

Development of a Homogeneous DNA Amplification Test for the Real-Time, Direct Detection of *Mycobacterium tuberculosis* DNA on the BDProbeTec™ ET* Instrument

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ABSTRACT

■ *Mycobacterium tuberculosis* (Mtb) infections pose a serious threat to public health, with immunocompromised and HIV positive individuals representing a significant portion of those susceptible to infection. Growth-based culture testing (e.g. BACTEC® systems) for the presence of the pathogen requires many days for detection and identification. As the rise in Mtb infections occurs in various patient groups, the need for a rapid and direct method for detecting Mtb continues. The development of a real-time assay that utilizes strand displacement amplification (SDA) for the direct detection of *Mycobacterium tuberculosis* complex, including an internal amplification control to monitor inhibitory samples, has been developed in a homogeneous/closed format for use on the BDProbeTec™ ET instrument. Amplification of the Mtb genome (95 bp region of the *IS6110* DNA sequence) and an internal amplification control is detected by monitoring changes in fluorescence. The fluorescence emanates from a Mtb specific DNA probe labeled with the dye combination rhodamine/dabcyl and an internal control detector labeled with fluorescein/dabcyl. Statistically designed experiments have been performed to optimize the chemistry (e.g. DNA primers/detectors, fluorescent dyes, buffers, and cosolvents) to achieve a limit of detection of 3.04 cfus/reaction. This amplification system allows for specificity with lack of cross-reactivity. Genomic DNA from mycobacterium belonging to the *M. tuberculosis* complex were found to be positive when tested at 100 copies/reaction, while other mycobacteria and non mycobacteria tested at 10⁷ copies/reaction were negative for amplification. The benefits of this Mtb assay include the following: high sensitivity, an ease of use compared to other technologies currently used in clinical labs, the advantage of having an internal control capable of verifying negative results, and a homogeneous chemistry format allowing for real-time testing of processed samples in one hour using the BD ProbeTec™ ET instrument.

INTRODUCTION

In recent years *M. tuberculosis* infections have seen increases worldwide, especially in Eastern Europe and Asia. The rise of HIV infections in certain geographical populations has had a co-effect on Mtb cases reported, with up to 33% increases in certain regions in the last decade¹. The worldwide increase in *M. tuberculosis* cases and appearance of drug-resistant strains continues to maintain tuberculosis as a great health risk to the population. Growth based technology (e.g. BACTEC® systems) is an established means to aid in diagnosing patients; however, the time required for positive identification has the potential to take from days to weeks. A methodology that allows for rapid time to results would be beneficial to clinicians and the overall health of the greater population.

Nucleic acid amplification technologies possess the ability to increase sensitivity and reduce the time required for the clinician to make a diagnosis. Strand displacement amplification is such a technology, and has previously been shown to be effective in a diagnostic instrument format².

Here we describe the development of a SDA based diagnostic test for use on the BDProbeTec™ ET System. The Mtb Direct Detection test utilizes technology allowing for real-time fluorescent detection of both the target organism DNA and an internal amplification control (IAC) in one well³. The IAC allows for the discrimination of true negative samples from potentially inhibitory⁴ or improperly processed ones.

A major requirement for a Mtb Direct Detection test is high sensitivity, and this has been achieved in a simple assay capable of detecting Mtb DNA in one hour after samples are tested on the BDProbeTec™ ET Instrument.

METHODS

Target Region

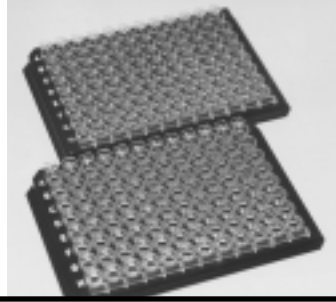
The amplification region for the Mtb Direct Detection Assay is a 95 base pair region of the *IS6110* insertion-like element present in members of the *M. tuberculosis* complex. Figure 1. indicates the SDA bumpers (teal), amplification primers (yellow), and the internal detection regions (purple and orange) for the *IS6110* region of the Mtb genome. The internal amplification control (IAC) sequence shares the SDA amplification primer sequence minus the bumpers, and contains a detection region different than the Mtb *IS6110* sequence.

SDA Mechanism

SDA amplification can be broken down into two phases. The first phase, the Target Generation step (Figure 2), creates the amplicon that feeds into the second phase, the Exponential Amplification step (Figure 3). Before annealing can occur, the sample is mixed with an excess of two primers (S₁ and S₂), two bumpers (B₁ and B₂), and a detector probe (Figure 2, steps 1 and 2). Primers consist of a Mtb DNA hybridizing region at the 3' end, a non-hybridizing tail at the 5' end, and a recognition site for the restriction enzyme *BsoBI* (CTC GGG) between these two regions (Figure 2). Polymerase and restriction enzyme are added



BD ProbeTec™ ET



after the priming step, causing the amplification primers and bumpers to be extended (Figure 2, step 3). In the process of extending the bumpers, the polymerase displaces the primer extension products. These extension products will hybridize to complementary primers and bumpers (Figure 2, step 4) and additional extension and displacement follows. The resulting strands can be hybridized to complementary primers, and these primers can be extended. The end products (Figure 2, step 5) contain the detector probe annealing region flanked by nickable *Bso*BI sites.

The primers contain the restriction sequence CTC GGG. During extension, the polymerase incorporates alpha thio-dCTP to create the complement of the restriction site. The result is a hemiphosphorothioated restriction site, which can only be nicked, not completely cut. The products of Figure 2, step 5 can only be nicked by *Bso*BI. The polymerase will extend from the nicked site, displacing the fragments designated as T_1 and T_2 , which will feed in to the second phase, Exponential Amplification (Figure 3, left side).

Detection

Amplification and detection occurs simultaneously. The detector probe consists of a target DNA specific hybridization region at the 3' end, and a hairpin structure at the 5' end. The loop of the hairpin contains the *Bso*BI recognition sequence CCC GAG. The 5' base is conjugated to a Donor Dye (i.e. Rhodamine), while the 3' base of the hairpin stem is conjugated to the Acceptor (i.e. Dabcyl). In its native state, the hairpin maintains the Donor and the Acceptor dyes in close proximity. When the Donor is excited, the fluorescent energy is transferred to the Acceptor molecule, and little fluorescence is observed. As the hairpin anneals to the target amplicon, it is extended by the polymerase and then displaced by the extension of an upstream primer (Figure 3, step 1-3). The resulting detector extension

product is complementary to the downstream primer S_2 (step 4). The primer (S_2) is extended (step 5) and the hairpin is linearized. This creates a double stranded restriction site capable of being cleaved by *Bso*BI. This process frees the Donor from the quenching effects of the Acceptor and allows fluorescence to be observed by the BDProbeTec™ ET instrument.³

System Optimization

Optimization occurred in a liquid format before the test was transformed into a dried format in which reagents were dried in either a priming microwell or amplification microwell. Priming wells containing primers, bumpers, fluorescent detectors, IAC and other SDA components were rehydrated with lysed or boiled target in sample buffer. Wells were incubated at room temperature for 20 minutes, then warmed to 70°C on a heat block. Amplification wells containing the restriction enzyme (*Bso*BI), polymerase (Bst), nucleotides, and the remaining SDA components were pre-warmed at 52°C as the priming wells were heated. After ten minutes, the samples were transferred from the priming microwells to the amplification wells, sealed, then placed in the BDProbeTec™ ET instrument for one hour. The thermally controlled fluorescent reader within the instrument monitors each reaction for the generation of amplified products, and data is converted to a metric known as MOTA—a measurement of the area underneath a relative fluorescent unit curve.

Development of the Mtb Direct Detection test has utilized statistically designed experiments for optimization of the essential SDA chemistry components. Figure 4. shows a comparison of two different buffer systems—40mM potassium phosphate, pH 7.6 and 100mM bicine, pH 8.7. The original Mtb assay utilized a potassium phosphate buffer for amplification; however, this was found not be compatible with the improved sample processing protocol. The higher pH bicine buffer was found to yield better amplification/sensitivity for Mtb and was more suitable with the

Figure 2. Mechanism of SDA—Target Generation Step

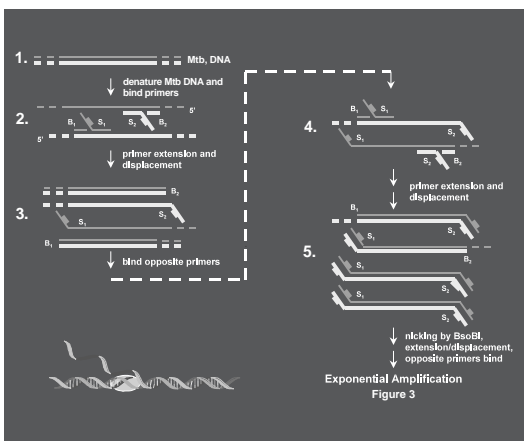
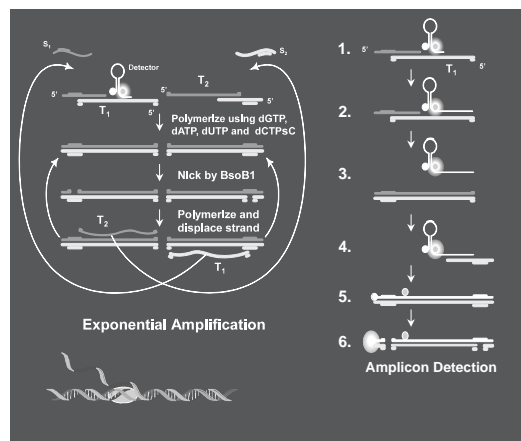


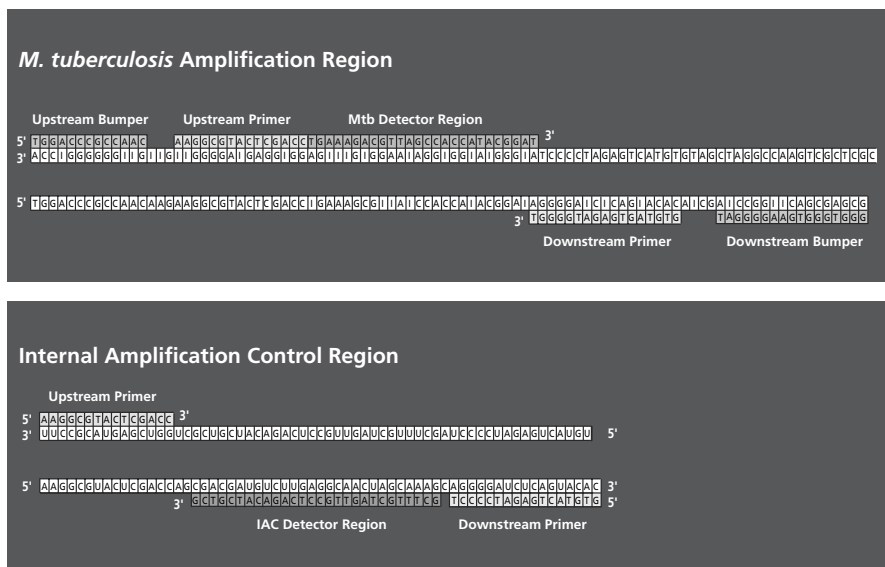
Figure 3. Homogeneous SDA Detection Test



sample processing chemistry⁴. The improved amplification performance is theorized to be a result of the Bst polymerase's requirement for a basic pH. Additionally, the dye pairs rhodamine/dabcyl and fluorescein/dabcyl were examined for both DNA targets. Rhodamine/dabcyl was found to yield increased amplification/detection for both Mtb and the IAC, and for this reason was chosen for the Mtb detector for future experiments.

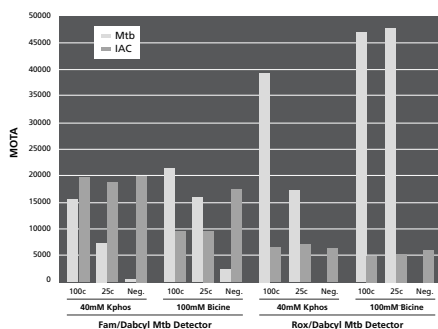
All other SDA components were optimized for obtaining increased Mtb sensitivity and an IAC robust enough to yield low numbers of indeterminate results. Multiple statistically designed factorial experiments were performed to optimize DMSO, glycerol and BsoBI/Bst concentrations (Data not shown). Additionally, the optimal pH range and potassium concentrations for bicine sample buffers for Mtb and IAC amplification/detection have been performed (Figure 5). The Mtb Direct Detection test can tolerate pH variations from 8.4 to 9.0. The potassium concentration affected the IAC performance more than the Mtb target DNA. A balance in Mtb and IAC performance was achieved at a buffer pH of 8.7 and 62 mM potassium (Data not shown). BsoBI and Bst concentrations were examined to determine the best combination for Mtb and IAC: 25.5 BD units of BsoBI and 8 BD units of Bst yielded high MOTA values, no dropouts, and low variability (Figure 6). Specificity and cross-reactivity were determined using 100 genomic copies of DNA from 5 members of the *M. tuberculosis* complex and 1x10⁷ genomes of other mycobacteria and non mycobacteria species. The fluorescent, real-time SDA chemistry resulted in positive results for all of the Mtb complex members and negative ones for all of the potential crossreactants (Table 1). Optimization of the SDA chemistry was finalized and used to determine a limit of detection (LOD) using a double stranded plasmid containing a portion of the *M. tuberculosis* genome (K10) and *M. tuberculosis* H37Rv cells spiked into BBL® MycoPrep™ phosphate buffer, which is normally used for resuspension of NALC pellets. The K10 DNA was denatured by boiling for 5 minutes and added to the appropriate bicine buffer, while the cells were taken through the Sample Processing procedure⁴. An LOD of 11 genome copies of plasmid DNA/reaction was achieved in the clean chemistry format and 3.04 cfus/reaction was the LOD for the H37Rv cells (Figure 7).

Figure 1. Amplification Regions for *M. tuberculosis* and IAC DNA Sequences



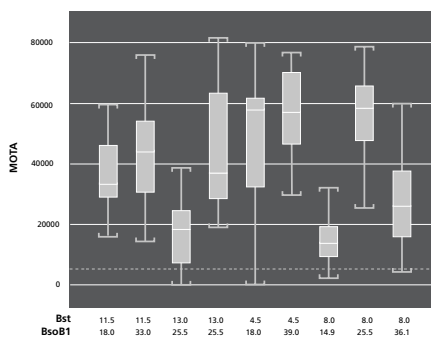
Positions of SDA Bumpers, Amplification Primers and Detectors for genomic Mtb and synthetic IAC target DNA. In the case of the Primers and Detectors, the colored sequences represent the annealing regions of those oligonucleotides, hence the tail regions and BsoBI sites are not shown. The Mtb target DNA and IAC will both be amplified by the SDA Primers, but differentiate by the use of Rox/Dabcyl and Fam/Dabcyl dye pair labeling on the Detectors.

Figure 4. Comparison of Mtb and IAC Performance with Different Dye Pair Combinations and Buffer Conditions



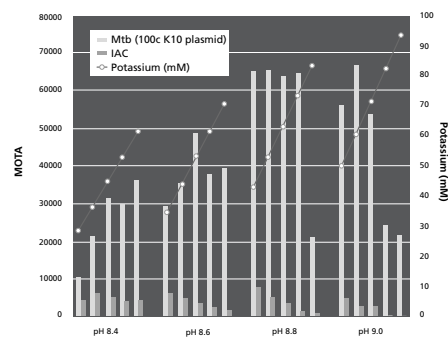
Comparison of Rox/Dabcyl and Fam/Dabcyl dye pairs for use on the Mtb specific and IAC specific detectors. This was examined in combination with potassium phosphate and bicine buffers for amplification. The dye pair used for the Mtb detector probe is specified, thus the opposite dye pair is used for the IAC in those samples. A plasmid DNA clone containing a portion of the *M. tuberculosis* genome (K10) was used for Mtb amplification. Mean values of 4 replicates of 100, 25 and zero copies of the K10 plasmid were tested in conjunction with the IAC. Amplification is calculated by measuring the area underneath the relative fluorescent unit curve (MOTA).

Figure 6. Optimization of BsoBI and Bst Concentrations for Mtb Direct Detection



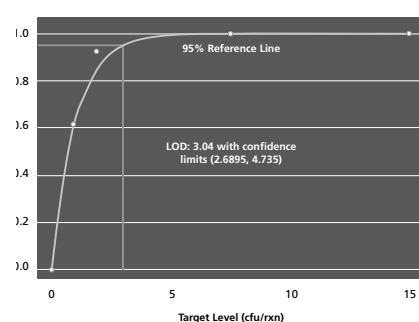
Statistical analysis of data from a BsoBI and Bst optimization experiment for Mtb. 24 replicates of 100 copies of K10 plasmid per enzyme combination was tested in the Mtb Direct Detection test. A cut-off of 5000 MOTA was used to determine successful amplification/detection of reactions. An combination of 25.5 BD units of BsoBI and 8 BD units of Bst was found to be chosen to be optimal. This is based on this data and that from an identical experimental design performed on the IAC (Data not shown).

Figure 5. Bicine pH and Potassium Tolerance



pH and salt robustness is critical for sample processing, since NALC samples will bring variation to the final amplification reaction. pH ranges of 8.4 to 9.0, and potassium ranges of 28 to 93mM were shown to yield sufficient Mtb amplification, but high salt did decrease the IAC. The graph above represents the mean value for four replicates of 100 copies of K10 plasmid and the IAC. Further optimization indicated that a Bicine Buffer of pH 8.7 and 62mM potassium produced robust Mtb sensitivity.

Figure 7. Analytical Sensitivity for Mtb Direct Detection



A Limit of Detection (LOD) of 3.04 cfus/reaction was determined for *M. tuberculosis* strain H37Rv using the Mtb Direct Detection test on the BDProbeTec™ ET System.

Table 1. Specificity and Cross-reactivity Testing

<i>M. tuberculosis</i> Complex Species			Mycobacteria Species			Non Mycobacteria Species			
Organism	Strain	Mtb MOTA	Organism	Strain	Mtb MOTA	Organism	Strain	Mtb MOTA	
<i>M. tuberculosis</i>	VA44	26643, 27260	<i>M. aichiense</i>	ATCC 27280	Negative	<i>A. israeli</i>	ATCC 10049	Negative	
<i>M. africanum</i>	ATCC 35711	38346, 35722	<i>M. aurum</i>	ATCC 23366	Negative	<i>A. auranticolor</i>	ATCC 15330	Negative	
<i>M. microti</i>	LCDC 203	28662, 27260	<i>M. avium</i>	ATCC 25291	Negative	<i>C. diphtheriae</i>	ATCC 11913	Negative	
<i>M. bovis</i>	CDC 52	20102, 31379	<i>M. celatum</i>	ATCC 5131	Negative	<i>C. pseudodiphtheriticum</i>	ATCC 10700	Negative	
<i>M. bovis BCG</i>	CDC 4	23690, 17292	<i>M. chelonae</i>	ATCC 1543	Negative	<i>C. xerosis</i>	ATCC 373	Negative	
<p>All species belonging to the <i>M. tuberculosis</i> complex were tested at 100 genomic copies and found positive by detection. Positivity is defined as a MOTA value greater than 5000. All potential cross-reactant mycobacteria and non mycobacteria species were tested in the Mtb Direct Detection test at 1x10⁷ genomic copies of DNA. All non <i>M. tuberculosis</i> complex mycobacteria and non mycobacteria species were found to be negative by the assay (MOTA values less than 5000). The IAC was positive for all negative cross-reactant sample reactions, indicating that amplification was not inhibited in those samples. This data is not included. Samples were tested in duplicate on the BDProbeTec™ ET System.</p>			<i>M. chitae</i>	ATCC 19627	Negative	<i>E. lentem</i>	ATCC 43055	Negative	
				<i>M. chlorophenolicum</i>	ATCC 49826	Negative	<i>N. asteroides</i>	ATCC 3308	Negative
				<i>M. confluentis</i>	ATCC 49920	Negative	<i>N. brasiliensis</i>	ATCC 19296	Negative
				<i>M. fortuitum</i>	ATCC 2808	Negative	<i>N. orientalis</i>	ATCC 19795	Negative
				<i>M. gadium</i>	ATCC 27726	Negative	<i>P. acnes</i>	ATCC 6919	Negative
				<i>M. gastri</i>	ATCC 15754	Negative	<i>R. equi</i>	ATCC 6939	Negative
				<i>M. gilvum</i>	ATCC 43909	Negative	<i>R. rhodochrous</i>	ATCC 13808	Negative
				<i>M. gordonae</i>	ATCC 14470	Negative	<i>S. albus</i>	ATCC 3004	Negative
				<i>M. hemophilum</i>	ATCC 43160	Negative	<i>S. gedanensis</i>	ATCC 4880	Negative
				<i>M. interjectum</i>	ATCC 51457	Negative	<i>S. griseus</i>	ATCC 10137	Negative
				<i>M. intermedium</i>	ATCC 51848	Negative	<i>S. somaliensis</i>	ATCC 13201	Negative
				<i>M. intracellulare</i>	ATCC 13950	Negative	<i>S. viridialbum</i>	ATCC 33328	Negative
				<i>M. kansasii</i>	TMC 1201	Negative	<i>S. alboverticillatum</i>	ATCC 29818	Negative
				<i>M. komossense</i>	ATCC 33013	Negative			
				<i>M. malmoense</i>	ATCC 29571	Negative			
				<i>M. marinum</i>	BD 2324	Negative			
				<i>M. neoaurum</i>	ATCC 25795	Negative			
				<i>M. obuense</i>	ATCC 27023	Negative			
				<i>M. paratuberculosis</i>	ATCC 19698	Negative			
				<i>M. scrofulaceum</i>	ATCC 19981	Negative			
				<i>M. simiae</i>	ATCC 25273	Negative			
				<i>M. simiae</i>	ATCC 25275	Negative			
				<i>M. smegmatis</i>	ATCC 19420	Negative			
				<i>M. sphagni</i>	ATCC 33027	Negative			
				<i>M. szugari</i>	ATCC 29716	Negative			
				<i>M. szulgai</i>	ATCC 23069	Negative			
			<i>M. xenopi</i>	ATCC 19250	Negative				

CONCLUSION

- A homogeneous, dried SDA chemistry has been developed for the direct detection of *M. tuberculosis* complex organisms, which utilizes fluorescent energy transfer for detection. This assay includes an internal amplification control (IAC) for discriminating inhibitory samples. Both target DNA and IAC’s amplification/detection are monitored simultaneously in one microwell on the BDProbeTec™ ET instrument.
- The Mtb and IAC amplification chemistries have been optimized for maximum sensitivity and lack of cross-reactivity. This has been accomplished by adjusting concentrations of oligonucleotides, buffers/pH, enzymes, co-solvents and fluorescent-labeled detectors, so that the assay is robust to the variability of chemical components that occurs in the processing of clinical respiratory samples.
- Five strains of mycobacteria belonging to the *M. tuberculosis* complex were amplified and detected at 100 genomic copies by performing this assay on the BDProbeTec™ ET instrument. Thirty-one non *M. tuberculosis* complex and 18 non mycobacteria species have been tested and found not to cross-react with the Mtb Direct Detection test.
- Analytical sensitivity of the Mtb Direct Detection test has shown that a limit of detection (LOD) of 11 genomic copies/reaction and 3.04 colony forming units (cfu)/reaction, with 95% confidence, is possible on the BDProbeTec™ ET System.
- The Mtb Direct Detection test is a simple test whose benefits are dried chemistry format, ease of use, and sealing of reactions to reduce contamination (sealed microwells).

References

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2. S. Ichiyama et al, “Diagnostic Value of the Strand Displacement Amplification Method Compared to Those of Roche Amplicor PCR and Culture for Detecting Mycobacteria in Sputum Samples”, *Journal of Clinical Microbiology*. 35:3082-3085, 1997
3. J. G. Nadeau et al., “Detection of Nucleic Acids by Fluorescence Quenching”, US Patent 5846726, 12/98
4. O. Llorin et al “Evaluation of the BDProbeTec™ ET System for the Direct Detection of Mycobacterium tuberculosis Complex from Smear Negative Respiratory Samples”, Poster U27