

# Evaluation of the BDProbeTec™ ET\* System for the Direct Detection of *Mycobacterium tuberculosis* Complex from Smear Negative Respiratory Samples

O. LLORIN, T. BRINK, S. BUSTOS, D. COPERTINO, C. FINNERTY, J. HARRIS, T. POTH, J. PRICE, AND K. YANSON

BD Biosciences • 54 Loveton Circle • Sparks, MD, USA 21152

## REVISED ABSTRACT

■ The nucleic acid amplification detection of *Mycobacterium tuberculosis* (M.tb) from acid fast bacilli (AFB) smear negative respiratory samples requires maximizing the volume of sample amplified, the removal of amplification inhibitors, efficient M.tb lysis, and robust amplification. We have developed a user-friendly sample processing method for respiratory samples in which strand displacement amplification (SDA) inhibitors are first removed from 500µl of digested/ decontaminated NALC sediment. SDA inhibitors are selectively solubilized by buffered chaotrope, leaving M.tb intact for centrifugal capture. Efficient M.tb lysis then occurs by subjecting pelleted cells to heat, alkali, and sweeping frequency ultrasonic energy. Statistical experimentation was implemented to determine optimal temperature, pH, and ultrasonic time parameters for maximal release of nucleic acids from M.tb cells. Processed samples are then simultaneously amplified and assayed in a homogeneous system for direct detection of both specific M.tb complex target sequence and an Internal Amplification Control (IAC) target sequence on the BDProbeTec™ ET instrument. The instrument allows for the detection of amplified products from processed samples in one hour. To evaluate the performance of the combined sample processing and assay systems for the detection of M.tb complex from smear negative clinical samples, 25 smear negative, M.tb culture positive and 25 smear negative, M.tb culture negative respiratory samples were tested. Sample types included sputa, bronchial washings, and bronchoalveolar lavages from eight clinical sites. Reference methods included the smear and culture results from the clinical site, along with patient chart history. The resolved sensitivity and specificity was 89.3% (25/28) and 100% (22/22), respectively. The (IAC) was positive in all samples tested, indicating that there was no inhibition of amplification in samples where M.tb target amplification did not take place. We conclude that the BDProbeTec™ ET System is both sensitive and specific for the direct detection of M.tb complex from smear negative respiratory samples.

## INTRODUCTION

In 1998, nearly 3 million people died of tuberculosis, more than any other year in history. The estimated 8.8 million new cases every year correspond to 52,000 deaths per week or more than 7,000 each day, which translates into more than 1,000 new cases every hour, every day.<sup>1</sup> Between 1993 and 1996 there was a 13% increase in TB cases worldwide. One third of the increase in the incidence of TB in the last five years can be attributed to the HIV pandemic.<sup>2</sup> Another factor contributing to the worldwide increase in TB infections is the emergence of multi-drug resistant strains. Tuberculosis continues to be a global public health problem.

Conventional methods for identification of M.tb from culture first require growth, which can take 2 to 3 weeks. Non-amplified probe tests can then identify M.tb within hours. At the very least, the time needed from sample collection to M.tb identification can take several weeks. The introduction of direct nucleic acid amplification tests has reduced this M.tb identification time to within hours of sample collection. In theory, these tests can detect a single organism in respiratory samples. Despite this, accurate detection of M.tb from AFB smear negative, M.tb culture positive samples with nucleic acid amplification tests continues to be problematic. Sensitivity obtained by commercial systems testing these sample types range between 29-85%.<sup>3,4</sup>

There are several reasons contributing to the poor sensitivity of M.tb amplification tests on smear negative samples. First, incomplete removal of amplification inhibitors from the respiratory sample can be a source of false negative results. Second, there are few organisms present in an AFB smear negative sample. The threshold for detection by AFB smear is approximately  $1 \times 10^4$  cfu/ml. Actual numbers of organisms present in a smear negative, culture positive sample can range from an upper limit of  $1 \times 10^4$  cfu/ml to a lower limit of 10 viable M.tb per ml for culture.<sup>5</sup> Third, M.tb grows in clumping, cable-like arrangements. This uneven distribution of M.tb compounds the sampling errors caused by the few organisms present to start with. Finally, nucleic acid extraction from M.tb cells is difficult. Target nucleic acid is entrapped in a thick waxy cell wall. Inefficient extraction can contribute to decreased sensitivity.

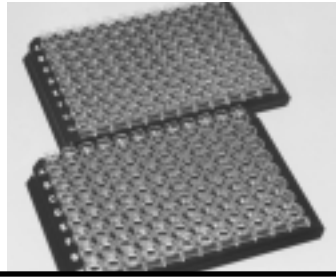
Here we report the development of a simple sample processing procedure designed to specifically overcome the challenges that AFB smear negative, culture positive samples present (Figure 1). The procedure has been coupled with the homogeneous SDA system for direct detection of both specific M.tb complex target sequence and an internal amplification target sequence (IAC) on the BDProbeTec™ ET system.

## METHODS

**RESPIRATORY SPECIMEN.** Seventy-four respiratory specimens (sputa, bronchial washings, and bronchoalveolar lavages) were submitted to nine clinical sites throughout the United States and one site in Brazil. At each clinical site, a portion of each specimen was digested, decontaminated by NALC/NaOH



# BDProbeTec™ ET



treatment.<sup>5</sup> The NALC sediment at each clinical site was cultured by routine methods including inoculation into BACTEC™ 12B, and onto 7H11 and/or Lowenstein-Jensen slants. AFB smears were prepared from each sample and stained with Auramine O, Ziehl-Neelsen, or Kinyoun stain, depending on the site. A portion of the unprocessed respiratory sample (ranging from 3 to 26 mls) was sent frozen to BD Biosciences for subsequent BDProbeTec™ ET analysis. At BD Biosciences, up to 10ml of the most purulent portion of each specimen was digested, decontaminated by NALC/NaOH treatment using BBL® Myco-Prep™.

**BDPROBETEC™ ET SPECIMEN PROCESSING (Figure 5).** The NALC sediments were briefly agitated on a vortex mixer. A 500µl volume of specimen was added to 1ml of Sample Wash Buffer, agitated on a vortex mixer for 5 sec., and centrifuged at 12,200x g for 3 min. The supernatant was discarded, the pellet was heated in the BDProbeTec™ ET Oven for 30 min. at 105°C, and quick spun for 10 sec. The pellet was resuspended in 100µl of Sample Lysis Buffer, agitated on a vortex mixer for 5 sec., and then sonicated for 45 min. at 65°C in the BDProbeTec™ Sonic Bath. The sample was then quick spun for 10 sec, followed by addition of 600µl of Sample Neutralization Buffer. The sample was agitated on a vortex mixer, and quick spun for 10 sec. The samples were either assayed immediately on the BDProbeTec™ ET instrument or frozen at -20°C for future analysis.

**BDPROBETEC™ ET SYSTEM ASSAY.** For a discussion of TB/IAC homogeneous SDA chemistry, please see poster U25, Harris, et al. "Development of a Homogeneous DNA Amplification Test for the Real-Time, Direct Detection of M.tb DNA on the BDProbeTec™ ET Instrument." Prior to the BDProbeTec™ ET testing, frozen samples were thawed at room temperature, heated in the BDProbeTec™ ET Oven for 30 min. at 105°C and then quick spun for 10 sec. No further preparation was necessary for specimens prepared and assayed the same day. For each BDProbeTec™ ET assay, at least one positive and negative control were prepared by adding 100µl of Sample Lysis Buffer and 600µl of Sample Neutralization Buffer to each control tube. Control tubes were then agitated on a vortex mixer for 5 sec., and then quick spun for 10 sec. Samples and controls were randomly distributed into the sample rack and the corresponding number of disposable Priming and Amplification microwells were placed into their respective metal microwell plate. Using a programmable eight-channel pipettor and aerosol resistant tips, 150µl of samples and controls were dispensed into the Priming microwells. The Priming microwell plate was covered with the Priming cover and incubated at room temperature for 20 minutes (plates can be held at room temp. for up to 6 hr.). At the end of 20 min., the cover was discarded and the Priming microwell plate was placed into the 71.5°C heat block. At this time, the Amplification microwell plate was immediately placed into the 53.5°C heat block for prewarming. After 10 min. of incubation, 100µl from each Priming well was transferred using the multi-channel pipettor to the corresponding Amplification microwell and was automatically mixed by the pipettor. After all samples and controls had been transferred, the Amplification microwells were sealed using the

Amplification plate sealer. The Amplification plate was then immediately placed into the BDProbeTec™ ET instrument. After one hour, MOTA (Metric Other Than Acceleration, a measurement of area under the relative fluorescent unit curve) values were provided by the instrument for each sample and control. Samples with M.tb MOTA values  $\geq 3,400$  were considered positive for M.tb complex DNA. If the M.tb MOTA was  $< 3,400$  and the IAC MOTA was  $\geq 5,000$ , then the specimen was considered negative for M.tb complex DNA. If the M.tb MOTA was  $< 3,400$  and the IAC MOTA was  $< 5,000$ , then the result was considered indeterminate.

**DISCREPANT ANALYSIS.** Discordant results were resolved using the reference methods of smear and culture results from the clinical site along with patient chart history. If the culture and BDProbeTec™ ET results were discordant, the discrepant sample was reassayed on the BDProbeTec™ ET instrument. Although repeat testing was done for informational use, only initial BDProbeTec™ ET results were used in the final tabulation of resolved results.

Figure 1.

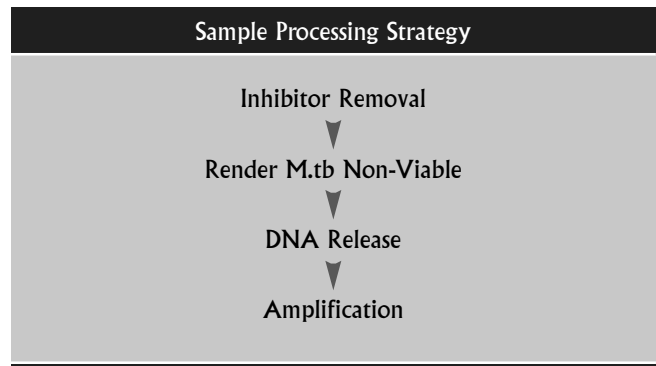


Figure 5.

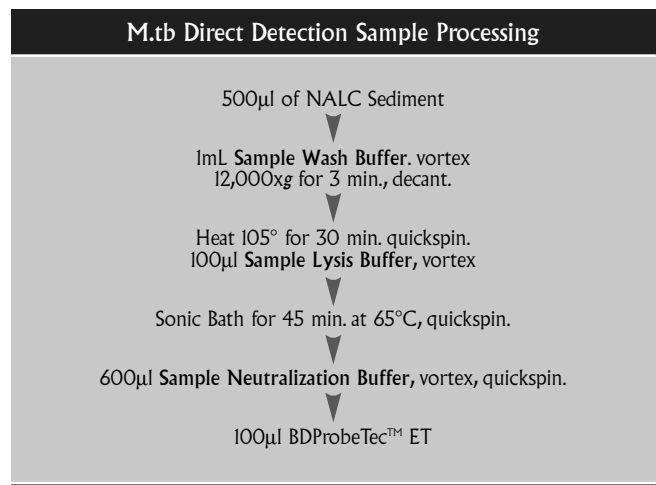
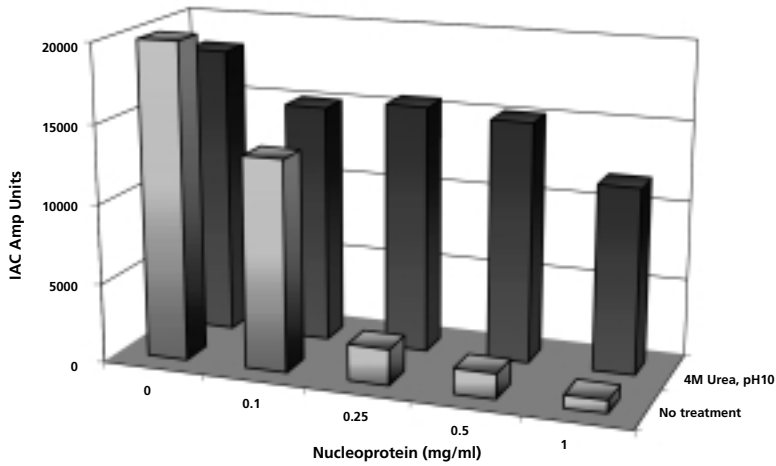


Figure 2. Inhibition Removal and the Internal Amplification Control (IAC)



In this model system, inhibition of the IAC is relieved by pretreatment of samples with an alkaline buffered chaotrope solution.

Figure 4. Bicine Buffer Improves Homogenous SDA

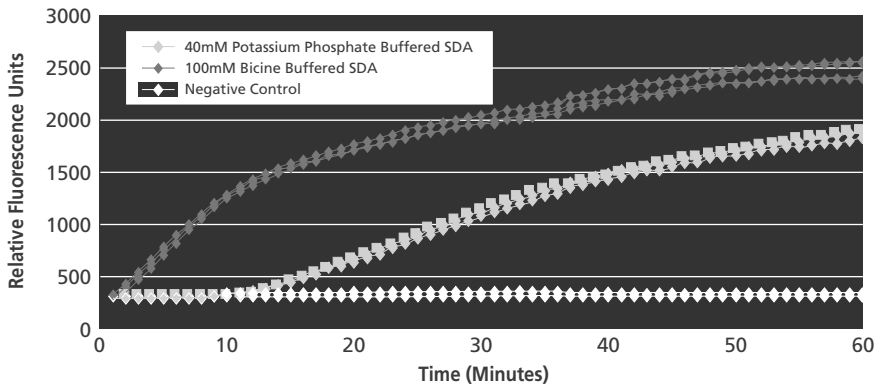
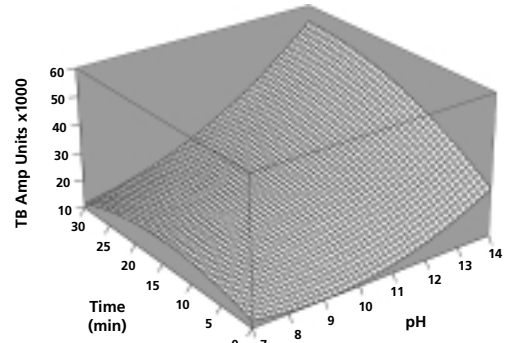


Figure 3. M.tb DNA Extraction by 65°C Heat, Alkali, and Ultrasonic Energy Exposure



Response surface plot was generated by face centered design experimentation. Efficiency of lysis was measured by thermophilic SDA of IS6110 target.

100 copies of IS6110 was amplified in SDA using two buffers. A higher bicine pKa closer to the optimal pH for TB/IAC homogeneous SDA improved amplification kinetics. KPO4 was inadequate for an alkaline based M.tb lysis protocol.

Table 1. Initial Results on R&D Study

Clinical Lab Results			BDProbeTec™ ET Initial Results		
AFB Smear	M.tb Culture	n=	TB Positive	TB Negative	Indeterminate
Positive	Positive	24	24	0	0
Negative	Positive	25	22	3 <sup>a</sup>	0
Negative	Negative	25	3 <sup>a</sup>	22	0

Initial Sensitivity	Initial Specificity	Indeterminate
Smear +, Culture + = 100%	Smear -, Culture - = 88%	0/74 samples tested = 0%
Smear -, Culture + = 88%		

<sup>a</sup> Upon repeat testing, one of 3 false negative samples remained negative. At clinical site, only one colony grew on Lowenstein-Jensen media; BACTEC™ I2B and 7HII cultures were negative. Remaining 2 false negative samples were TB positive on BDProbeTec™ ET repeat testing. Low sample load indicated in all three samples.  
<sup>b</sup> Discrepant analysis on the 3 initial false positives found that these samples were TB positive on repeat BDProbeTec™ ET testing. Two of three samples originated from an individual found to be infected with M.tb in 1991. Antibiotics were given for 9 months. When the two samples tested in this study were collected in 1998, this patient reported to the TB clinic coughing blood. The remaining false positive sample had a cohort BACTEC™ I2B, BDProbeTec™ ET Culture Confirmation analysis of this I2B culture, at a GI of 15, gave a positive TB result.

Table 2. Resolved\* Results on R&D Study

Clinical Lab Results			BDProbeTec™ ET Resolved Results		
AFB Smear	M.tb Culture and/or Patient History	n=	TB Positive	TB Negative	Indeterminate
Positive	Positive	24	24	0	0
Negative	Positive	28	25	3	0
Negative	Negative	22	0	22	0

Resolved Sensitivity	Resolved Specificity	Indeterminate
Smear +, Culture + = 100%	Smear -, Culture - = 100%	0/74 tested = 0%
Smear -, Culture and/or Patient History + = 89.3%		

\* Discordant results were resolved using the reference methods of smear and culture results from the clinical site along with patient chart history.

## References

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## DISCUSSION

The use of an alkaline buffered chaotrope solution is employed to remove inhibitors of amplification. This strategy solubilizes SDA inhibitors while leaving the M.tb target organism intact.<sup>6</sup> The solution has been shown to remove SDA inhibitors such as charged proteins (Figure 2). Glycoproteins and mucins are typical inhibitors found in digested, decontaminated respiratory samples. High levels of endogenous non-target DNA have also inhibited SDA. The inhibitor removal wash takes advantage of the refractory nature of M.tb by lysing non-M.tb cells in the sample. In this way, SDA inhibition by non-target DNA is reduced. The internal amplification control monitors amplification, thus ensuring that a negative result is indeed negative.

The clumping of M.tb cells and the few numbers of cells present can cause sampling errors. We have addressed this concern by increasing the volume of primary NALC sample that is amplified after processing. The NALC sample volume amplified on the BDProbeTec™ ET is relatively large in comparison to volumes tested in other commercial assays. By taking 500µl of digested and decontaminated sample, resuspending its centrifuged pellet in 700µl, and testing 100µl of the final resuspended volume, 71µl of the NALC sediment is amplified. In contrast, 10-25µl is the range of NALC sediment volumes tested in commercial systems available in the U.S. The limit of detection of 42 M.tb H37Rv cfu/ml for the BDProbeTec™ ET system, reflects the benefits of this increased primary NALC sample volume.<sup>7</sup> As discussed previously, M.tb can be present anywhere from 10-1x10<sup>4</sup> cfu/ml in a smear negative, M.tb culture positive sample.

To overcome the difficulty of extracting target nucleic acid that is entrapped within a waxy cell wall, statistically designed experiments were conducted to optimize the parameters of M.tb exposure to temperature, alkaline pH, and sweeping frequency ultrasonic energy (Figure 3). Conventional SDA, up to this point, had used ≤40mM potassium phosphate to buffer the sample. The incorporation of a 100mM bicine buffer afforded

SDA the ability to neutralize alkali treated samples to optimal SDA pH conditions. The higher pKa of bicine, closer to the optimal SDA pH condition, had the additional benefit of improving amplification kinetics (Figure 4).

The clinical data demonstrate that the sample processing procedure coupled with homogenous TB/IAC SDA on the BDProbeTec™ ET system allows for a sensitive and specific M.tb assay. If the assay is to be sensitive at detecting low positive, AFB smear negative samples, then the detection of M.tb from high positive, AFB smear positive, M.tb culture positive samples should be without flaw. This is indeed the case (Table 1). Of the 24 AFB smear positive, M.tb culture positive samples that were tested, all were correctly identified by the BDProbeTec™ ET system. In our R&D study evaluating the assay on 28 smear negative samples, the resolved sensitivity was found to be 89.3% (Table 2).

Of the three smear negative samples missed, one sample was found to have been called culture positive because of one colony which grew on a Lowenstein-Jensen slant. The cohort cultures in BACTEC™ 12B and solid media 7H11 slant had no growth. This indicates that low sample load probably contributed to this false negative result. Repeat testing of the two remaining smear negative false negatives resulted in the detection of M.tb. This also indicated low sample load as a probable cause for the initial results. The resolved specificity was found to be 100% and the IAC determined that all samples (n=74) were amplification competent.

## CONCLUSION

- We conclude that the BDProbeTec™ ET system is a sensitive and specific assay that allows for the simultaneous amplification and detection of M.tb from digested, decontaminated respiratory specimens. The benefits of a simple sample processing protocol designed for maximal low-positive sensitivity and specific M.tb detection has been demonstrated.