9.6 *Target DNA*—The target DNA to be amplified should be extracted from the detection material, for example, tissue or body fluid, in such manner and be sufficiently liberated from associated proteins, that amplification is possible under the PCR reaction conditions selected. See 6.1 for further discussion of target material preparation.

9.7 *Target RNA, When RNA is Subjected to PCR*—The desired RNA should be present in sufficient amount and purity to allow reverse transcription to cDNA to occur. The generated cDNA should be free of substances known to inhibit PCR. See 6.1 for further discussion of target material preparation. In addition to the considerations for preparation of DNA, using RNA as target material demands that special attention be paid to obtaining, transporting, storing, and preparing the sample material to preserve the integrity of the RNA. Use of RNAse-free supplies and equipment or addition of an RNAse inhibitor, or both, should be employed. Storage of prepared RNA should be at – 70°C.

9.8 *DNA Probe, Optional*—In general, selection of oligonucleotide probes is determined by the sequence conservation, base composition, and size of the target nucleic acid sequence. Oligonucleotide probes selected for PCR should have sufficient length, generally 20 to 35 bases long, and G:C content for the particular application. They should be free of extraneous oligonucleotide fragments that are formed during their synthesis. Many of the criteria used in choosing primers (see 9.5) are applicable to choosing and incorporating probes. The probes should not contain any sequences complementary to either of the primers. Labeled probes can also be used.

9.9 *Wax for Hot Start, Optional*—Commercially prepared sterile wax with a melting point at DNA denaturation temperature used to facilitate hot-start PCR. In hot-start PCR, all components for the reaction are mixed together as usual, but one critical component is left to be added after DNA denaturation. By using a hot-start wax, the mixture and key component are separated by a wax layer and are only mixed when the DNA denaturing temperature is reached and the wax melts.

10. Conditions Preliminary to the Performance of PCR

10.1 *Optimization*—Each PCR assay system should be optimized. When the system is properly optimized, the result will be efficient amplification only of the nucleic acid segment of interest. Optimization includes experimentation to choose the most effective enzyme(s) and its concentration(s), concentration of dNTPs, length, concentration and annealing temperature of (**9**) of primers, concentration of ions, such as Mg^{++}, Mn^{++}, K^{+}, concentration of buffer salt(s), and the correct cycling times and temperatures. The optimum number of cycles depends primarily on the starting concentration of target DNA. Too many cycles (for example, > 40 (**5**)) can result in nonspecific PCR products. Optimization usually is accomplished by varying one parameter in a series of reactions where all other components and parameters are held constant. Optimization studies are usually performed with pure nucleic acid as the target, that is, phenol-extracted, provided all the phenol is subsequently removed. After amplification, the resulting PCR products are usually examined by gel electrophoresis for signs of non-specific amplified products, primer-dimers, low yield, or the wrong product band size. Depending on the application, the parameters providing the sharpest, clearest, most visible amplified band are selected (see 11.2.4).

10.2 *False Positive Results*—One of the major concerns of the PCR procedure is the danger of false positive results. If one is not experienced with using the proper techniques for avoiding contamination/carry-over, and is furthermore if not extremely careful, one can readily obtain false positive results. It is well known that inexperienced PCR users often obtain carry-over/contamination in their reaction tubes. There are several major sources of contamination: cross contamination between samples, contamination with plasmid or phage DNA used as controls, and contamination with PCR product carried-over from a previous, positive reaction. Recommendations to avoid contamination are as follows:

10.2.1 As described in 7.1 of this guide, these different stages of the PCR procedure should occur preferably in separated rooms or at least in separated areas within one room: setting up of the PCR reaction mixture, sample preparation, instrumental amplification of the DNA, and subsequent analysis of the amplified DNA.

10.2.2 The danger of contamination/carry-over can further be minimized when suitable pipettes are used (see 8.2), when laboratory coat and gloves are changed for each of the procedural steps, and when pipetting of DNA samples and reaction mixture into PCR sample tubes is performed in a containment hood or biological safety cabinet. Additionally, there should be a unidirectional traffic flow of reagents and tubes from pre-amplification to post-amplification work areas within a laboratory. See Refs (**4, 14,15, 16, 17**) for further useful information concerning steps that should be taken to avoid on avoiding carry-over/contamination in a laboratory.

10.2.3 Chemical and enzymatic methods (**14-16**) of PCR product carry-over control can be applied to the basic PCR methodology, but cannot always be applied when using nested PCR. Nested PCR, therefore, should be used with caution.

10.3 *False Negative Results/Testing for PCR Inhibitors*—In addition to those substances known to inhibit PCR (**10, 11**), there may be substances inherent to a particular sample type or preparation process that introduces inhibitors. Strategies to test for inhibitors include the following:

10.3.1 Amplifying an endogenous gene or conserved part of the gene expected to be present in the sample. This can be done as a separate amplification or as a co-amplification.

10.3.2 Spiking-in the target nucleic acid at a copy number near the threshold of the assay in all negative specimens.

10.3.3 Spiking-in a target of different nucleic acid sequence and co-amplifying. Look for size or sequence differences by gel or probe.

10.3.4 Spiking-in a random nucleic acid sequence with the same primer sequence as the target, co-amplifying, and detecting by probe or size difference.

10.3.5 Testing several dilutions of the prepared sample. If amplification becomes more efficient, that is, greater amount of PCR product formed, as the dilution is increased, an inhibitor is probably present.

10.4 Use of the preceding strategies will depend on the test system being developed.

11. Performance of PCR

11.1 When a suitable PCR laboratory is available (Section 7), the target nucleic acids have been carefully prepared (6.1), PCR inhibitors have been removed or avoided as much as possible (6.1), the necessary equipment and supplies are accessible (Section 8), the appropriate chemicals and reagents have been obtained (Section 10) and the preliminary conditions have been met (Section 10), then PCR can be properly performed. However, several additional, important concerns should be addressed

11.2 *Assay*:

11.2.1 *Number of Amplified Segments*—A decision is necessary concerning how many different segments of the target nucleic acid sequence should be amplified, that is, how many different primer pairs should be used for a given assay. In many cases, amplification of a single genomic segment of the target DNA is suitable for identification of the target DNA if the primer pair used is properly selected and optimized. The diagnostic value, however, can be increased by amplification of several different target sequences of the nucleic acids to be detected.

11.2.2 *Protocol*—Many different protocols have been designed for the PCR detection of various nucleic acids. Because technology in PCR research and diagnostics is developing so rapidly, it is not possible to describe a single protocol or set of conditions that would apply to all situations. If you are a first-time user of PCR and need general advice for getting started, for example, information concerning amounts of reaction mix components necessary, order of addition of components, temperature cycle profiles, and so forth, it is recommended that you consult basic textbooks for example, **(3, 17)** and contact manufacturers of PCR reagent kits for research use.

11.2.3 *Instrument Calibration*—The automated cycling instrument should be calibrated periodically and the calibration should include such items as well-to-well temperature consistency within a cycle, length of cycle time, and number of cycles. For temperature calibration, the temperature should be accurate to ±1°C. Instrument calibration kits can be purchased from some manufacturers and should be used, if available. For further information, see CLSI MM3-A2.

11.2.4 *Detection–Analysis of the Amplified DNA Segment*—The amplified target DNA should subsequently be analyzed. Standard operating procedures for electrophoresis, HPLC, chromatography, solution hybridization, solid phase, or other detection system are readily available from vendors. The analysis of an amplified PCR product should include a step to ensure that the product is specific, such as hybridization with a specific DNA probe.

11.2.5 *Use of Nested PCR*—This procedure is useful for many PCR applications. An increased risk of carryover contamination, however, is associated with this procedure, and this can be a problem for clinical diagnostic applications.

11.3 *Inclusion of Controls*—The following controls are highly recommended, but are not limited to:

11.3.1 A low-level positive control that contains a definite number of the target sequence copies; the number of copies that gives a positive response depends on the detection system used.

11.3.2 A negative control that contains sufficient DNA for PCR (RNA for RT-PCR), but no detectable target DNA (RNA).

11.3.3 A reagent control free of contaminating DNA may be used.

11.3.4 An internal control that is co-amplified with the target sequence may be used for special purposes. This control is a nucleic acid sequence that is co-amplified with the target nucleic acid to identify specimens containing substances that may interfere with PCR amplification. An internal control can be a random nucleic acid sequence containing the same primer binding regions as the target or a unique nucleic acid sequence amplified by an independent primer pair. The internal control can be added to the amplification reaction or spiked into the specimen preparation reagents.

11.3.5 During the sample preparation stage, it is recommended that one set up an isolation control using pure, distilled water as a sample and proceed through all the steps as with actual samples. This negative control tests whether or not there is any contamination in the nucleic acid isolation reagents.

12. Evaluation and Interpretation of Results

12.1 The analysis of amplification products should be documented, for example, through photographs of gels, densitometer scans of gels, preserved hybridization filters, preserved autoradiograms, or photographs of these, or through printouts of absorbance or fluorescence measurements.

12.2 An algorithm for determining positivity and negativity of results should be established and validated; see page 24 of Ref **(3)**. Such algorithm may depend on the specific target to be detected and on the number of primer pairs employed for detection.

13. Report (for Diagnostic Applications)

13.1 In addition to the provisions of this guide previously described, a concise, written report of the assay should be prepared and should include the following information:

13.1.1 The gene segment that was amplified and the method that was used to specifically detect the amplification product.

13.1.2 If a step is included that involves reverse transcription of RNA to cDNA, this information should be provided.

13.1.3 Results of the assay including controls. A statement should be made as to whether the assay result is positive, negative, or questionable.

13.1.4 Information about the source of the nucleic acid sequence to be amplified should be given.

13.1.5 The sequences of all primers and probes used in the assay should be given.

13.1.6 The temperature, holding times, ramp times, number of cycles, type and units of polymerase(s) used, concentrations

of primers, Mg^{++} and dNTPs, presence of chaotropes, and so forth, should be given.

14. Quality Assurance

14.1 The technical, chemical, and enzymatic requirements associated with the course of the reaction should be tested through the use of suitable controls (see 10.3 and 11.3).

14.2 The reagents used for sample preparation and amplification should be monitored for the presence of target DNA that could lead to false positive results.

14.3 The methodology used to detect amplified DNA segments should be tested with regard to sensitivity and specificity.

14.4 Proficiency panels made up of known positive and negative samples that are blinded to the investigator may be used to determine if the investigator is capable of running the test and such panels are recommended for diagnostic applications.

14.5 Each instrument used should be routinely calibrated as recommended by the manufacturer to ensure optimum function.

15. Troubleshooting

15.1 There are many variables associated with PCR. Consequently, if extreme caution is not used, errors may occur, and these can lead to such results as weak PCR product, no PCR product at all, nonspecific product(s), smearing of bands, primer-dimer formation, false-positive results, false-negative results, and so forth. The use of suitable controls (see 10.3 and 11.3) will aid in the interpretation of unexpected results. It is beyond the scope of this guide to include a troubleshooting manual. Some textbooks discuss PCR technical problems that can occur (**4**). It is also recommended that contact be made with technical support representatives of PCR reagent vendors for PCR troubleshooting advice.

16. Keywords

16.1 amplification; diagnosis; DNA probe; hybridization; PCR; polymerase chain reaction; primer

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