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Standard Guide for Detection of Nucleic Acids of the Mycobacterium Tuberculosis Complex and Other Pathogenic Mycobacteria by the Polymerase Chain Reaction Technique[1]

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INTRODUCTION

This guide covers detection of nucleic acids (deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)) of mycobacteria and specific identification of the Mycobacterium tuberculosis complex (MTBC, which includes *M. tuberculosis, M. bovis, M. microti* and *M. africunum*), *M. avium, M. leprae* and *M. intracellulare* nucleic acids by the polymerase chain reaction (PCR) technique.

PCR is a widely known molecular biology procedure that involves the amplification of a piece of DNA by as much as a million-fold over the course of several hours. It is also possible to use PCR to detect RNA by first copying RNA with the enzyme reverse transcriptase to produce a complementary DNA molecule, which is then amplified by PCR; the combined process is known as RT-PCR. The amplified DNA fragments can then be detected, identified and quantitated by classical procedures of biochemistry/molecular biology. As few as only several molecules of DNA in a biological test specimen can be rapidly and accurately identified. PCR is used as a tool in molecular biology laboratories for basic and applied research, in clinical laboratories to aid in the diagnosis of genetic, neoplastic and infectious diseases, and in biotechnology laboratories for the preparation of biotechnology products and to test for contaminants.

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis (TB) in humans, and TB is one of the leading causes of human death in the world (**1, 2**)[2]. About one third of the present world's population is infected with MTB (**1**). The definitive test for tuberculosis, isolation and specific identification of MTB in culture, requires several weeks. Microscopic examination of acid-fast smears is rapid, but non-specific and relatively insensitive. The value of using PCR or other DNA/RNA amplification procedures for TB diagnosis is rapidity; clinical specimens can be evaluated within a day. Thus, patient care and treatment can be initiated more rapidly when a specific diagnosis has been determined.

This guide was developed by ASTM in collaboration with DIN (German Institute for Standardization) Subcommittee E3/E9 on Molecular Biological Detection of Mycobacteria, Department for Medical Standards (NAMed). It is recommended that this mycobacteria-specific PCR guide be used in conjunction with ASTM's general PCR Guide E 1873. The combination of the two guides provides recommendations, basic considerations, criteria, and principles that should be employed when developing, utilizing or assessing PCR-specific protocols for the detection of the DNA or RNA of specific mycobacteria.

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

1. Scope

1.1 This guide covers basic considerations, criteria, principles and recommendations that should be helpful when developing, utilizing, or assessing PCR-specific protocols for the amplification and detection or identification of mycobacterial nucleic acids. This guide is not a specific protocol for the detection of specific mycobacteria. It is intended to provide information that will assist the user in obtaining high quality

[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.

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

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and reliable data. The guide is closely related to and should be used concurrently with the general PCR Guide E 1873.

1.2 This guide has been developed for use in any molecular biology or biotechnology laboratory. It may be useful for the detection of mycobacteria in clinical, diagnostic laboratories.

1.3 This guide does not cover details of the various methods such as gel electrophoresis that can be utilized to help identify PCR-amplified mycobacterial nucleic acid sequences, and it does not cover details of instrument calibration.

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

1.5 **Warning**—Laboratory work involving certain clinical specimens and microorganisms can be hazardous to personnel. **Precaution**: Biosafety Level 2 facilities are recommended for potentially hazardous work, and Biosafety Level 3 facilities are required for propagating and manipulating Mycobacteria tuberculosis cultures (**3**). Safety guidelines should be adhered to according to NCCLS M29-T2, I17-P and other recommendations (**3**).

2. Referenced Documents

2.1 *ASTM Standards:* [3]

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

2.2 *NCCLS Standards:*[4]

M29-T2 Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue-Second Edition; Tentative Guideline

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

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

3. Terminology

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

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *internal control*, *n—in PCR*, a control used to assess the amplifiability of the reaction, that is, to determine whether or not PCR inhibitors may be present in the reaction; for example, an internal control can be a synthetic DNA segment that can be added to the sample prior to amplification, that is of similar length and base composition to the target gene sequence, and that contains primer binding regions identical to those of the target sequence.

3.2.2 *restriction enzymes*, *n*—naturally occurring proteins (also called restriction endonucleases) that are purified from bacteria and that recognize specific nucleic acid sequence patterns (sites) and cleave the nucleic acid at or near that sequence (site).

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[4] Available from the National Committee for Clinical Laboratory Standards, 771 E. Lancaster Ave., Villanova, PA 19085.

4. Significance and Use

4.1 This guide is intended for use in any laboratory utilizing PCR or RT-PCR to amplify and detect nucleic acid sequences of mycobacteria from a biological preparation and to identify the species of origin.

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 MTB.

5. Background Information about TB, MTB, Other Mycobacteria, and Detection of Mycobacteria by PCR

5.1 The mycobacteria are acid-fast, non-motile, rod-shaped, aerobic bacteria that do not form spores. They contain several species pathogenic to humans. The primary human pathogens are members of the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum* and *M. microti*) and *M. leprae*. Other mycobacteria, such as the *M. avium* complex (*M. avium* and *M. intracellulare*) and *M. kansasii*, cause disease in immunocompromised individuals.

5.2 Tuberculosis has been a major public health problem in the world for many centuries, particularly in overcrowded city areas. In the 18th and 19th centuries, TB was responsible for a quarter of all adult human deaths in European cities (**4**). Several public health measures that finally led to a considerable improvement include the pasteurization of milk, and the antibiotics streptomycin (introduced in 1944) and p-aminosalicylic acid (introduced in 1946) (**4**). However, the emergence of drug-resistant MTB strains is becoming a serious problem. For this and other reasons, TB is presently on the rise again and is one of the leading infectious causes of death of adult humans in the world.

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5.2.1 The isolation of a species from the MTBC is required for the definitive diagnosis of tuberculosis. Routine cultures are time-consuming and can take up to eight weeks. Microscopic examination of acid-fast smears is the most rapid method for the detection of mycobacteria, but it is insensitive and non-specific. Immunological and serological techniques are limited, in general, due to poor sensitivity or specificity, or both (**5, 6**). Species-specific nucleic acid probes have significantly improved the opportunity for rapid confirmation of culture results for several mycobacterial species (**7**).

5.3 The *M. avium* complex (MAC) consist of 28 serovars (subspecies) of two distinct species, *M. avium* and *M. intracellulare* (**8**). The criteria used to distinguish *M. avium* from *M. intracellulare* are now well established (**9-11**). MAC organisms are ubiquitous in nature and have been isolated from water, soil, plants, and other environmental sources (**12**). These organisms are of low pathogenicity and frequently colonize healthy individuals without causing disease. Person-to-person transmission is thought to be unlikely, but many investigators believe that pulmonary disease may result from inhalation of infectious environmental aerosols. MAC infections have become increasingly more common in the United States, and *M. avium* is the most common non-tuberculous mycobacterial species associated with human disease. The greatest increase in MAC infections during the past decade has been in AIDS patients for whom MAC has become the third most common

opportunistic disease (**13**). In one report, 98 % of MAC infections in 45 AIDS patients were due to *M. avium*, whereas 40 % of MAC infections in patients without AIDS were due to *M. intracellulare* (**14**).

5.3.1 The isolation of MAC by culture is required for definitive diagnosis of MAC infection. However, routine cultures are time consuming and can take up to eight weeks for final diagnosis. Additionally, culture does not distinguish between *M. avium* and *M. intracellulare* infection. Several of the therapeutic agents used currently in the treatment of MAC disease have different in vitro activity against *M. avium* and *M. intracellulare* (**15**). As additional therapeutic agents become available, the ability to distinguish between *M. avium* and *M. intracellulare* may become more important.

5.4 *Mycobacterium leprae*, the causative agent of leprosy, remains a serious health problem worldwide (**16**). Diagnosis of *M. leprae* infections is a problem due to the fact that this organism cannot be cultured by conventional methods. Classical methods of diagnosis, such as microscopic examination of skin biopsies and antibody testing lack sensitivity and specificity (**17**). However, PCR can be used for rapid and sensitive diagnosis of *M. leprae* infections.

5.5 PCR has proven to be a useful procedure for the detection of mycobacteria and the specific identification of the species present. Mycobacterial DNA in a clinical sample is typically extracted and amplified, and the PCR product is then identified. Depending on the PCR amplification target employed, the analytic sensitivity of amplification assays ranges from about 1 to 100 mycobacteria (**17**). Primers used in the PCR amplification from mycobacterial genomes can be either species-specific or genus-specific. PCR-based tests specific for mycobacteria have been shown to improve the rapid diagnosis of tuberculosis and other mycobacteria-caused diseases by allowing the direct detection of mycobacteria in clinical specimens.

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5.5.1 Clinical studies have demonstrated that PCR-based assays accurately detect MTB in respiratory specimens (**18-22**). The specificity of these tests ranges from 95 to 100 %. Furthermore, these MTB-specific tests do not cross-react with other mycobacteria. Clinical specimens that yielded non-tuberculosis mycobacteria when cultured gave negative results when tested by MTB-specific PCR assays. The MTB PCR assays generally exhibit greater than 90 % sensitivity for the subset of clinical specimens that are positive for acid-fast bacilli (AFB) positive by microscopy. These assays exhibit lower sensitivity, ranging from 50 to 75 %, for the subset of clinical specimens that are AFB-negative. The limited sensitivity is attributed to the low organism load in AFB-negative specimens. Because the volume of specimen used to inoculate cultures is usually much greater than the volume tested by PCR, mycobacteria may be delivered to the culture, but not to the PCR reaction.

5.5.2 Clinical studies have shown that PCR assays can detect MAC directly in respiratory specimens and in blood (**23-27**). Many of these assays can distinguish which of the two MAC species, *M. avium* or *M. intracellulare*, is present in the specimen. The specificity of these tests ranges from 97 to 100 %. Furthermore, these MAC-specific tests do not cross-react with other mycobacteria. Clinical specimens that yielded *M. tuberculosis*, or other non-MAC mycobacteria, when cultured gave negative results when tested by MAC-specific PCR assays. Sensitivity for MAC is generally greater than 90 %.

6. Principle of the PCR Method

6.1 See Guide E 1873 (Section 5) for a description of the PCR method.

6.2 In addition to 6.1, the amplification of target sequences takes place in vitro. Procedures for detection of specific amplified products that allow a differentiation to be made between species of the MTBC and ubiquitous mycobacterial species should be used preferentially.

7. Target Material

7.1 For general information see Guide E 1873 (Sections 6 and 9).

7.2 In addition to 7.1, for the detection of nucleic acid sequences of the MTBC, immediate transport of the test material to the laboratory is recommended. It is possible that transport periods of >48 h can interfere with the nucleic acid detection.

7.3 Typical biological specimens for the detection of mycobacteria or their nucleic acid sequences include:

7.3.1 *Specimens Used in Biotechnology or Basic Molecular Biology Research Laboratories*—Cultures of mycobacteria, purified preparations of mycobacteria or mycobacterial nucleic acid.

7.3.2 *Specimens Used in Clinical, Diagnostic Laboratories in the Case of Suspected MTB Infection*—Respiratory sample material (for example, sputum, bronchoalveolar lavages), gastric juice, stomach aspirate, urine, sperm and prostatic secretion, puncture exudate, cerebrospinal fluid, bone marrow and biopsy material (tissues).

7.4 Methods currently used for extraction of the target sequence from mycobacteria are:

7.4.1 *Alkaline Lysis*—In the presence of sodium hydroxide and detergent with increased temperature and subsequent neutralization of the reaction mixture.

7.4.2 *Guanidium Isothiocyanate*—Extraction.

7.4.3 *Enzymatic Extraction*—Of nucleic acid using proteinase K plus lysozyme or detergent treatment, or both.

7.4.4 *Mechanical-Physical Pretreatment*—For disruption of the bacterial cells, for example, ultrasound, repeated shock freezing/boiling.

7.4.5 A combination of the procedures described in 7.4.1 to 7.4.4 is possible. Methods used should be tested in the laboratory with respect to their efficiency and reproducibility through the introduction of appropriate control bacteria.

7.5 Substances that inhibit PCR such as heparin, hemoglobin, etc., should be considerably reduced in amount through the extraction procedures used. When extraction procedures do not reduce inhibitors sufficiently, typical methods for reducing the amount of inhibitors, such as solid phase adsorption of the nucleic acids or inhibitors, phenol-chloroform extraction, etc., may be used to remove inhibitors. Purification of the extracted nucleic acids by solid phase adsorption, for example, to silicates or resins, can lead to increased purity, but may also result in considerable losses of nucleic acids. This guide

recommends that an internal control nucleic acid sequence be added to each sample to monitor amplification and detect samples that inhibit PCR (**28**).

8. PCR Laboratory Design

8.1 Follow instructions for PCR laboratory design according to Guide E 1873 (Section 7) and NCCLS MM3-A (Section 12).

9. Special Equipment/Supplies

9.1 *General*—See the recommendations of Guide E 1873 (Section 8) and NCCLS MM3-A (Section 12).

9.2 In addition to 9.1, equipment that ensures safe disruption of mycobacteria, such as programmed incubators, enclosed sonicators, or the equivalent is needed.

9.3 Nucleic acids from mycobacteria of the MTBC and from clinical specimens containing *M. tuberculosis, M. avium* or *M. intracellulare* should be isolated in Biosafety Level 2 (BSL2) or BSL3 laboratories.

10. Chemicals and Reagents

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

10.2 *Mycobacteria PCR Primers*—The selected target DNA sequence should be bounded by nucleic acid sequences specific to mycobacterial DNA; these sequences will serve as primer binding sites. The primer binding sequences shall be unique to mycobacteria to ensure that DNA of other organisms is not amplified along with the mycobacterial DNA. The primer binding sequences also shall be highly conserved among all mycobacterial species that need to be detected. If the primer binding sequences are not well-conserved, certain mycobacterial species with substantially divergent sequences might not get amplified.

10.2.1 Several primer pairs may be used to enhance specificity. Each primer pair can simultaneously amplify a different target sequence within the mycobacterial genome. Alternatively, one target sequence can be amplified using a single primer set to generate an initial amplification product. Then, a second set of nested primers, which recognize sequences within the initial amplification product, can be used to generate the shorter, final amplification product (see 10.2.1.2).

10.2.1.1 *Multiple Targets*—The likelihood of false-positive results is reduced by defining a positive result as the presence of multiple, specific amplification products. Reactions inadvertently contaminated with a single DNA target sequence will contain only a single amplified target and not be classified as positive. Reactions containing non-specifically amplified DNA that mimics one of the target sequences will lack the other specific targets and not be classified as positive.

10.2.1.2 *Nested Amplification*—The primary target is amplified using an outer set of primers to generate an intermediate that serves as the target for the second set of internal primers. This two-stage amplification process can generate more final product, thereby increasing analytical sensitivity. Specificity will be enhanced, because the final amplification product will be formed only if the correct intermediate is formed. Products formed by non-specific interactions between the first set of primers and extraneous DNA will not contain binding sites for the second set of primers and, thus, will not generate the characteristic final product. Caution should be taken when using nested PCR, according to the general PCR Guide E 1873.

10.2.2 The target sequence recognized by the primers may be present in a single copy in the mycobacterial genome or may be present in multiple copies. Because they are present in multiple copies in each organism, repetitive target sequences provide a natural amplification that may enhance the analytical sensitivity of the assay.

10.2.3 The primer binding sequences may be specific to one species of mycobacteria or may be conserved among several species of mycobacteria. Species-specific primers should be used when the assay is required to detect only one species of mycobacteria. Primers that are conserved among several species should be used for applications where it is desirable to detect any, or all, of the different species of mycobacteria that may be present in a sample. An example of the latter application is clinical diagnosis. Specimens from patients suspected of having mycobacteria infection may contain any one of several pathogenic species. A diagnostic test shall be able to detect all of the species and identify which of them is actually present.

10.2.3.1 *Species-Specific Primers*—The sequences recognized by these primers shall be unique to the species of interest and shall not be found in other mycobacterial species. Table 1 lists several species-specific primers for the amplification of the MTBC.

10.2.3.2 *Multiple Species Primers*—These primers should contain sequences that are conserved in all of the mycobacterial species of interest. If it will be necessary to identify the particular species present in a sample, the primers should be positioned so that a portion of the sequence between them differs among the target species. A set of species-specific probes that are homologous to this unique region can be hybridized to the amplified DNA for species identification. Table 1 lists several primer pairs that amplify DNA from multiple mycobacterial species and yield products that can contain unique sequences for species identification.

10.3 *Detection of Amplification Products*—The method used to visualize reaction products shall distinguish amplified target DNA from DNA generated from non-specific interactions between primers or between primers and non-target DNA.

10.3.1 For amplification products generated by species-specific primers, gel electrophoresis can be used to demonstrate that the product matches the expected size of the amplified target DNA. Alternatively, more specific detection techniques, such as restriction enzyme digestion or hybridization to a DNA probe, can be used to show that the products contain sequences characteristic of amplified target DNA and should be used in clinical laboratory practice.

10.3.2 Amplification products generated by primers that interact with multiple species may be detected using generic techniques that do not identify the individual species present, or they may be visualized using species-specific detection methods. Generic detection techniques can be used to screen for specimens that contain mycobacteria. This can be accomplished by gel electrophoresis or by using probes or restriction

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