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Real-time polymerase chain reaction assay for the detection of M. tuberculosis

Karen Lynn Morgan
San Jose State University

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REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF *M. TUBERCULOSIS*

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William Murray

Dr. William Murray

Chris Brinegar

Dr. Chris Brinegar

Ruthann Kibler

Dr. Ruthann Kibler

Gerry Guibert

Mr. Gerry Guibert, MPH Director of Montery County Public Health Laboratory

APPROVED FOR THE UNIVERSITY

Thea L. Williamson 07/26/05

Abstract

Real-time Polymerase Chain Reaction for the Detection of *M. tuberculosis*

by Karen Lynn Morgan

The resurgence of tuberculosis is a serious public health issue in the United States and a leading cause of death worldwide. The causative agent, *M. tuberculosis*, is a slow growing bacterial pathogen. Consequently, diagnostic testing based on conventional culture techniques is delayed. The Center for Disease Control recommends nucleic acid amplification testing on clinical specimens to speed the diagnosis of tuberculosis, thereby enabling prompt treatment. This thesis describes the development of a real-time polymerase chain reaction (RT-PCR) assay which yields rapid, sensitive and specific detection of *M. tuberculosis* in clinical specimens. The RT-PCR assay was designed to target the ITS region of the 16S rRNA gene of *M. tuberculosis* using Taqman hybridization probes for detection of amplicons. Analyzing 231 respiratory specimens composed of 76 specimens from culture-confirmed tuberculosis cases and 155 culture negative specimens, the developed RT-PCR assay achieved a sensitivity of 85.5% and specificity of 100%.

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Introduction

The resurgence of drug-susceptible and multi-drug-resistant tuberculosis is a serious public health issue in the United States and in developing nations (8). The causative agent of tuberculosis, *Mycobacterium tuberculosis,* is a slow-growing bacterium requiring two to eight weeks for growth in broth or culture media (28). This extended growth period delays the detection, diagnosis and effective treatment of patients with tuberculosis. Development of a rapid and specific assay for the detection of *M. tuberculosis* infection would be of great public health value, as prompt diagnosis expedites patient treatment and limits transmission to others. Specific, sensitive, and rapid detection of *M. tuberculosis* can be achieved by nucleic acid amplification (NAA) testing methods. This research describes the literature review and subsequent development of a NAA assay using the real-time polymerase chain reaction (PCR) method to detect *M. tuberculosis* in respiratory tract specimens.

The Public Health Significance of Tuberculosis

Today, over eight million new cases of tuberculosis (TB) develop each year worldwide. Annually, three million people die due to *M. tuberculosis* infection, earning it the title of the most lethal bacterial pathogen (48, 55). In 2003, over 15,000 new cases of tuberculosis occurred in the United States. The Centers for Disease Control (CDC) recognized a resurgence of TB beginning in 1985 (11). Cases of TB among immigrants from TB-endemic nations contributed to this resurgence in the U.S. Outbreaks of TB among the homeless and in crowded settings like hospitals, correctional facilities, and

hospices facilitated further spread of the disease. The increased number of TB infections among the immunocompromised population infected with HIV also promoted the resurgence of TB. Additionally, delays in recognizing, the emergence of multiple drug-resistant strains of *M. tuberculosis* allowed these strains to spread (11). TB remains well entrenched in the immigrant, homeless and immunocompromised populations, thus the CDC has called for more rapid and specific methods of diagnosing TB (9).

Table 1 shows the distribution of new TB cases found in different ethnic populations in 2003 (8).

TABLE 1. New TB cases in the U.S. population, 2003.

Race/Ethnicity	New TB Cases/100,000 people	Percentage of New TB Cases 2003
American/Alaskan Indian	149	1
Asian	3,421	23
Black, non-Hispanic	4,165	28
Hispanic	4,165	28
White	2,826	19
Hawaiian or Pacific Islander	149	1
Total	14,874	100

Source: CDC: Division of tuberculosis elimination, Surveillance report, 2003, (8).

Strategies to combat the resurgence of tuberculosis. In 1989, the CDC published the *Strategic Plan for the Elimination of TB in the United States*. This plan set a goal of reducing TB to one new case per million people by 2010 (9). The interim goal was three and a half cases per 100,000 people by 2000. However, with the resurgence of TB the CDC's TB elimination efforts were set back by a decade (9). The CDC strategy to combat the TB resurgence involves improving and accelerating laboratory diagnosis, strengthening surveillance, increasing direct observation of therapy (DOT) and

expediting the investigation and treatment of close contacts of TB patients (11). The CDC national TB surveillance system revealed a baseline of five new cases per 100,000 people in 2002. The revised target set for 2010 is one new case per 100,000 people (11).

In order to reduce the incidence of new TB cases by 2010, the U.S. Department of Health and Human Service's goal is to have 90% of new TB patients completing their curative therapy within 12 months (47). The present baseline is 74% of patients completing therapy within a year (47). TB control programs must ensure that TB patients complete their therapy, since patients who do not complete the full course of antimicrobial therapy often become ill and contagious again. These inadequately treated patients may be the reservoir for the development of multi-drug-resistant mycobacteria (47). In addition to treating active cases of TB, persons with latent TB infections should also receive therapy since five to ten percent of these patients will develop TB later in life (36).

A report published by the United States Department of Health and Human Services, *Healthy People 2010,* outlined strategies for improving laboratory diagnosis of *M. tuberculosis* by reducing the time needed for a laboratory to confirm and report TB cases (47). The desired target for 2010 is to identify and report at least 75% of positive TB cases within two days. To identify and report positive TB cases presently, it takes an average of two to six weeks (28, 47). This goal constitutes a 90% improvement and requires using rapid NAA techniques. Commercially available NAA tests are capable of detecting *M. tuberculosis* in a clinical specimen within 48 hours of receipt. However, these diagnostic tests require advanced laboratory staff training and upgraded molecular

laboratory capabilities. Nonetheless, the use of NAA testing in the U.S. would facilitate rapid laboratory diagnosis, enable doctors to start therapy sooner thus reducing morbidity from *M. tuberculosis* infections (16).

During the continuing TB resurgence in the U.S., California has led the nation with 20% of the new cases. With the need for renewed TB surveillance in the state, the California Department of Health Services drafted a TB prevention plan (6). The primary goal of this plan was to increase and improve the identification and reporting of all TB cases in the state. Even with the obvious need for the rapid and accurate laboratory diagnosis of *M. tuberculosis* in clinical specimens, only 12% of reported TB cases in California were diagnosed using rapid nucleic amplification methods in 2003 (16).

The national and state recommendations encouraging the use of rapid and specific NAA testing methods, the availability of PCR instrumentation, and the need to rapidly diagnose the tuberculosis patients in Monterey County, California, led to the development of a real-time PCR assay to detect *M. tuberculosis* in clinical specimens.

The Biology of *M. tuberculosis*

Characteristics of *Mycobacterium* species. Mycobacteria are non-spore forming aerobic bacilli with a lipid rich cell wall. Over fifty *Mycobacterium* species are described most of which are non-pathogens normally found in the environment, while others are opportunistic pathogens. In contrast, *M. tuberculosis* is a strict pathogen with humans as the only natural reservoir (36). Mycobacteria are weakly Gram-positive bacilli but do not stain well with Gram stain reagents. The lipid and mycolic rich cell

wall (60% dry weight), causes the organisms to be resistant to many stains as well as disinfectants. The most frequently used staining method, the Ziehl-Neelson stain, uses heat to facilitate the uptake of carbol fuschin, the primary dye, by the complex cell wall. The bacilli cannot be decolorized with acid alcohol solutions and thus retain the carbol fuschin. Therefore, mycobacteria are referred to as "acid fast bacilli" or AFB (36).

Due to their complex cell wall and fastidious growth requirements, mycobacteria are slow growing compared to most other bacteria. The generation time for *M. tuberculosis* is 18 to 20 hours compared to 20 minutes for *Escherichia coli* (36). Colonial morphology of *Mycobacterium* is species variable. For example, the colonies of *M. avium* are transparent, domed and smooth compared to the friable, cord-like, and rough colonies characteristic of *M. tuberculosis.* Some *Mycobacterium* species are pigmented independent of light exposure (scotochromogens), some require light to develop pigment (photochromogens), and some species are non-pigmented or buff colored like *M. tuberculosis* (34).

(i) *M. tuberculosis* complex. Four species comprise the *M. tuberculosis* complex: *M. tuberculosis, M. microti, M. bovis,* and *M. africanum.* These species can be differentiated with difficulty by biochemical testing (28). However, clinically they cause the same disease and respond to the same antimicrobial drug therapy so as a practical matter they are treated as a single entity. However, epidemiological evidence indicates that the majority of tuberculosis cases in North America are caused by *M. tuberculosis* (13).

(ii) Genetics of *M. tuberculosis*. Another defining characteristic of mycobacteria is the high guanosine and cytosine (G+C) content of their DNA. The G+C content is approximately 61-71% which is a characteristic of *Mycobacterium* (34). The most widely studied genome sequences of *M. tuberculosis* are the chromosomally located insertion sequence IS6110 and the 16S rRNA gene. These genetic sequences can be used to differentiate mycobacteria from other microbial flora in clinical specimens (33).

(iii) IS6110. Many laboratories have developed in-house PCR tests based upon the IS6110 insertion sequence of mycobacteria (7, 25). Several genetic elements in IS6110 are unique to *M. tuberculosis* (29). Between one and 25 individual copies of IS6110 are found in various regions in the genome of *M. tuberculosis* (29). The variations in copy number are used for identification and epidemiological tracking of *M. tuberculosis* strains using restriction fragment-length polymorphisms (RFLP) analysis (29). IS6110 is mobile and is known to insert itself in various places in the genome, disrupting coding regions 64% of the time, which leads to errors in the reading frame (29). Therefore, other genomic sites have been investigated in search of more stable target regions for identification purposes (29).

(iv) 16S rRNA. While the IS6110 insertion element of *M. tuberculosis* has been used extensively, some researchers consider the analysis of the 16S rRNA gene to be the "gold standard" for bacterial identification (20). In this case, a "gold standard" is defined as the test which provides the most accurate bacterial identification. It is the standard by which the specificity of any other identification method is compared (18). For accurate identification of the *M. tuberculosis* complex, the 16S rRNA sequence is an

ideal target to amplify (30). Analysis of the 16S rRNA gene by the Basic Local Alignment Search Tool (BLAST), using an extensive computerized database of nucleotide sequences available at the website, **www.ncbi.nlm.nih.gov/BLAST** was performed. Portions of the 16S rRNA gene sequence analyzed demonstrated 99% homology with the *Mycobacterium tuberculosis* complex nucleotide sequence (43). The 16S rRNA gene contains conserved sequence regions specific for the *Mycobacterium* genus, flanked by highly variable regions that can be used to specifically differentiate *Mycobacterium* species (20). The *M. tuberculosis* complex, *M. avium* complex, and *M. kansasi* can all be differentiated using this target sequence (31).

Pathogenesis of *M. tuberculosis*

TB is a highly infectious disease, with as few as three bacilli needed to cause infection (36). Generally, TB is transmitted person-to-person by inhalation of infectious aerosols resulting in a primary infection of the lung. While a complete understanding of *M. tuberculosis* virulence is lacking, it is known that when aerosolized *M. tuberculosis* reach the alveoli the alveolar macrophages phagocytize the organisms. During this initial stage of disease, the infected macrophages can spread the organism to adjacent lymph nodes and beyond to other tissues of the body via the blood stream (36). Intracellular survival of *M. tuberculosis* is facilitated by a cell wall associated lipid of *M. tuberculosis*, sulfolipid, that inhibits fusion of lysosomes to the phagocytic vacuoles of the macrophages thus preventing phagololysosome formation (44). These virulence mechanisms enable *M. tuberculosis* to evade destruction in the phagosome. Moreover,

the bacilli survive and multiply in the infected macrophages (44). Although *M. tuberculosis* invades and multiples in the alveolar macrophages, circulating macrophages and lymphocytes are attracted to the site of infection by chemotactic factors. The mycobacterial infection stimulates T helper cells (CD4+) and T cytotoxic cells (CD8+). Cytokine release by T cells (CD4 cells) activates the macrophages' ability to phagocytize and destroy mycobacteria (36). The cytotoxic T cells lyse infected macrophages allowing the release of bacilli leading to subsequent bacterial killing by the activated macrophages. Antibody produced by activated B cells is ineffective against the intracellular mycobacteria, but once the bacilli are released, opsonization by antibody can aid in the subsequent phagocytosis of the organisms.

It is unclear why some activated macrophages can kill *M. tuberculosis* and other activated macrophages are infected by the organism (36). Differences in cellular immunity mechanisms of individuals may explain this difference. Recently, CD44, a macrophage-associated adhesion molecule was found to act as a receptor site for attachment of *M. tuberculosis* leading to subsequent phagocytosis of the mycobacteria (32). Furthermore, CD44 mediates macrophage recruitment to an infection site, and is associated with increased destruction of mycobacteria by the macrophage (36).

The pathology of a TB infection is due to the immune response of the host rather than the virulence factors of the mycobacteria (36). Cellular immune responses resulting in cytokine toxicity, activation of the complement cascade, effects of macrophage-derived hydrolytic enzymes, and reactive oxygen intermediates can cause tissue necrosis (36). The amount of tissue damage is dependent on the dose of mycobacteria infecting

the host and the strength of the host immune response. Well-organized microscopic granulomas, called tubercles form in infected tissues. The tubercle consists of multinucleated giant cells formed by the fusion of several macrophages, activated macrophages or epithelioid cells, and a surrounding collar of lymphoid cells (44). Tubercle granulomas can wall off small foci of mycobacteria and prevent their spread (32). However, large necrotic granulomas can be surrounded by fibrin, which protects the mycobacteria from being eliminated by the host immune response. The mycobacteria inside the tubercle can remain viable but dormant for years, until the host's immune system weakens due to old age or an immunosuppressive disease, causing re-activation of infection (36).

Clinical disease. The clinical symptoms of TB varies widely depending on the virulence of the *M. tuberculosis* strain infecting the patient and the immune response of the host. As the patient's cellular immunity improves, mycobacterial replication stops three to six weeks after infection (13). The patient may have a positive tuberculin skin test with purified protein derivative (PPD), but no other indication of active disease (13). It is estimated that 1.9 billion people (one third of the world's population) are infected with TB (36). Five to ten percent of patients infected develop active disease within two years of exposure and another ten percent within their lifetime (36). If left untreated, half of the patients who develop pulmonary TB will die within five years (13).

The clinical symptoms of active pulmonary TB include malaise, weight loss, cough and night sweats. The sputum may be scant or purulent and bloody. The production of bloody sputum (hemoptysis) is associated with tissue destruction and

cavitation in the lung (36). Radiographic evidence of disease, a positive skin test with PPD, and laboratory diagnosis of mycobacteria by smear and/or culture confirm a diagnosis of TB.

Persons with advanced HIV infection are likely to have a primary infection progress to active pulmonary TB in 50% of the cases (13). This is due to the progressive destruction of the immune system caused by HIV, particularly the loss of cell mediated immunity and the reactivation of latent TB infection. Furthermore, in HIV positive patients, TB often disseminates by hematogenous spread to extra-pulmonary body sites, or produces systemic infection (11).

Diagnostic Laboratory Methods to Detect *M. tuberculosis*

Conventional methods. The standard method of *M. tuberculosis* diagnosis is accomplished by examining sputum smears for the presence of AFB stained by the Ziehl-Neelson method. However, smear examination for AFB is only 51-70% sensitive and not specific for *M. tuberculosis* (16, 28). Although the sensitivity of a smear examination is limited, when positive for AFB, it is the first indication of a possible case of TB. An alternative staining method is auramine-fluorochrome staining which is based on the fluorescent dye binding to the mycobacterial cell wall. The sensitivity of sputum examination is greater with this method as it is easier to detect fluorescent bacilli compared to the standard Ziehl-Neelson AFB stain, thus educing smear examination time (28).

Concomitant with the smear examination, mycobacteria have traditionally been detected by culture in liquid and solid media, such as Lowenstein-Jensen agar or Middlebrook 7H11 agar (28). Culture is more sensitive and specific than AFB smear and recovery of the organism allows the precise identification of the pathogen. Culture isolates can be identified by biochemical tests like niacin accumulation , catalase production, and antimicrobial susceptibility. Typically, *M. tuberculosis* grows after 21 days of incubation on a solid media such as Middlebrook 7H11. Commercially available liquid culture systems can reduce culture time, producing detectable growth in 14 days (16). In the past, the liquid media systems for *Mycobacterium* detection used broth with radiolabeled ^{14}C-palmitate. Growth of *Mycobacterium* was detected by the release of ^{14}C labeled CO_2 from the broth. More recently, non-radiometric growth detection systems have been used to detect growth, fluorimetrically or colorimetrically, based on changes in gas pressure, carbon dioxide production, or oxygen consumption respectively (2). The Monterey County Public Health Laboratory (MCPHL) uses the Bactec MGIT 960 instrument (Becton Dickinson, Sparks, MD), that operates on the principle of oxygen consumption combined with a fluorescent sensor that responds to decreasing oxygen concentration. Photodetectors in the instrument measure the level of fluorescence, determining the amount of consumed oxygen, and calculate the increase of microbial growth (2).

M. tuberculosis recovery rates as high as 97% from culture are achieved using both solid media and broth culture systems (16). However, even under optimal

conditions, recovery of *M. tuberculosis* by culture takes 14 days thus delaying definitive diagnosis, effective patient treatment and infection control.

High performance liquid chromatography (HPLC). High performance liquid chromatography (HPLC) has been applied to several well-characterized mycobacterial species (15). Butler analyzed characteristics of the mycolic acids in mycobacteria using the HPLC Beckman System Gold Instrument (Beckman Instruments Inc., San Ramon, CA) and gradient elution chromatography (5). Locations of the identifying peaks were compared to peak patterns of known mycobacteria. The *M. tuberculosis* complex was identified with 98% accuracy. HPLC was found to be a rapid and accurate assay for mycobacteria. However, a disadvantage of the method is the need to culture the organism first resulting in diagnostic delays. Furthermore, few clinical laboratories have the equipment or trained personnel to routinely perform the HPLC analysis.

Molecular Diagnostics

Molecular diagnostic methods are based on testing for the presence of specific nucleotide sequences found in the DNA or RNA of a target organism. In order to be useful, the nucleotide sequence chosen must be unique or specific for the particular organism under investigation. Finding a specific nucleotide sequence of *M. tuberculosis* in a respiratory specimen in a background of normal flora aids in the detection of the pathogen. Moreover, the use of molecular diagnostics greatly reduces the detection time for *M. tuberculosis* complex and increases the specificity of identification (16).

When too few gene copies of a pathogen are present to be detected, molecular techniques are available to amplify the identifying target sequence of the pathogen to

detectable levels. Genetic analysis of pathogen-specific nucleic acid sequences are also performed by restriction fragment or sequencing techniques. These procedures can provide the characterization of gene sequences and subsequently provide pathogen identification.

The advantages of molecular diagnostics are speed, accuracy, specificity, and reducing exposure of laboratory workers to pathogens. Disadvantages of these techniques include possible contamination by environmental nucleic acids or endonucleases and the difficulty of developing an assay for unprocessed clinical specimens that is sensitive and specific enough to be of diagnostic use.

Sensitivity and specificity. Various molecular methods used to identify the *M. tuberculosis* complex can be compared in terms of sensitivity and specificity. The sensitivity of an assay is the measure of its efficiency in detecting a positive result. The specificity of an assay is a measure of its ability to yield negative results in the absence of disease or the analyte for which the test was designed (17). These criteria are used to validate the clinical usefulness of the real-time PCR (RT-PCR) diagnostic method described here.

Hybridization probes. Probes are short nucleotide sequences that are designed to bind or hybridize to a complementary target sequence. Detection molecules like fluorochromes or enzymes conjugated to the probe allow detection of the target sequence after it hybridizes to the probe. The use of hybridization probes achieves the specific detection of *M. tuberculosis* more rapidly than conventional techniques (16).

(i) Accuprobe. Accuprobe is a hybridization assay produced by GenProbe, Inc. (San Diego, CA). Various probe types used in assays can specifically differentiate *M. tuberculosis* complex, *M. avium* complex, *M. gordonae,* and *M. kansasi* as they target ribosomal RNA (rRNA). Each probe consists of single-stranded DNA tagged with a chemiluminescent label that is complementary to the rRNA of the target organism (19). After releasing the rRNA from the organisms, the tagged DNA probe combines with the target rRNA to form a stable DNA:RNA hybrid. The chemiluminescent signal from the hybridized probe is detected and measured by a luminometer.

An advantage of the Accuprobe assays is their ability to provide rapid detection by targeting rRNA which may be present in many more copies than DNA in a bacterial cell. The Accuprobe assay achieves 90% sensitivity for identifying *M. tuberculosis* complex (16). The major disadvantage of the Accuprobe assay is that samples must first be grown in culture to provide enough organisms with rRNA copies that hybridize with the probe. In addition, the efficacy of these assays has not been demonstrated on clinical specimens such as sputum.

Nucleic acid amplification methods.

(i) Polymerase chain reaction (PCR). Nucleic acid amplification methods like the PCR, have revolutionized biology. PCR is an integral part of diagnostic testing for bacterial pathogens like the *M. tuberculosis* complex, as it can exponentially amplify a few gene copies of *M. tuberculosis* in a patient specimen to detectable levels within hours rather than relying on culture to amplify the detectable amount of organism. Therefore, PCR is of great value for the diagnosis of TB.

To multiply target sequences of a specific pathogen, PCR utilizes the process of DNA replication found *in vivo*. PCR begins by denaturing or separating double stranded DNA (dsDNA) to produce single stranded DNA (ssDNA). Then Taq polymerase uses both ssDNA strands as a template for synthesizing new complementary nucleotide sequences. Taq polymerase is a DNA polymerase that originates in *Thermus aquaticus*, a bacterium that lives in hot water springs. Taq polymerase is used in PCR as it is heat stable and remains functional even after the heating of the PCR reaction mix to 95°C during the denaturation process.

To synthesize new complementary strands of the pathogen-specific target sequence, Taq polymerase requires a short segment of dsDNA to initiate synthesis. These short dsDNA segments are provided by single stranded oligonucleotide primers which are designed to anneal at points flanking the desired DNA target sequence. Starting from the primers, elongation of the new complementary strands of the target region occurs as Taq polymerase adds free deoxyribose-nucleotides (dNTPs) to the strands. The repeated cycles of denaturation, primer annealing, and complementary strand elongation produce millions of copies of the desired target sequence. These PCR copies are called amplicons. The amplicons of the target region are specific for the organism being identified (51). In this manner, a few gene copies of a pathogen present in a clinical specimen can be amplified to a detectable level.

The PCR assay is carried out in a thermocycler, an automated device that rapidly cycles the reaction mixture through three different temperatures in a series of discrete steps enabling the denaturation, annealing, and elongation processes to form amplified

products. Denaturation of the dsDNA occurs momentarily at 95°C. The primers anneal to the ssDNA at lower temperatures (50-60°C) depending on the design of the primer. The elongation of the complementary strands occurs at 72°C, a temperature optimal for Taq polymerase activity (51).

Any PCR assay requires several controls to ensure the accurate amplification of the target sequence present in a specimen. During the DNA extraction process, a control should be run concurrently with the patient specimen (38). This control consists of nuclease free water in place of the specimen and the reagents that are used to extract the DNA from the specimen. If the extraction control produces amplicons, contamination has occurred. Several sources of contamination include nucleic acids from other patient specimens or from reagents that have been contaminated. If contamination has occurred, all positive results are suspect (38). Contamination may also occur during the PCR assay, and therefore a negative water amplification control is included. If this control produces amplicons, then extraneous DNA has contaminated the amplification process and again all positive results are suspect. Conversely, a positive control that consists of a known sample of the target DNA must be included in the PCR assay. If the positive control does not produce amplicons, then some inhibitory factor preventing amplification is present in the mixture or a critical reagent is missing. Thus, all negative results would be suspect. Finally, to ensure that there are no inhibitory factors preventing amplification in the specimen itself, a PCR assay is run concurrently on the patient specimen that will amplify a target sequence common to all human specimens (38). For example, the beta-globin gene is found in all human cells, and its amplification ensures that no inhibitory

substances are present. The lack of amplicons of beta-globin DNA in a clinical specimen indicates that there are PCR inhibitors present in the specimen or no human cells are present, which is unlikely in respiratory specimens. Therefore, a negative beta-globin result for a PCR assay on a patient's specimen would render the patient's assay invalid.

With the ability of PCR to take one gene copy of a desired target sequence and amplify it to millions of detectable copies, even pathogens present in low numbers in a clinical specimen can be detected within 48 hours. This constitutes a great advantage as there is no need to wait for the organisms to grow in culture. Additionally, when the assay is properly designed the amplified DNA target sequence is extremely specific for a particular pathogen. Thus, the sensitivity and specificity of pathogen detection can be greatly enhanced by PCR techniques (48).

A disadvantage of PCR is that in order to amplify genes of a particular microbe, a target gene sequence must be known so that specific and complementary primers can be designed (48). If the target sequence or primers chosen are not selective enough to detect the pathogen, a great deal of non-specific amplification can occur providing no definitive identification. In addition, laboratory staff must have the requisite training, PCR instrumentation and facilities necessary for PCR-based diagnostics.

(ii) Real-time PCR (RT-PCR). Conventional PCR assays occur in steps at three different temperatures and transition times, generally requiring an assay time of one to three hours. When a PCR system based on the use of closed thin-walled capillary tubes for the reaction mixture and air temperature control was developed, rapid-cycle PCR was

created. The high surface area to volume ratio of the capillary tubes and the low heat capacity of air made cycle times of less than 30 seconds feasible (53). Therefore, PCR reactions did not need to reach equilibrium over longer periods at specific temperatures, but rather, they achieved a kinetic character with quick temperature transitions. Denaturation and annealing times were effective in less than one second, and the entire rapid-cycle PCR assay was completed in 35-50 minutes (53).

With conventional PCR assays, the detection of amplicons requires separate methods, like gel electrophoresis, to assess the size and purity of the PCR products. RT-PCR technology is based upon using an on-going rapid-cycle PCR reaction with the simultaneous detection of the PCR products as the reaction occurs. During automated temperature cycling, the accumulation of the amplicons is measured by the hybridization of a fluorochrome-tagged probe to the PCR product strands. The resulting fluorescence can be detected by the LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany), and the entire process is performed within a closed capillary tube (53). The closed tube system significantly reduces chances of contamination as no other manipulation of amplicons occurs before amplified products are detected.

The fluorescence of the hybridization probe is proportional to the amount of PCR product and can be detected in real time at the end of each PCR cycle. When the fluorescence level is plotted against the PCR cycle number, the resulting amplification curve is similar to a bacterial growth curve (53). The amplification curve consists of an initial lag phase, exponential log phase and the final plateau phase. The lag phase occurs when the amount of fluorescence produced by the hybridized probe-amplicon complex is

below the background fluorescence or the sensitivity of the fluorometer (photodetector) in the instrument. The log phase occurs when there are abundant ssDNA templates of the target DNA sequence, Taq polymerase, free dNTPs, and probe molecules in the reaction mixture allowing for exponential amplification of each gene copy present. As the resulting amplicons hybridize with the fluorochrome tagged probe, the amplicons reach detectable levels. The plateau phase occurs when the PCR amplification can no longer be sustained since template or reactant concentrations are exhausted (53). In a RT-PCR assay, the plateau phase usually occurs around 45 cycles (41). Figure 1 demonstrates RT-PCR amplification curves of *M. tuberculosis* present in clinical specimens.

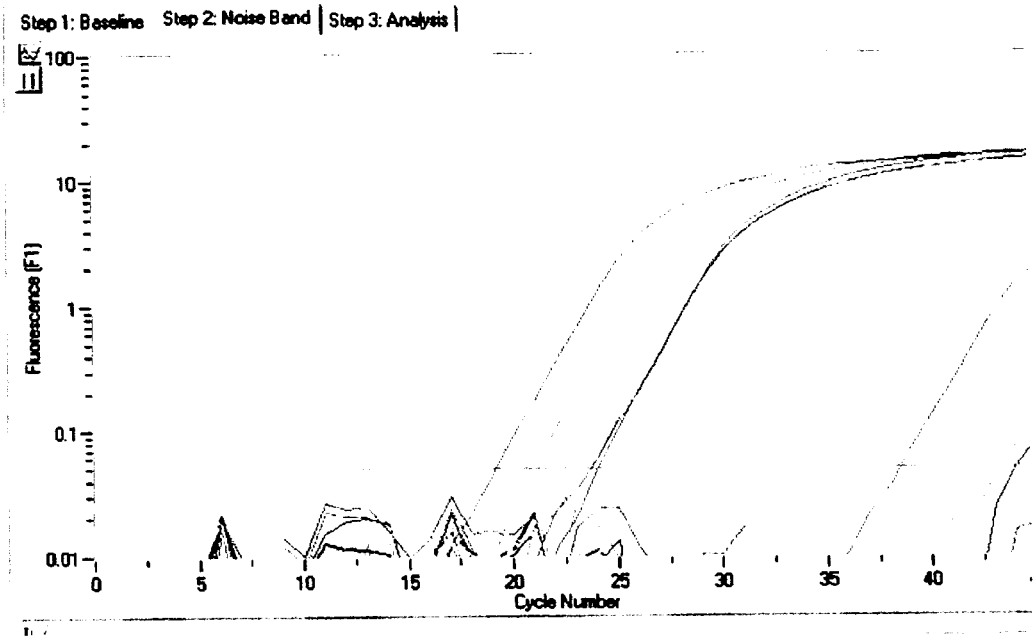

FIG. 1. Amplification curves of *M. tuberculosis* in a RT-PCR Assay
Source: The research LightCycler-PCR assay for *M. tuberculosis* performed by the author. Note the horizontal red threshold line that excludes background fluorescence. The colored sigmoid curves represent amplicons of *M. tuberculosis* detected in various patient specimens.

The red threshold line in Figure 1 is set at the lowest possible point on the log amplification curve for all PCR reactions excluding all background fluorescence (41). The threshold cycle (Ct), is the cycle number at which amplicon fluorescence levels cross over the baseline threshold and are detected. A calibration curve can be established by determining Ct values of standards containing known concentrations of target DNA. Comparison of an unknown sample with the calibration curve allows the determination of the concentration of DNA in the unknown sample (41). Thus, the Ct value provides a measure of the concentration of template initially present. The earlier the Ct value occurs and the lower its number, the greater the initial amount of target DNA or the number of gene copies present in the specimen (41). Generally, the RT-PCR assay is highly sensitive, as it is able to amplify five initial gene copies to a detectable Ct value (41).

Fluorogenic hybridization probes used in RT-PCR. The hybridization probes used in RT-PCR are the fluorescence resonance energy transfer (FRET) type, Taqman probes and molecular beacons (30).

(i) FRET. FRET probes involve energy transfer over a short distance between two fluorochrome molecules attached to probes (14). Two probes are labeled with different fluorochrome dyes. The probes are designed to hybridize head to tail with approximately a five base pair separation. The $3'$ end of the upstream probe is labeled with fluorescein, which acts as the fluorescence resonance energy transfer donor. The $5'$ end of the downstream probe is labeled with an acceptor dye, usually LC Red 640 or LC Red 705. Upon hybridization of the two probes to the target sequence, the fluorescein is

excited by the light source and emits fluorescent light energy that excites the acceptor dye. Subsequently, the acceptor dye will emit fluorescent light at the wavelength that the LightCycler instrument detects (14). Figure 2 depicts a schematic of the FRET probe mechanism.

(ii) Taqman probes. Taqman probes are a type of FRET probe involving dual fluorochrome hybridization. However, Taqman probes are designed as a single hybridization probe with a fluorochrome on either end. Instead of transferring additional energy, one fluorochrome is inhibited by a second fluorochrome that acts as a quencher dye. The close proximity of the quencher moiety to the fluorochrome suppresses the fluorescent signal. Taqman probes employ a reporter fluorochrome at the 5' end and a quencher on the 3' end of the probe. During the amplification and hybridization phase of PCR, the probe hybridizes to the target region. Next, the 5'-3' endonuclease activity of the Taq DNA polymerase hydrolyzes the bound probe, releasing the reporter flurochrome from proximity to the quencher allowing the released fluorochrome to fluoresce. The resulting increase in a fluorescent signal is detected by the LC-PCR instrument, signifying the amplification of target DNA. A schematic of the action of Taqman probes is shown in Figure 3.

(iii) Molecular beacons. Molecular beacons are also dual labeled fluorogenic probes similar to the Taqman probe system. The essential difference between the two systems is that molecular beacon probes are designed with self-complementary sequences at either end. Moreover, molecular beacons contain a reporter dye on one end and a quencher dye on the other. In the absence of the target sequence, the molecular beacon

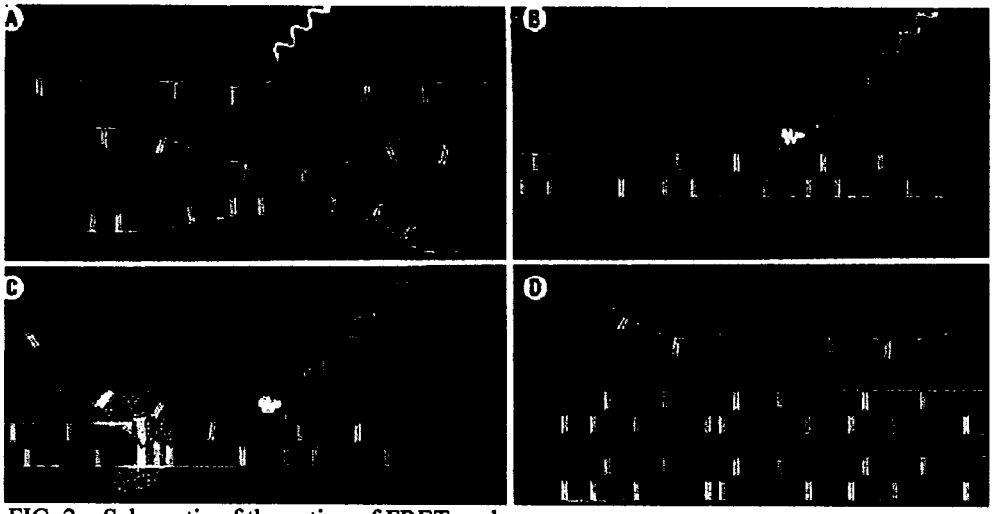

FIG. 2. Schematic of the action of FRET probes
Source: Cockerill, 2002 (14).

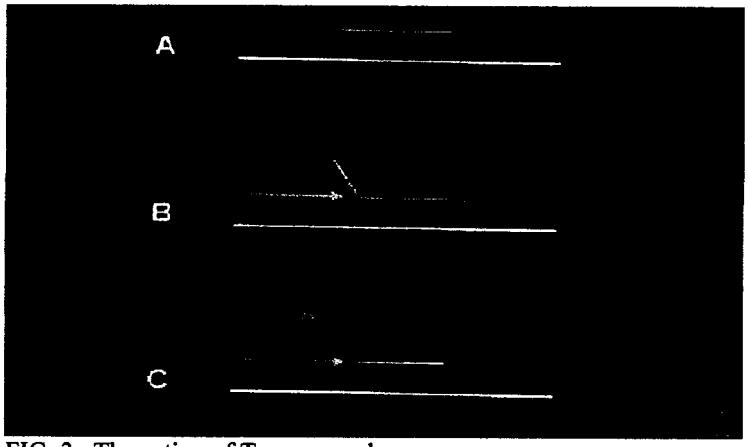

FIG. 3. The action of Taqman probes
Source: Cockerill, 2002 (14).

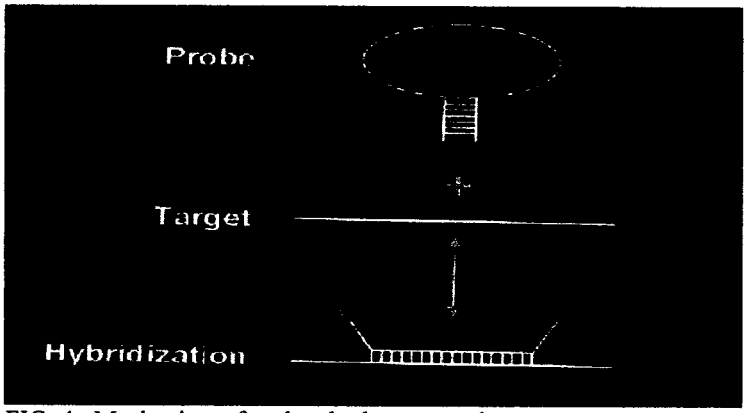

FIG. 4. Mechanism of molecular beacon probes
Source: Cockerill, 2002 (14).

probe will fold into a stem-loop structure due to the self-complementary sequences. In this state, the quencher is in close proximity to the reporter dye and no fluorescence occurs. If hybridization to the target sequence occurs, the stem-loop structure unfolds becoming linear as it binds to the target DNA. Unfolding removes the reporter dye from the influence of the quencher and allows fluorescence to occur. An advantage of molecular beacons is the ability to discriminate between target sequences that have single base pair changes making them effective in investigating single nucleotide polymorphism (14). Figure 4 demonstrates the mechanism of molecular beacon probes.

Although molecular beacons have great discriminatory power, the probe-target duplex of the Taqman probes is more stable than the stem-loop conformation of molecular beacons (14). For this reason, the Taqman probe design was chosen as the diagnostic tool for development in the diagnostic assay described in this thesis. In addition to a background of normal flora, clinical specimens are notoriously contaminated, a fact that demands PCR-based diagnostic methods to be as robust as possible in order to detect a *M. tuberculosis* target sequence. Another consideration was the relative simplicity of Taqman probe design compared to FRET probes and molecular beacons.

Primer and probe design. Primers for RT-PCR assays should be as close as possible to the probe but not overlapping it. Primers and Taqman probes are sensitive to G-C (guanine-cytosine) content and nucleotide base sequences. Their G-C content should be in the 50-60% range. In addition, runs of identical nucleotides, especially runs of four or more guanine repeats on the 5′ end should be avoided. The five nucleotides at

the 3′ end should have no more than two guanine or cytosine bases to avoid the annealing of primers to primers (primer-dimers). Strands that give the probe a higher cytosine than guanine content should be selected (38).

Selection of the melting temperature, T_m, is critical to the successful annealing of the primers or probe to target sequences. The T_m is defined as the temperature at which 50% of the primers or probes are annealed to the target strand. The primer T_m should be 58-60°C and the probe T_m should be 68-70°C (38). Resulting amplicons should be optimally, 50-150 base pairs in length.

Direct testing of clinical specimens by nucleic acid amplification. Two commercially available nucleic acid amplification (NAA) assays for the detection of *M. tuberculosis* from clinical specimens are the *M. tuberculosis* direct test (MTD) by Gen-Probe Inc. (San Diego, CA) and Amplicor by Roche (Roche Ltd. Indianapolis, IN). Initially, the Food and Drug Administration (FDA) approved these NAA tests for smear positive respiratory specimens only as the initial trials did not adequately assess the specificity of smear negative specimens (14). However, the CDC and FDA approved both of these assays in 2000 for testing smear positive or negative specimens (10).

(i) MTD/MTD2. The MTD by Gen-Probe Inc. (San Diego, CA) is based upon transcription-mediated amplification (TMA). In this method, target RNA is released from the mycobacteria by sonification or enzymatic methods and then serves as a template for *in-vitro* replication (23). The first step of amplification involves a promoter-primer hybridizing to a particular sequence of the target RNA. Secondly, reverse transcriptase creates a DNA copy of the target RNA by extension from the promoter-

primer. The resulting RNA:DNA duplex is degraded by RNase H and a second primer binds to the DNA copy. Reverse transcriptase then forms a dsDNA copy. Next, RNA polymerase recognizes the promoter sequence in the DNA and makes more RNA strands initiating another round of transcription and amplification (19). An acridinium ester-labeled DNA probe that binds to the PCR products detects the target amplicons. A developing agent is added to produce the probe's chemiluminescent signal which varies in intensity based upon amplicon concentration (25). Luminescence is measured by a luminometer in the final step.

The advantage of the MTD assay lies in the fact that *M. tuberculosis* has 2000 copies of RNA per cell. This provides many target sequences for sensitive detection even if low numbers of *M. tuberculosis* are present in a specimen (50). An advantage of the RNA target is that it is associated with viable mycobacteria indicating active infection. In contrast, a disadvantage of DNA methodologies is that DNA copies may be detected even if the mycobacteria are non-viable. Hence, the MTD assay is more indicative of an active case of TB than a DNA-based assay (3). The MTD assay takes only five hours to perform, a much shorter time than is needed for conventional PCR.

Piersimoni investigated the MTD assay and found that it achieved a sensitivity rate of 95.9%, while another researcher, Vuorinen, determined an 86.2% sensitivity rate. Piersimoni and Vuorinen also determined the specificity of the MTD assay to be 99.6% and 100%, respectively. These researchers concluded the MTD test to be both very sensitive and specific, as well as being easy to perform on direct specimens (38, 50). However, there are disadvantages in the MTD assay. First, there is no internal

amplification control included in the assay to detect specimens with amplification inhibitors. The CDC recommends performing an amplification control with each PCR assay (10). Secondly, the assay is performed only on respiratory specimens that are AFB positive on the initial smear contributing to the high sensitivity of the results (12). Other disadvantages are that the assay requires a luminometer and is expensive to perform for laboratories with a small test volume (19).

In 1998, the FDA approved the use of a second generation enhanced MTD2 test (Gen-Probe, San Diego, CA) that incorporates modifications to make it more sensitive and faster to perform (four hours). Like the MTD, this assay is not dependent upon a thermocycler. Researchers have found the MTD2 to be 100% sensitive and 99.6% specific with less false positive results than found with the original MTD assay (12). Although this assay is very sensitive, specific and is feasible for a large reference laboratory, it is cost prohibitive ($60/test) for smaller laboratories with fewer test requests (19).

(ii) Amplicor. The Amplicor assay by Roche (Indianapolis, IN) consists of DNA extraction followed by amplification and detection using PCR technology. Amplicor is used to directly amplify and detect *M. tuberculosis* from clinical specimens. In the assay, mycobacteria must first be lysed in an alkaline solution and then the mixture is neutralized. A 50 µl volume of extracted DNA is added to a master mix, a mixture containing free nucleotides, primers, a biotinylated probe, and Taq polymerase. The primers amplify a 584 base pair sequence which is part of a highly conserved 16S rRNA gene. The PCR assay is carried out in a thermocycler and amplicons are detected by

hybridization with a DNA probe specific for *M. tuberculosis*. The hybridized probe – amplicon complex is detected by a colorimetric detection system comprised of a peroxidase conjugate and tetramethylbenzidine substrate system (38).

The advantage of the Amplicor assay is the amplification of only a few gene copies of *M. tuberculosis* resulting in detectable and specific amplicons that differentiate *M. tuberculosis* from other *Mycobacterium* species. Piersimoni and Vuorinen found the sensitivity of Amplicor to be 85.4% and 86.2%, respectively (38, 50). They also determined the specificity of the Amplicor assay to be 99.6% and 100% (38, 50). Positive and negative amplification controls are included in this assay, which takes four hours to perform. Although the Amplicor assay is less sensitive than the MTD2 assay, it has clinically relevant specificity and is of great value in diagnostic testing. The Amplicor assay costs approximately $40 per test and is therefore less expensive than MTD2 (1).

A major disadvantage of the Amplicor assay is that due to its basis on amplification of DNA, both viable and non-viable mycobacteria may be detected, thus the presence of amplified products may not indicate an active infection (38). Another disadvantage is that the assay is proprietary and must be carried out in a specialized thermocycler. Furthermore, the assay also requires separate detection of the amplicons by colorimetric probe hybridization in a microwell plate. Procedural manipulation between amplification and detection steps can lead to possible contamination with extraneous DNA.

Real-time PCR assays.

(i) Artus Biotech. Artus Biotech Inc. (San Francisco, CA) has developed a real-time assay for the *M. tuberculosis* complex by using FRET probes for probe hybridization and detection. Advantages of the Artus Biotech assay are a sensitivity of 94.7% and a specificity of 100% (1). Since the assay uses the LightCycler instrument with its one step real-time amplification and detection, the time of diagnosis is reduced as well as the possibility of environmental contamination. After DNA extraction (four hours) the Artus assay takes one hour to perform. The main disadvantages of the Artus Biotech assay are its expense ($39/test), and the fact that the FDA has not yet approved it for use in the United States (24).

(ii) LightCyler-PCR assay. In an effort to overcome the disadvantages of the Artus Biotech assay, the work presented here describes the development of an in-house RT-PCR assay for *M. tuberculosis* at the MCPHL. Since this inexpensive assay ($14/test) uses the LightCycler instrument, it has been named the LightCycler PCR assay (LC-PCR). The development of this assay and a discussion of its sensitivity and specificity are presented in Chapter 2. Currently, including the LC-PCR described here, there are four common PCR based *M. tuberculosis* direct detection assays that can be preformed directly on respiratory tract specimens (Table 2).

TABLE 2. Comparison of direct detection tests for *M. tuberculosis* complex

Tests	MTD/MTD2	Amplicor	Artus Biotech	LC-PCR
Principle	TMA	PCR	PCR	PCR
Target	16S rRNA	16S rRNA	Proprietary	16S rRNA
Detection	Luminescent Probe	Probe Hybridization	FRET Probes	FRET Probe Taqman
Run Time (hours)	4	6	5	5
Sensitivity (%)	100	85.8	94.7	85.5
Specificity (%)	99.6	100	100	100
Instrument	Luminometer	Thermocycler/ Cobas	LightCycler	LightCycler
Advantages	Isothermic Detects viable AFB	Few gene copies needed Amplification Control	Sensitive to 1 gene copy per µl PCR and detection in single tube Amplification control	Sensitive to 5-10 gene copies per µl PCR and detection in single tube Amplification control Drug therapy/AFB negative specimens tested
Disadvantages	50µl DNA needed	Two step, separate PCR and Microwell Hybridization Probe detection Possible contamination	Detects viable and non-viable AFB Not yet approved by the FDA	Detects viable and non-viable AFB Not yet approved by the FDA
Cost per test	$60	$40-200[1]	$39[2]	$14[2]
Manufacturer	Gen-Probe	Roche	Artus Biotech	MCHD

(1) Assumes 1 to 4 patients per run, 4 controls, @ $25/well
(2) Cost includes $2.16 extraction cost

Further discussion of these methods can be found in the text.

Nucleic acid amplification testing of *M. tuberculosis* culture isolates.

(i) Inno-LiPA Rif TB. The Inno-LiPA Rif TB test (Innogenetics, Ghent, Belgium) can be used for the detection of *M. tuberculosis* from culture. The LiPA assay can detect *M. tuberculosis* and resistance to the antimicrobial rifampin using the same assay. Clinically when resistance to rifampin develops, there is often treatment failure (45). The LiPA assay consists of ten oligonucleotide probes with one probe specific for the *M. tuberculosis* complex. Additionally, the assay involves five probes for the overlap regions of the *rpoB* gene associated with rifampin resistance, and four probes that cover specific target sequences for common *rpoB* mutations (45). Researchers found the LiPA assay to be 98.6% sensitive in detecting both *M. tuberculosis* and resistance to rifampin (45). This level of sensitivity would be of great value in the identification of *M. tuberculosis* and the concomittant detection of rifampin resistance. However, the test is currently not available in the United States.

The main disadvantage to the LiPA assay is that unless there is a great deal of rifampin resistant TB in a community, the ten probe format of the assay is too costly for routine diagnostic use in a clinical laboratory. Moreover, since the assay is performed on culture isolates of mycobacteria, there is a two to six week lag time until results are obtained.

(ii) LCR. The Ligase Chain Reaction (LCR) (Abbott Laboratories, Oxnard, CA) is another assay used to detect *M. tuberculosis* in culture isolates. The LCR is a probe-based amplification test in which the amplification of the probe occurs through polymerization of the target sequence (16). In this assay, two probes are used for each

strand of DNA which are ligated together to form a single probe by the action of DNA polymerase and DNA ligase. Antigen-antibody reactions are used for detection of the ligated probe. Antibodies that form a complex with the probe are conjugated to magnetic particles which act as a platforms on which the antigen-antibody reactions can take place in solution. The magnetic particles also aid in the capture of the complexes. After incubation, excess antibody is washed off and a second antibody with a fluorescent label is added, which binds to the probe-antibody complex. The fluorescence resulting from the bound fluorochrome tagged antibody is measured by a fluorometer and constitutes a positive result. A positive LCR assay demonstrates the presence of a *M. tuberculosis* target sequence to which the probe is hybridized.

The advantages of the LCR diagnostic test are a sensitivity of 97-99%, a specificity of 90-100% and speed as it takes only 90 minutes to complete the assay (16). The disadvantages of the LCR assay are the requirements for a thermocycler and other specific LCR equipment. The assay is also culture dependent resulting in a delay of two to six weeks until diagnostic results are obtained.

Restriction endonuclease analysis and DNA fingerprinting.

(i) RFLP. Once amplicons are produced by PCR or reverse transcriptase PCR, the amplicons can be subjected to digestion by restriction endonucleases and studied by a technique known as restriction fragment length polymorphism (RFLP) analysis. Investigators have studied the IS6110 and the 16S rRNA sequences of mycobacteria using RFLP analysis. Roth selected and analyzed the internally transcribed spacer (ITS) portion of the 16S-23S ribosomal DNA (rDNA) of several species of mycobacteria (43).

The 16S rRNA alone has a low number of polymorphic sites within mycobacterial genes. However, the ITS portion showed divergent nucleotide sequences that discriminated between species of *Mycobacterium*. Roth digested the amplified products of the 16S rRNA ITS sequence with restriction enzymes *Itae*III, *Cf*I, *Msp*I, and *Dde*I . The resulting fragments were gel electrophoresed. The fragments were visualized by ethidium bromide staining and the fragment band sizes were visually compared to similarly digested known species of mycobacteria and a 100-bp marker ladder. The RFLP analysis showed discriminatory interspecies power identifying 100% of the samples to the *Mycobacterium* genus level. The RFLP pattern analysis was able to correctly identify 84% of the *Mycobacterium* isolates to the species level (43). Researchers concluded that the high intraspecies sequence stability and good discriminatory ability of the RFLP analysis made the method advantageous and cost effective for identifying mycobacteria species especially in contrast to DNA sequencing (43).

RFLP is of value in an epidemiological cluster analysis of an outbreak, tracking individual or multi-drug resistant strains, and for specifically identifying *M. tuberculosis*. RFLP techniques do provide sensitive and specific identification for a university or reference laboratory. However, most clinical or public health laboratories do not have the gel electrophoresis capabilities or trained staff for this type of analysis. Another disadvantage is that RFLP analysis is also culture dependent resulting in a two to six week diagnostic delay. Additionally, RFLP based upon fewer than five gene copies of *M. tuberculosis* cannot be accurately performed (43)

Blots and hybridization probe analysis. DNA or RNA amplicons can be transferred ("blotted") to a solid substrate such as nitrocellulose or nylon. Hybridization probes specific for the amplified target sequence are labeled with fluorescent, chemiluminescent or radioactive compounds. During incubation these labeled probes hybridize to the target amplicons attached to the substrate surface. Detection signals from these hybridized probes are measured by a luminometer, a photodetector, or by film respectively (52). Southern blots and Northern blots are both examples of nucleic acid transfer techniques utilizing probe hybridization methods for amplicon detection.. Based upon DNA polymorphism of insertion sequences (IS), Van Soolinger restricted mycobacterial IS6110 and IS986 (49). After performing a Southern blot and probe hybridization, the resulting fingerprints were characterized by computer analysis with known mycobacterial patterns. The analysis of both IS sequences specifically identified *M. tuberculosis* and specified particular strains of epidemiological interest (49). While these techniques provide specific pathogen identification, these procedures are time consuming and research oriented limiting their use for routine *M. tuberculosis* diagnostic testing.

Microarrays. Microarrays for *M. tuberculosis* are constructed by arranging PCR amplicons of mycobacterial genes on a glass slide. The amplicons are robotically spotted on glass microscope slides at high density. Two or more fluorochrome-labeled probes, complementary to mycobacterial target sequences, are then added. A high concentration of bound probe hybridizing to a particular amplicon on the microarray detects its presence. A confocal laser scanner measures the fluorescence intensities of the

hybridized probes. (29). To identify *M. tuberculosis* based upon its thirty-nine repetitive IS6110 sequences, Kivi used the microarray procedure (29). If unknown DNA sequences match the hybridization probe density of *M. tuberculosis,* a positive test for *M. tuberculosis* is established.

Microarray analysis can specifically identify the *M. tuberculosis* complex and can be used to map unknown portions of complex genome. However, microarrays are more applicable to research, require highly specialized instrumentation and thus are beyond the scope and budget of a clinical laboratory attempting to identify *M. tuberculosis* in a patient specimen.

Sequencing. Sequencing involves the use of a sequencer, an automated instrument that determines the nucleotide sequence of amplicons. Once the sequence of the test organism is determined, the sequence is compared to known sequences stored in cDNA libraries or databases. For example, a BLAST search in the Gen Bank database can be run looking for similarities between the test sequence and known sequences of *Mycobacterium*. Thus, gene sequencing can be a highly sensitive and specific method for identifying mycobacteria. Additionally, the detection of mutations in the normal target sequence of the test organism may indicate possible drug resistance (37). The sequencing process involves PCR amplification of the target gene segment, gel electrophoresis of the amplicons followed by sequencing and analysis. Sequencing is also culture growth dependent. Sequencing is a reference method used for research or performed in specialized laboratories and therefore is not applicable to screening patient specimens on a routine basis. Set up costs for DNA sequencing instruments range from $45,000 to

130,000 and cost an additional $40 per test. These costs make this method beyond the scope of most clinical laboratories (37).

Of the *M. tuberculosis* diagnostic methods described, it is clear that RT-PCR methods provide the most rapid, specific and sensitive diagnostic assay results. The description of the development of a cost-effective RT-PCR based diagnostic assay for *M. tuberculosis* follows in Chapter 2.

CHAPTER 2

Design and Development of a Real-Time PCR Assay for the Detection of *M. tuberculosis*

Experimental Design

Real-time PCR methodology. Many public health laboratories in California have begun extensive training of personnel in molecular diagnostics to prepare primarily for bioterrorism agent identification. To increase molecular capabilities and expertise, the MCPHL acquired a LightCycler PCR instrument (Roche Inc., Mannheim, Germany). While expensive ($57,000) it has utility for bioterrorism agents and other pathogens of public health significance like *Bordetella pertussis*, food borne pathogens, and *M. tuberculosis.* The advantages of RT-PCR methodology and the availability of the LightCycler instrument made possible the development of a RT-PCR assay for the detection of *M. tuberculosis* in respiratory specimens. The assay described in this research is hereafter referred to as the LightCycler-PCR (LC-PCR) assay. Successful implementation of this assay would fulfill the Federal and State goals of diagnosing TB within two days (47).

Research design. Respiratory specimens (sputa, bronchiolar lavage) from patients in Monterey County, CA were split into two aliquots (18). One aliquot was used to prepare smears and cultures for conventional AFB examination and the second aliquot was frozen at –20°C for subsequent PCR testing. Clinical specimens with AFB positive and negative smear results were selected for extraction of mycobacterial DNA and testing with the LC-PCR assay.

Splitting of the specimens allowed comparison of results obtained from the LC-PCR assay and from conventional smear and culture methods. The sensitivity and specificity of the LC-PCR assay was determined using growth of *M. tuberculosis* in

culture. The Accuprobe assay (GeneProbe, San Diego) was used to verify culture isolates as *M. tuberculosis* complex. When LC-PCR assay results did not agree with the conventional culture and smear results, the Artus Biotech (San Francisco, CA) RT-PCR assay or patient clinical history was used to judge the discrepancies. Thus, the specificity and sensitivity of the LC-PCR assay for *M. tuberculosis* was evaluated.

Target sequence. The 16S rRNA gene was investigated for the development of the RT-PCR assay because of its stability and highly conserved regions characteristic of *M. tuberculosis.* However, to gain discrimination between species of *Mycobacterium* the internal transcribed spacer (ITS) region within the 16S rRNA gene was chosen as a possible target sequence. Researchers demonstrated that the ITS region of the 16S rRNA gene had variability which resulted in significant interspecies differentiating ability and clear intraspecies discrimination for the genus *Mycobacterium* (43).

Miller performed a real-time PCR assay using a partial ITS sequence from the 16S rRNA gene (35). The results confirmed sensitive and specific identification of the *M. tuberculosis* complex from known broth cultures. Because the ITS sequence is highly specific for *M. tuberculosis*, it can be used to differentiate it from other slow-growing species of mycobacteria with acceptable diagnostic reproducibility (35).

Published sequences of the 16S rRNA gene of *M. tuberculosis* were searched at **www.ncbi.nlm.nih.gov.** This search yielded several publications that reported the ITS region of the 16S rRNA gene of *M. tuberculosis* was a strategic target for amplification (35, 43, 46). In analyzing the ITS portion of the 16S rRNA gene of *M. tuberculosis*, Miller used the accession number L15623 from the national data bank for the nucleotide

sequence. For this project, the ITS portion of the 16S rRNA gene (L15623) was subjected to BLAST analysis. The L15623 sequence is 433 base pairs long and the ITS portion is located from base pairs 137 to 411 (35). BLAST analysis of this ITS nucleotide sequence produced 99% homology with the *M. tuberculosis* complex. Therefore, the ITS sequence was selected as the target sequence for the *M. tuberculosis* LC-PCR diagnostic assay.

Probe and primer selection. The primers and Taqman probe combination selected for this research was obtained from Integrated DNA Technologies (Coralville, CA), **www.idtdna.com/biotools/primer_quest**. The probe sequence was evaluated for homology to *M. tuberculosis* compared to other organisms using BLAST alignment analysis. BLAST analysis of the selected probe sequence demonstrated a homology of 50-80% for the *M. tuberculosis* complex. Besides *M. tuberculosis,* the organism with the closest homology to the probe nucleotide sequence was *Xanthomonas axonopedis*. However, this bacterium is a plant pathogen and not commonly associated with the human respiratory tract (26). No other *Mycobacterium species* showed homology to the probe nucleotide sequence. The LC-PCR primers and the probe sequences are listed in Table 3.

TABLE 3. Nucleotide sequence of primers and probes

Oligonucleotide	**Sequence**
Sp1	5' - GAC AAC AAA GTT GGC CAC CAA CAC - 3'
Sp2	5' - GCG CCC TTA GAC ACT TAC AAA CAC - 3'
Probe	5' FAM-AGC GGT GGC GTG TTC TTT GTG CAA TA-BHQ -3'

FAM= fluorescein, BHQ= Black Hole Quencher

Materials and Methods

Conventional identification of *M. tuberculosis.*

(i) Specimen processing. Patient specimens were processed according to standard decontamination and concentration methods using N-acetyl-L-cysteine and 2.5% NaOH (28). The processed specimens were inoculated into the Bactec/MGIT broth detection system (Becton Dickinson, Sparks, MD) and 7H11 agar plates. Culture media were incubated for six weeks. (28). Auramine-O and Rhodamine-B fluorochrome stained smears of the clinical specimens were examined. The remaining specimen aliquots were frozen at -20°C and processed later by DNA extraction for the LC-PCR assay.

(ii) Fluorochrome smear results. Auramine stained smears were read the same day as the specimens were processed. The number of fluorescent AFB on the smear was graded in the following manner at the MCPHL.

None = 0 - 3 AFB per slide

Rare = 3 - 9 AFB per slide

Few = 10 - 50 AFB per slide

Numerous = 1 or more AFB per field (500 total magnification)

(iii) AFB Culture. Acid-fast organisms producing typical rough colony morphology on 7H11 culture media and broth cultures of AFB were identified using Accuprobe hybridization probe (GenProbe Inc., San Diego, CA) specific for *M. tuberculosis* complex. These culture results were compared to the LC-PCR results of the same specimens.

LC-PCR assay DNA extraction procedures.

(i) DNA extraction and purification control. Previous studies have shown that silicon spin filter kits efficiently remove inhibitory substances that may be present in respiratory specimens collected for *M. tuberculosis* detection (4). For this study, DNA was extracted and purified from 76 TB smear and culture positive respiratory specimens, and 155 smear and culture negative respiratory specimens using commercially available silicon spin filters from the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). Specimens were treated with a combination of lysozyme and proteinase K according to the manufacturer's modified protocol D to lyse the mycobacterial cell wall (24, 40). Following extraction, the DNA was precipitated by the addition of 100% ethanol. Next, the precipitated DNA was transferred to a silicon spin column for purification (40). After the spin column was washed twice with Qiagen kit buffers, an extra spin for 5-10 minutes was added to insure the removal of all ethanol which can interfere with the PCR assay (24, 40). The spin filter was eluted with 100 µl buffer and the DNA filtrate collected for RT-PCR analysis. A negative extraction control composed of TB buffer was included with each group of extracted specimens to detect DNA contamination during the procedure.

(ii) Extraction of broth cultures. Culture isolates (e.g. MGIT 960 broth tubes) were not processed with spin columns for the DNA extraction/purification process. It was found that spin column extraction of broth cultures produced overwhelming concentrations of template DNA that interfered with specificity for *M. tuberculosis* in the LC-PCR assay. Instead 0.5 ml of broth suspension was transferred to a microcentrifuge

tube and heat inactivated for 20 minutes at 95°C. This heat inactivation extraction method produced sufficient concentrations of DNA for the LC-PCR assay.

Optimization of the LC-PCR assay. The final concentrations of primers, probe, $MgCl_2$, and template DNA were optimized by varying each component of the reaction mix to achieve the greatest concentration of PCR products.

(i) Template DNA concentration optimization. A stock solution of *M. tuberculosis* broth culture (strain H37rv; ATCC 27294) was adjusted to a McFarland standard of 1, to yield a sufficient concentration of DNA for testing. The resulting DNA template standards were quantified by diluting (10^0-10^{-5}) the *M. tuberculosis* broth culture and comparing the dilutions' LC-PCR results to the DNA quantification standards from the Artus Biotech assay (San Francisco, CA). This comparison established a standard curve for the developed LC-PCR assay of 10^0–10^5 gene copies of *M. tuberculosis* template DNA. The serial dilutions of *M. tuberculosis* template DNA were tested against the primer concentration of 0.5 μM (midrange), the probe concentration of 0.2 μM, and a 3 mM $MgCl_2$ concentration which were all held constant in the template-PCR optimization reactions. Additionally, the *M. tuberculosis* standards and a concentration of *M. avium* were used as positive and negative amplification controls respectively for the LC-PCR assay.

(ii) $MgCl_2$ concentration optimization. Holding the primers and probes at midrange concentrations of 0.5 μM and 0.2 μM, respectively, and the DNA template at a 10^{-2} dilution (1000 gene copies), the $MgCl_2$ concentrations were varied from 1.0-5.0 mM in the optimization PCR assays.

(iii) Primer concentration optimization. $MgCl_2$ concentration of 3 mM and probe concentration of 0.2 μM were tested against the DNA template concentration of 1000 gene copies using a mid-range primer concentration of 0.5 μM during the optimization trials. This mid-range primer concentration produced detectable amplicons so the primer concentrations were not varied in later PCR assays.

(iv) Probe concentration. In the optimization PCR trials, the probe concentration was not varied but held at 0.2 μM as good PCR products were produced at that concentration in all previous trials.

The experimental parameters of LC-PCR reactants for all optimization trials of the LC-PCR assay are summarized in Table 4.

TABLE 4. Summary of LC-PCR reactant concentrations varied for optimization trials

Trial	***M.tuberculosis* Template in Gene Copies**	**$MgCl_2$ Concentration**	**Primer Concentration**	**Probe Concentration**
Template Concentration	10^1 –10^4	3 mM	0.5 μM	0.2 μM
MgCl2 Concentration	10^3	1-5 mM	0.5 μM	0.2 μM
Primer Concentration	10^3	3 mM	0.5 μM	0.2 μM

(v) Gel electrophoresis of LC-PCR assay amplicons. The LC-PCR products were run on high-resolution EL-300 gels (Elchrom Scientific, Chum, Switzerland) using a tenth concentration (1/10) of the M3 marker (Elchrom Scientific, Chum, Switzerland) to look for the maximum amplicons. The PCR products of the initial optimization reactions were electrophoresed on a high-resolution agarose gel stained with ethidium

bromide. The electrophoresis patterns were analyzed for the presence of the 123 base pair amplicon, (the length of the *M. tuberculosis* target sequence) and possible primer-dimers. Additionally, analysis of the LC-PCR assay amplicons on this high-resolution gel established the optimal reactant concentrations. The gel electrophoresis results are shown in Figure 5.

(vi) LC-PCR assay temperature. The optimum annealing temperature (T_m) for the LC-PCR was selected based upon the calculated T_m for the primers using the Integrated DNA Technologies program. Reaction T_m was set at 5^0C below (56^0C) the forward primer T_m of 61^0C (42). The denaturation temperature was 95°C and the elongation temperature was set at 72°C, the optimum temperature for *Taq* polymerase.

(vii) Optimization of LC-PCR assay reaction temperature. Electrophoretic analysis of the LC-PCR products formed at various annealing temperatures demonstrated that annealing temperatures of 54-58 °C were all equally effective in *M. tuberculosis* amplicon production. The original annealing temperature selected was 56 °C. However, in analyzing ten cultures of mycobacteria other than TB (MOTT), amplicons with Ct values of 37-45 cycles were produced. To increase the stringency of the LC-PCR assay and achieve better specificity, the temperature for annealing was increased to 59°C. This reduced false positives in MOTT samples by 30%. Stringency of the reaction was further increased by reducing the $MgCl_2$ concentration to 2 mM. At 2 mM $MgCl_2$ and 59° C, 70% of the known MOTT broth cultures were negative. The remaining broth culture DNA extracts produced Ct values of 40-45. An overwhelming concentration of mycobacterial template DNA (>10^6 gene copies) in these extracts was

thought to be responsible for the loss of specificity. It was determined that broth culture extracts had sufficient amounts of "free" DNA to be detected without extraction and purification with Qiagen spin filters. Subsequently, all the broth cultures were inactivated in 0.5 ml aliquots at 95 °C for 20 minutes using a DNA heat extraction procedure (39). This extraction technique produced gene template in concentrations suitable for the LC-PCR assay. The subsequent LC-PCR assays of MOTTs gave no false positive results for AFB (refer to Table 10).

To increase assay stringency even further, a "step down temperature" protocol was tried. The step down parameters involved decreasing the temperature from 61° C for 5 cycles, to 60°C for 5 cycles, and finally to 59 °C for the remaining 35 cycles. This makes annealing more difficult, reducing nonspecific binding. The step-down protocol was used to test a battery of specimens with known high, low and negative concentrations of *M. tuberculosis* template DNA. The results demonstrated a loss of sensitivity, as low concentrations of *M. tuberculosis* DNA were not detected as consistently as when the 59°C protocol was employed. Therefore, the step-down procedure was not used.

(viii) Reaction buffer used in optimization. LightCycler Fast Start DNA reaction mix was used as the standard hot start reaction buffer (42). This reaction mix buffer contains free deoxyribose nucleotide tri-phosphates (dNTPs), uracil tri-phosphates (UTP), buffers, and *Taq* polymerase. The *Taq* polymerase in the Fast Start reaction mix is inactive at room temperature, thus there is little non-specific primer binding during reaction mix preparation. The Fast Start *Taq* polymerase is activated by removal of blocking groups at 95°C (42).

(ix) Uracil-DNA-glycosylase optimzation. This enzyme is recommended for use in PCR reactions by the California Microbial Disease Laboratory. It is used in PCR reactions to destroy contaminating amplicons (DNA produced i*n vitro*) or non-specific binding such as primer-dimers. The enzyme cleaves DNA wherever a dUTP (Fast Start reaction mix) has been incorporated during previous PCR assays (42). When the denaturation temperature of 95°C is reached during a PCR assay, this enzyme is inactivated (heat labile) so it will not destroy desired amplicons (42).

(x) Beta-actin amplification control. Clinical specimens were also tested with an internal amplification control to assure that there was no inhibitory substance present that would interfere with amplification (39). The amplification control consisted of the master mix, primers, and a probe to detect beta-actin. The beta-actin PCR assay (Applied Biosystems, Foster City, CA) was performed on every LC-PCR sample at the same time as the *M. tuberculosis* assay was performed. Failure to produce a beta-actin PCR product indicates some substances in the sample, may have inhibited PCR amplification, thus invalidating the *M. tuberculosis* LC-PCR assay as well.

(xi) LightCycler parameters for *M. tuberculosis* and beta-actin LC-PCR assays. The first step in the LC-PCR assay is a 10-minute incubation of the template DNA and reaction mix at room temperature to allow the action of uracil-DNA-glycosylase to degrade any extraneous contaminating DNA. Next, the denaturation, amplification, elongation and cooling steps of the LC-PCR assay occur in the LightCycler instrument. The LC-PCR protocol includes an initial *Taq* polymerase activation step at 95°C for 10 minutes, followed by 45 cycles of amplification. Each

amplification cycle consists of the following segments: denaturation at 95°C for 5 seconds, annealing at 59°C for 15 seconds and elongation at 72°C for 15 seconds. The assay culminates with a cooling step at 40°C for 30 seconds. The total time of amplification, detection and analysis using this protocol is 30 to 35 minutes. Fluorescent measurements are taken at every cycle to detect the threshold cycle (Ct value).

Results of the LC-PCR Assay for *M. tuberculosis*

Results of the LC-PCR assay optimization. Analysis of the initial optimization LC-PCR assay results and visual examination of the gel electrophoresis of initial *M. tuberculosis* amplicons, established the optimal reactant concentrations for the developed assay.

(i) Results of the template concentration optimization. Ct values resulting from the initial LC-PCR optimization trials for the various template concentrations demonstrated that earlier (i.e. lower) Ct values were associated with greater concentrations of PCR products. Thus, lower Ct values reflected optimal conditions for the LC-PCR as summarized in Table 5.

TABLE 5. Ct values of various *M. tuberculosis* DNA template concentrations

Number of gene copies TB DNA Template	**Ct value**
100,000	20.45
10,000	24.12
1,000	28.08
100	31.04
10	34.82
1	38.02

Increasing the dilution of the template gives progressively larger Ct values. When the 10^{-5} dilution (i.e. one gene copy) was run in the LC-PCR assay, it gave a Ct value of 38.02 cycles. Subsequent assays with the 10^{-5} dilution produced no Ct values, therefore this template concentration is too small for consistently accurate results. The results for DNA template concentrations of 10^{1} -10^{5} gene copies gave optimal LC-PCR results as judged by Ct values in the range of 20 –35 Ct cycles.

Analysis of the gel electrophoresis patterns of the various DNA template concentrations assayed (10^2 and 10^3 gene copies) determined that these concentrations produced equally dense amplicons (see Figure 5).

(ii) $MgCl_2$ concentration optimization results. The LC-PCR assay was performed using $MgCl_2$ concentrations varying from 1 mM to 5 mM. The resulting Ct values of the various products were examined to determine the $MgCl_2$ concentration yielding the greatest amplicon concentration as shown in Table 6.

TABLE 6. Ct values compared to $MgCl_2$ concentrations.

$MgCl_2$ Concentration (mM)	**Ct Values (number of cycles)**
1	0
2	28.98
3	26.78
4	25.99
5	25.61

The results of the Ct values from the LC-PCR optimization demonstrate that 1 mM of $MgCl_2$ produces no PCR product. The Ct value for 2 mM is 28.98 and the Ct values for 3 mM, 4 mM and 5 mM are all essentially the same (approximately 26). Therefore, $MgCl_2$ values of 2-5 mM would give optimal PCR conditions. Gel electrophoresis results confirm that no amplicons are produced using 1 mM of $MgCl_2$, however 2 mM to 5 mM give equivalent amplicons. The most stringent concentration of 2 mM was selected for the LC-PCR assay.

(iii) Primer and probe optimization results. The mid-range primer concentration of 0.5 μM and the probe concentration of 0.2 μM were held constant in all LC-PCR assays. Gel electrophoresis analysis and the corresponding Ct values established that the primer concentration and probe concentration of 0.5 μM and 0.2 μM respectively demonstrated optimum amplicon production without the formation of any primer-dimers (Refer to Figure 5).

(iv) Interpretation of gel electrophoresis results. Analysis of the electrophoresis patterns of the PCR products confirmed the migration of the amplicon bands just below the 124 base pair band of the M3 marker (at 1/10 concentration) (Elchrom Scientific, Chum, Switzerland). Since the amplified target sequence is 123 base pairs long, the alignment of the amplicon bands near the 124 base pair marker indicates the amplification of the target sequence occurred during the LC-PCR assay. No smaller base pair primer–dimers appear on the gel. The gel electrophoresis assay is found in Figure 5.

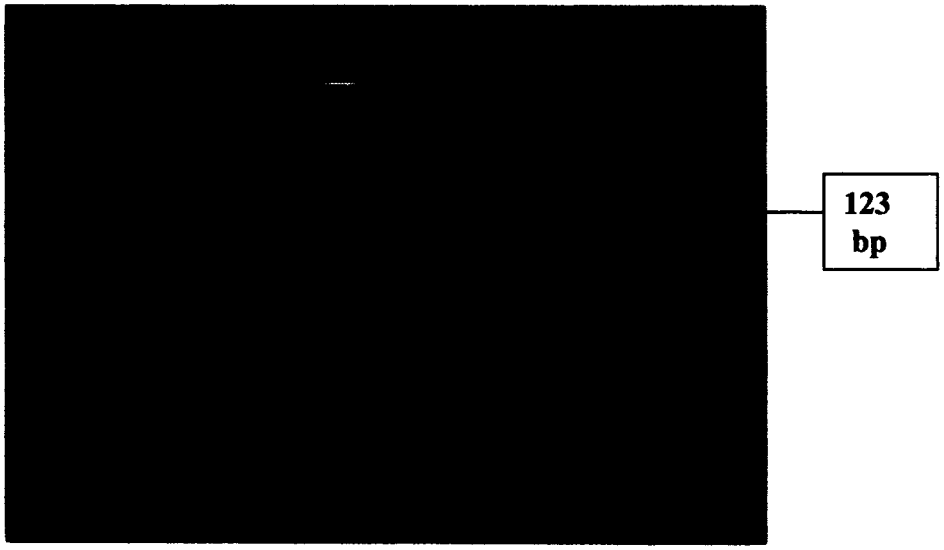

Fig. 5 Gel electrophoresis of LC-PCR optimization assays.
The gel electrophoresis was run in 0.75x TAE buffer, at 100 volts for 97 minutes. The gel was stained with ethidium bromide for 15 minutes. The base pair marker was at a 1/10 M concentration.

TABLE 7. Gel lane map of PCR products at various temperatures and reactant concentrations

Lane	1	2	3	4	5	6	7	8	9	10	11	12
TB DNA # of Gene Copies	100	1000	100	1000	100	1/10 M3	1000	1000	1000	1000	1000	1000
$MgCl_2$ in mM	3	3	3	3	3	1/10 M3	3	1	2	3	4	5
Temp. °C	56	56	58	58	54	1/10 M3	54	56	56	56	56	56

1/10 M3 is a one tenth concentration of the M3 marker defining distance of base pair migration

Results of the LC-PCR assay on clinical specimens. The LC-PCR assay results on 76 AFB culture positive respiratory specimens and 155 AFB culture negative specimens are summarized in Table 8. Culture specimens positive for *M. tuberculosis* generally produced detectable amplicons before 40 cycles (Ct <40) and this was chosen as the cutoff value for a positive test. Amplicons appearing between 40 and 45 cycles were seen with specimens from treated TB cases and rarely with patients who had no evidence of TB. Specimens that yielded amplicon between 40 and 45 cycles, were re-tested. If the repeated test yielded no Ct values the result was considered negative. If amplicon was repeatedly detected between 41 and 45 cycles the result was considered an indeterminate result, thus no verifiable result could be obtained. In such a case, another specimen was requested for further testing. The resulting LC-PCR report protocol is diagrammed in Figure 6.

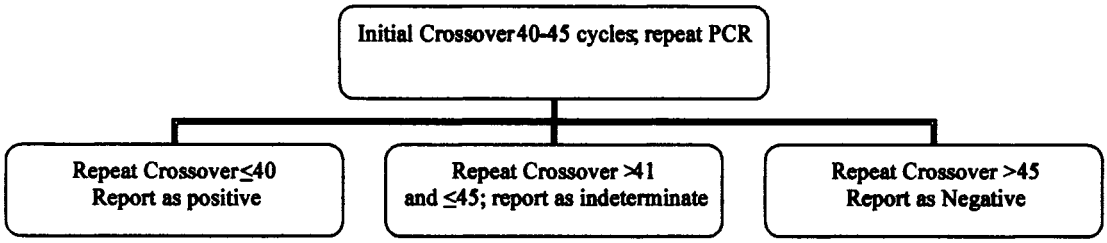

FIG. 6. LC-PCR assay report protocol

Using the above criteria, this LC-PCR assay produced a positive result in 65 of 76 AFB culture positive specimens. Nine specimens repeatedly produced a Ct value of 40-45 and were considered indeterminate. Two specimens from culture positive specimens produced no detectable amplicon at 45 cycles and were considered false negatives. One

of the false negatives had produced an initial Ct value of 43, but upon repeat analysis no *M. tuberculosis* DNA was detected. The second false negative was from an AFB positive smear and culture positive TB patient. When this specimen was tested with the Artus Biotech assay (San Francisco, CA) as the verifying test, no DNA from *M. tuberculosis* was detected. The *M. tuberculosis* false negative specimen was also tested with the beta-actin assay, which demonstrated a positive result. This proved there were no amplification inhibitors present. An error in the extraction process was the most probable cause of the false negative LC-PCR result (refer to Table 8).

Many researchers have commented on additional factors possibly responsible for false negative AFB specimens (16, 24). One factor is the clumping characteristic of *M. tuberculosis* and the difficulty of obtaining even distribution of mycobacteria in positive specimens to be analyzed (24). It would be possible to inadvertently discard the bacterial pellet from the initial extraction step as well. A DNA denaturing process possibly responsible for false negatives could occur during the heat inactivation process in the biological safety hood. Accidental use of the UV light in the hood during inactivation would damage any DNA present.

One hundred fifty-five specimens from patients with negative cultures were tested with the LC-PCR assay. Twelve of the 155 specimens from individual patients yielded a Ct value of 41-45 on initial testing; eleven of these were negative upon repeat testing. Some of these specimens were found to be from previously treated TB patients who had negative smears and cultures. It was thought that residual *M. tuberculosis* DNA was responsible for the Ct values at 41-45 cycles which were not detectable on repeat

testing (22). Three culture negative specimens with initial 41-45 Ct values which were negative on re-testing, were near specimens with great amplicon concentrations during the LC-PCR assay. The initial amplicons detected may have been due to contaminating amplicons or other factors in the dynamic system. One specimen from this culture negative group repeatedly produced a Ct value of 40-45 and was considered an indeterminate result. Though this was a specimen from a negative culture, the presence of *M. tuberculosis* DNA was confirmed as a positive result when tested by the Artus Biotech assay (San Francisco, CA). In review of the test order, this specimen followed that of a strongly positive TB patient during the extraction process. Since the culture negative specimen contained *M. tuberculosis* DNA, it is probable that during the extraction process it became contaminated.

To improve the specificity of the assay, specimens were considered positive only if the amplicon appeared before 40 cycles. None of the 155 culture negative specimens produced significant levels of amplicon at ≤ 40 cycles. The indeterminate specimen was not judged a positive result as it showed amplicon at 43 cycles (Table 8).

TABLE 8. LC-PCR assay results in clinical specimens compared to culture growth

		Culture Results for ***M. tuberculosis***	
	PCR	Positive	Negative
LC-PCR Results	Positive (Ct<40)	65	0
LC-PCR Results	Negative (Ct>45)	2 (1)	154 (2)
LC-PCR Results	Indeterminate (Repeatable, Ct >40, <45)	9	1 (3)

(1) Includes one specimen with Ct values 40-45 on initial testing but negative on repeat testing and one specimen that produced no amplicon.
(2)Includes 11 specimens that had Ct values 40-45 on initial testing but were negative on repeat testing.
(3) Specimen was also positive in a second PCR assay using different primers and probe; probable contamination during extraction.

Sensitivity and specificity of the LC-PCR assay. The sensitivity of an assay is computed by the following formula (17):

# of true positives / # of true-positive + false-negatives X 100% = % Sensitivity

Computation of the LC-PCR assay sensitivity:

65 true positives / 65 true positives + 11 indeterminant/false negatives X 100% = **85.5%**

Specificity is computed by the following formula:

# of true negative results/ negative results + false-positive results X 100% = % Specificty

Computation of the LC-PCR assay specificity:

155 true negatives / 155 negative/indeterminates + 0 false positives X 100%= **100%**

Results of the LC-PCR assay for mycobacterial broth cultures. In addition to detection of *M. tuberculosis* in clinical specimens, the LC-PCR assay was also evaluated as a method to identify AFB from broth cultures. *M. tuberculosis* was detected in eight culture broths using Accuprobe hybridization probes for verification (Genprobe Inc., San Diego, CA). All eight *M. tuberculosis* broth cultures were positive in the LC-PCR assay (100% sensitivity). MOTT were recovered from 20 broth cultures. All 20 cultures were negative by the LC-PCR assay (100% specificity). The results of the LC-PCR assay of various broth cultures are summarized in Table 9.

TABLE 9. LC-PCR for identification of AFB recovered in broth cultures.

	Results with LC-PCR	
Organisms Cultured	**Positive**	**Negative**
M. tuberculosis	8	0
MAI (1)	0	6
M. gordonae	0	6
MOTT (2)	0	4
M. scrofulaceum	0	2
M. chelonae/absessus	0	1
M. kansasii	0	1

(1) *M.avium-intracellulare* complex (2) Mycobacteria other than TB

Correlation of the LC-PCR assay results with MOTT isolates in clinical specimens. To further test the specificity of the LC-PCR assay, 18 smear-positive and

smear-negative clinical specimens were tested which later grew MOTT in culture. The LC-PCR assay was performed on these patient specimens and the results were summarized in Table 10.

TABLE 10. LC-PCR results from MOTT isolates in clinical specimens

Organism cultured	**Results with LC-PCR**	
	Positive	**Negative**
M. avium complex	0	8
M. gordonae	0	7
MOTT(1)	0	3

(1) Mycobacteria other than *M. tuberculosis*

Analyzing the results of TABLE 11, the LC-PCR assay achieved 100% specificity in clinical specimens containing MOTT.

Correlation of AFB smear results and the LC-PCR assay. Generally, AFB smears are less than 70% sensitive in indicating the presence of mycobacteria in a specimen (16). However, the AFB smear has traditionally been the quickest diagnostic tool available to a clinician. Outside of nucleic acid amplification tests, the AFB smear still is the first indicator of a TB infection but is even less sensitive for MOTT infections (28). Table 11 correlates smear and the LC-PCR assay results.

TABLE 11. AFB smear and culture results correlated to results of the LC-PCR

Smear/Culture Results	PCR Positive	Indeter-minate	PCR Negative
AFB positive smear Culture positive	52	7	1
AFB negative smear Culture positive	13	2	1
AFB positive smear TB culture negative MOTT(1) culture positive	0	0	4
AFB negative smear Culture negative	0	1	150

(1) Mycobacteria other than TB

Table 11 shows that all specimens which had AFB positive smears and positive cultures, with one exception, were LC-PCR positive. Overall, the LC-PCR assay is more sensitive than AFB smears in detecting *M. tuberculosis* culture positive specimens. The LC-PCR assay demonstrated 85.5% sensitivity (65 positive LC-PCR assay results to 76 positive cultures) while AFB positive smears were 78.9% sensitive (60 AFB positive smears to 76 positive cultures) in detecting AFB. Furthermore, the LC-PCR assay is specific for the *M. tuberculosis* complex not just AFB. The LC-PCR assay detected *M. tuberculosis* DNA in 13 out of 16 smear negative, culture positive specimens. Four AFB smear positive specimens grew MOTT (*M. avium*) in culture. However, none of these specimens were positive by the LC-PCR assay. No other MOTT positive culture specimens produced a positive result with the LC-PCR, verifying 100% specificity. The one smear and culture negative specimen with the indeterminate result was the one previously discussed which was thought to be contaminated with external DNA during extraction. In summary, the LC-PCR assay was more specific than AFB smear results (100% to 94% respectively).

Additionally, AFB smear results are used to generally monitor the response to antimicrobial drug treatment by showing a decrease in the number of AFB on subsequent smears as treatment progresses. There are many variables in the specimen collection and processing procedures, but the smear can provide a rough estimation of drug efficacy. A comparison of LC-PCR values indicating the concentration of *M. tuberculosis* organisms present versus the number seen on the AFB smear is presented in Table 12.

TABLE 12. LC-PCR Ct values compared to AFB smear quantified results

Smear Results	**19-25 Cycles**	**26-30 Cycles**	**31-35 Cycles**	**36-39 Cycles**	**40-45 Cycles**
Rare	1	5	2	6	4
Few	3	0	8	7	3
Numerous	2	6	11	3	0
Negative	6	0	5	1	3

In Table 12, smears quantified as having few or numerous AFB correlated with Ct values 31-35 and 26-35 cycles, respectively, reflecting good detection by smear and the LC-PCR assay. A greater number of smears with rare numbers of AFB were associated with later Ct cycles (36-45) or smaller *M. tuberculosis* DNA concentrations which is expected. However, the LC-PCR assay is substantially more sensitive than the AFB smears as six smears were negative, but the corresponding specimens showed low Ct values (19-26) and great concentrations of *M. tuberculosis* DNA.

Discussion

The availability of RT-PCR instruments like the LightCycler in public health laboratories makes it feasible to detect *M. tuberculosis* in clinical specimens by molecular methods. The accurate results of the LC-PCR assay provides a better basis for rapid diagnosis and treatment monitoring than previously provided by AFB smear results. The LC-PCR assay described in this study detected *M. tuberculosis* from clinical specimens within the recommended 48-hour period (47). Positive LC-PCR assay results from 76 specimens that grew *M. tuberculosis* in culture demonstrated the LC-PCR assay's ability to detect *M. tuberculosis* with 85.5% sensitivity which is comparable with commercially available nucleic acid amplification tests (43).

The 155 culture negative specimens produced 154 LC-PCR negative results and one indeterminate result requiring a new specimen. No false positive TB results (≤ 40 Ct) were generated by the LC-PCR assay described, thus achieving 100% specificity. There have been few published reports describing *M. tuberculosis* detection by PCR assay on culture negative specimens (35, 43).

To validate the use of an in-house molecular test, public health laboratories must meet validation requirements. Test validation parameters require twice as many negative specimens as positive ones to be tested which yield verifiably accurate results (39). The LC-PCR assay met these validation criteria as 155 *M. tuberculosis* negative specimens and 76 positive specimens were successfully tested with verifiably accurate results. According to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines, greater than 90% agreement is needed between reference assays and those

developed in-house (17). The specificity and sensitivity values of this experimental LC-PCR assay were within the reference value range for validation of the assay (90-100% specificity and 85-95% sensitivity).

The predictive value of a diagnostic test is the probability that a positive or negative result accurately defines a true positive or negative condition (17). Prevalence is used in calculating the predictive value of a clinical test. The prevalence of *M. tuberculosis* positive specimens in this study was 3.75 per 100 patients The positive predictive value of the LC- PCR results was 77.3% in the clinically suspect (AFB positive smears) population. The predictive negative value of the LC-PCR was 99.9%. Thus, a negative LC-PCR result reflected a true negative condition 99.9% of the time.

Like a smear exam, the LC-PCR detects nonviable organisms and is not necessarily predictive of a positive culture in antimicrobial drug treated patients. This study included patients who had already started antimicrobial drug treatment. Only ten of the 76 AFB positive cultures were from patient specimens collected before the initiation of treatment. Drug therapy may have an effect on the performance of diagnostic tests as the drugs cause unpredictable changes in *M. tuberculosis* (38). Thus, prior drug treatment may have caused the indeterminate results found in this study. Defining the indeterminate result category (specimens with reproducible Ct values in the 40-45-cycle range) optimized the sensitivity of the assay without sacrificing specificity allowing for repeat assays on indeterminate specimens. With positive Ct values (less than 40 cycles) providing results with 85.5% sensitivity and 100% specificity, the LC-PCR assay

demonstrated accuracy and reproducibility in detecting *M. tuberculosis* in clinical specimens.

The CDC developed an algorithm for the use of NAA testing like the LC-PCR assay in detection of *M. tuberculosis* in respiratory specimens (10). The CDC recommended NAA testing of all first-time AFB smear positive specimens and smear negative specimens with a high index of suspicion (i.e. symptoms and radiologic images). A positive AFB smear and positive NAA test result constitute a presumptive diagnosis of TB. If a patient specimen is smear negative but NAA test positive, additional specimens (not to exceed three) are tested with the NAA assay. When subsequent specimens test NAA positive, the patient is presumed to have TB. If repeated NAA assay results are negative, the patient is presumed to be free of infection with *M. tuberculosis*. However, clinical judgment is the final criteria for patient treatment (10). The recommended algorithm for NAA testing emphasizes the diagnostic value of the LC-PCR assay on clinical specimens.

This study validated the use of the LC-PCR as an adjunct to smear and culture. Though the LC-PCR assay made it possible to shorten the time for diagnosis of *M. tuberculosis* infections to less than 48 hours, the assay cannot replace the culture since culture isolates are essential for drug susceptibly and epidemiological typing studies. Furthermore, culture is needed for detection of positive cultures with gene copies below the LC-PCR assay detection limit of five gene copies. However, in addition to early detection of TB in smear positive patients, the LC-PCR assay can be effectively used to detect *M. tuberculosis* in AFB smear negative patients with suspicious clinical

symptomology. This can be achieved due to the 100% specificity and 99.9% negative predictive value of the LC-PCR assay. Thus the LC-PCR assay provides an accurate and rapid basis for diagnosis. The LC-PCR assay is a valuable tool that will improve efforts to diagnose and control TB by facilitating accurate and prompt patient treatment.

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