

Detection of *Mycoplasma pneumoniae* by Strand Displacement Amplification on the BDProbeTec™ ET System

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ABSTRACT

■ *Mycoplasma pneumoniae* causes approximately 15-20% of all cases of pneumonia. Standard laboratory methods for diagnosis of *M. pneumoniae* include culture and serology. While *M. pneumoniae* organisms are fastidious and require up to 3 weeks to culture, serological methods are insensitive and non-specific. Nucleic acid amplification methods for the detection of *M. pneumoniae* offer the advantages of speed, sensitivity and specificity. We have developed a method for the detection of *M. pneumoniae* using strand displacement amplification (SDA) and real-time detection. This assay is part of a respiratory panel currently under development for the BDProbeTec™ ET system, including tests for *Legionella pneumophila* and *Bordetella pertussis*, that utilize a universal fluorescence energy transfer detector probe. Included in each assay is an internal control to enhance confidence in results. The analytical sensitivity of the BDProbeTec™ ET *M. pneumoniae* assay is less than 100 copies of target DNA per reaction. The assay detects all strains of *M. pneumoniae* tested and does not exhibit significant cross-reactivity with any other relevant members of the genus *Mycoplasma* or other bacteria evaluated to date.

INTRODUCTION

■ *Mycoplasma pneumoniae* is predominantly a pathogen of the human respiratory tract and can cause bronchitis, pharyngitis and walking pneumonia. It most commonly infects older children and young adults and usually occurs in fall and early winter. Symptoms include headache, low-grade fever, malaise, dry cough and sore throat.¹

Pneumonia caused by *M. pneumoniae* cannot be distinguished clinically from that caused by other bacteria and viruses. Traditional laboratory methods of diagnosis include culture and serology. *M. pneumoniae* is fastidious, requiring special media and several weeks to grow; while serological methods are insensitive, non-specific and also cumbersome to perform. Nucleic acid amplification techniques offer the potential for improved sensitivity and specificity as well as rapid results, allowing for easier and more accurate diagnosis.

A method has been developed for the detection of *M. pneumoniae* DNA using strand displacement amplification (SDA) and real-time detection on the BDProbeTec™ ET System. This assay amplifies a region within the P1 cytoadhesion gene of *M. pneumoniae*. A universal detector probe is utilized for real-time fluorescence energy transfer detection. An internal amplification control (IAC) has been incorporated, that is co-amplified with native DNA, to identify samples that may contain inhibitors of the SDA reaction. The dual-dye capabilities of the BDProbeTec™ ET System allow for detection of both native target and IAC detector probes to occur within 60 minutes (Figure 1). The assay described here comprises a simple workflow (Figure 2).

The *M. pneumoniae* assay is part of a respiratory panel of tests currently under development for the BDProbeTec™ ET System. The other tests in this panel include assays for *Legionella pneumophila*, *Bordetella pertussis* and the *Chlamydiaceae* family. The *M. pneumoniae* SDA assay is sensitive and specific and combines a simple workflow with rapid results.

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1 Mahon, CR and Manuselis G, (1995) *Textbook of Diagnostic Microbiology*. pp. 623-624. W.B Saunders Company, Philadelphia, PA.

METHODS

DNA TARGET AND INTERNAL AMPLIFICATION CONTROL. A segment of the *M. pneumoniae* P1 gene was cloned into pUC19 vector and used as the target during assay development. The internal amplification control (IAC) was prepared by mutation of the sequence within this clone that corresponds to the SDA amplicon. Analytical quantification of plasmid stocks was performed using the Picogreen assay (Molecular Probes, Inc.). Diplex SDA reactions contained 100 copies of the IAC plasmid.

SPECIFICITY. One-hundred fold dilutions of 8 ATCC stocks, each representing a different *M. pneumoniae* strain, were prepared and tested in the diplex assay. (Table 1).

CROSS-REACTIVITY. Diplex SDA reactions were seeded with a variety of organisms at a concentration of $\sim 10^6$ organisms or viral particles per reaction. Three replicates of each organism were examined in the presence of the IAC to validate negative results (Table 2).

LIMIT OF DETECTION. To determine the analytical sensitivity of the *M. pneumoniae* assay in both monoplex (no IAC) and diplex (with IAC) systems, SDA was performed on dilutions of the cloned target nucleic acid sequence. Sixteen replicates were tested at each target level. (Figure 3).

INTERNAL AMPLIFICATION CONTROL EFFECTIVENESS. The ability of the BDProbeTec™ ET *M. pneumoniae* assay IAC to identify a potentially inhibitory sample was evaluated by performing diplex SDA reactions in the presence of increasing amounts of non-specific DNA. (Figure 4).

COMPATIBILITY WITH QIAGEN EXTRACTION TECHNOLOGY. Compatibility of the Qiagen extraction technology in conjunction with SDA was demonstrated by spiking a sputum pool with varying concentrations of *M. pneumoniae*. The parental stock of *M. pneumoniae* (ATCC# 15531) was quantitated by electron microscopy and stored frozen. Two hundred microliters of sputum, seeded and unseeded, was processed using the QIAamp® DNA Blood Mini Kit (Qiagen, Inc.) and the blood and body fluid spin protocol, according to the manufacturer's instructions. Sixteen replicates were assayed per spike level in the diplex BDProbeTec™ ET *M. pneumoniae* assay (Figure 5).

DATA ANALYSIS. All experiments were performed using the BDProbeTec™ ET System. Data were analyzed using the Time to Threshold (T3) algorithm developed for this instrument. Negative samples never achieve the threshold value and are assigned a T3 value of 60. Positive samples have a T3 < 60.

RESULTS

Table 1. Specificity

Organism	ATCC Strain
<i>Mycoplasma pneumoniae</i>	15492
<i>Mycoplasma pneumoniae</i>	15531
<i>Mycoplasma pneumoniae</i>	29342
<i>Mycoplasma pneumoniae</i>	15293
<i>Mycoplasma pneumoniae</i>	15377
<i>Mycoplasma pneumoniae</i>	29085
<i>Mycoplasma pneumoniae</i>	39505
<i>Mycoplasma pneumoniae</i>	49894

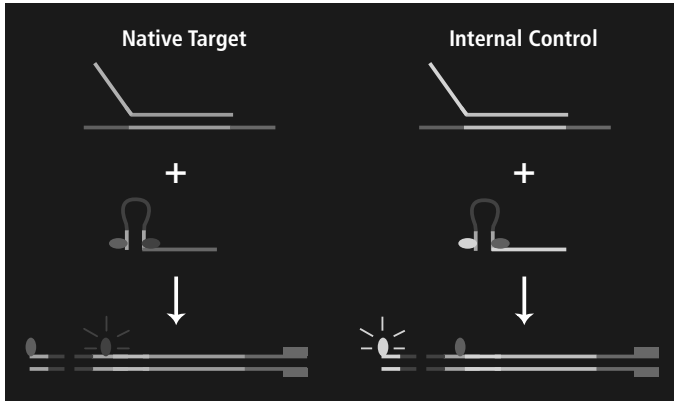
All strains of *M. pneumoniae* tested were detected at 100-fold dilutions of the ATCC stocks ($\sim 10^3$ - 10^4 organisms/reaction).

Table 2. Cross-Reactivity (All organisms tested at $\sim 10^6$ organisms/reaction)

<i>Mycoplasma</i> species	Non- <i>Mycoplasma</i> species	Non- <i>Mycoplasma</i> species	Non- <i>Mycoplasma</i> species
<i>Acholeplasma laidlawii</i> ATCC 23206*	<i>Acinetobacter calcoaceticus</i> ATCC 13339	<i>Neisseria gonorrhoeae</i> ATCC 19424	Adenovirus-5 ABI Type 5
<i>Mycoplasma arginini</i> ATCC 23838	<i>Actinomyces israelii</i> ATCC 10049	<i>Neisseria meningitidis</i> ATCC 13077	<i>Blastomyces dermatitidis</i> ATCC 4292
<i>Mycoplasma buccale</i> ATCC 23636*	<i>Aeromonas hydrophila</i> ATCC 7966	<i>Neisseria mucosa</i> ATCC 19696	<i>Chlamydia pneumoniae</i> ABI AR-39
<i>Mycoplasma faucium</i> ATCC 25293	<i>Bordetella bronchiseptica</i> ATCC 10580	<i>Prevotella oralis</i> ATCC 33269	<i>Chlamydia trachomatis</i> ABI LGV2
<i>Mycoplasma fermentans</i> ATCC 19989	<i>Bordetella parapertussis</i> ATCC 15311	<i>Salmonella choleraesuis</i> serotype enteritidis ATCC 13076	<i>Coccidioides immitis</i> ATCC 7366
<i>Mycoplasma gallinarum</i> ATCC 15319	<i>Bordetella pertussis</i> ATCC 9797	<i>Salmonella choleraesuis</i> serotype typhi ATCC 19430	<i>Cryptococcus neoformans</i> ATCC 36556
<i>Mycoplasma gallisepticum</i> ATCC 19610	<i>Branhamella catarrhalis</i> ATCC 25238	<i>Serratia marcescens</i> ATCC 8100	Cytomegalovirus-ABI AD-169
<i>Mycoplasma genitalium</i> ATCC 33530	<i>Candida albicans</i> ATCC 44808	<i>Staphylococcus aureus</i> , protein A-producing ATCC 12598	Enterovirus (Echovirus) ABI 11
<i>Mycoplasma hominis</i> ATCC 23114	<i>Citrobacter freundii</i> ATCC 8090	<i>Staphylococcus aureus</i> , non-protein A-producing ATCC 25923	Herpesvirus-1 ABI MacIntyre
<i>Mycoplasma hyorhinis</i> ATCC 17981	<i>Corynebacterium diphtheriae</i> ATCC 11913	<i>Staphylococcus epidermidis</i> ATCC E155	<i>Histoplasma capsulatum</i> ATCC 12700
<i>Mycoplasma lipophilum</i> ATCC 27104	<i>Corynebacterium jeikeium</i> ATCC 43734	<i>Stenotrophomonas maltophilia</i> ATCC 13637	Influenza virus A- BDAD PR8
<i>Mycoplasma orale</i> ATCC 23714	<i>Enterococcus faecalis</i> ATCC 29212	<i>Streptococcus</i> group B ATCC 12386	Influenza virus B-BDAD HK/5/72
<i>Mycoplasma penetrans</i> ATCC 55252	<i>Eikenella corrodens</i> ATCC 23834	<i>Streptococcus pneumoniae</i> ATCC 6303	Parainfluenza 1 virus-BDAD Sendai
<i>Mycoplasma primatum</i> ATCC 15497	<i>Enterobacter aerogenes</i> ATCC 13048	<i>Streptococcus pyogenes</i> ATCC 19615	Rhinovirus ABI 80-015
<i>Mycoplasma salivarium</i> ATCC 23064	<i>Enterobacter cloacae</i> ATCC 13047	<i>Streptococcus mutans</i> ATCC 25175	Resp. Syncytial virus, Long strain ABI 74-093
<i>Mycoplasma synoviae</i> ATCC 25204	<i>Enterococcus faecium</i> ATCC 19434	<i>Porphyromonas asaccharolytica</i> ATCC 25260	
<i>Ureaplasma urealyticum</i> ATCC 27618	<i>Escherichia coli</i> ATCC 11775	<i>Peptostreptococcus anaerobius</i> ATCC 27337	
	<i>Fusobacterium nucleatum</i> ATCC 25586	<i>Veillonella parvula</i> ATCC 10790	
	<i>Haemophilus influenzae</i> ATCC 33533		
	<i>Haemophilus parainfluenzae</i> ATCC 7901		
* 1:10 dilution required	<i>Klebsiella pneumoniae</i> subsp ozaenae type 4 ATCC 11296		
	<i>Klebsiella pneumoniae</i> subsp pneumoniae ATCC 13883		
	<i>Lactobacillus acidophilus</i> ATCC 4356		
	<i>Moraxella osloensis</i> ATCC 19976		
	<i>Mycobacterium tuberculosis</i> ATCC 27294		

None of the organisms tested cross-reacted in the BDProbeTec™ ET *M. pneumoniae* Assay

Figure 1. Diplex Universal Detection



Internal Amplification Control

- Verifies negative results and identifies inhibitory samples
- Same priming sequences as native target but with mutated internal region
- Native target and IAC detected using probes labeled with different dyes

Figure 2. BDProbeTec™ ET Liquid SDA Workflow

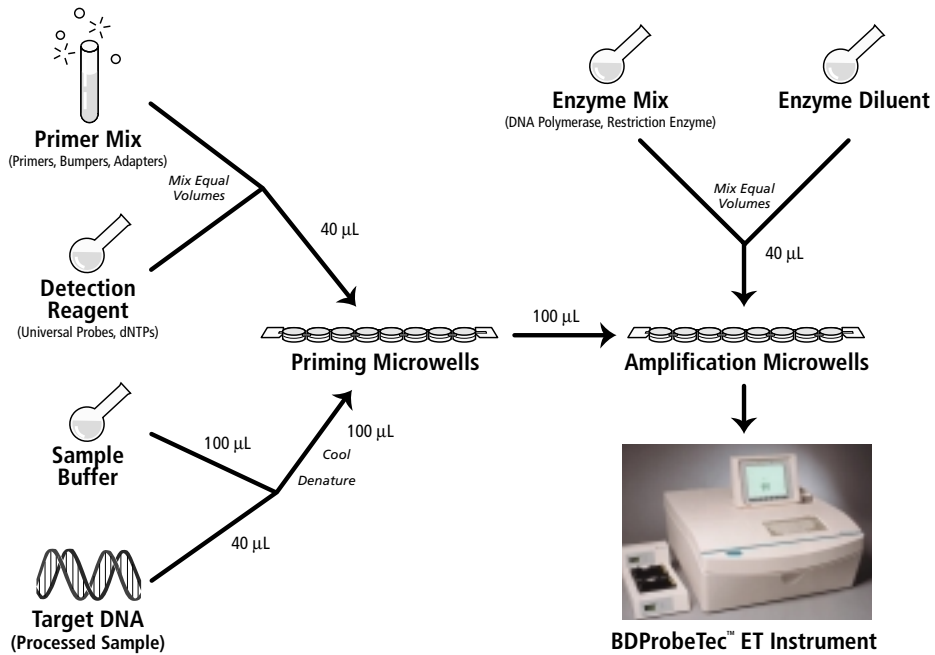
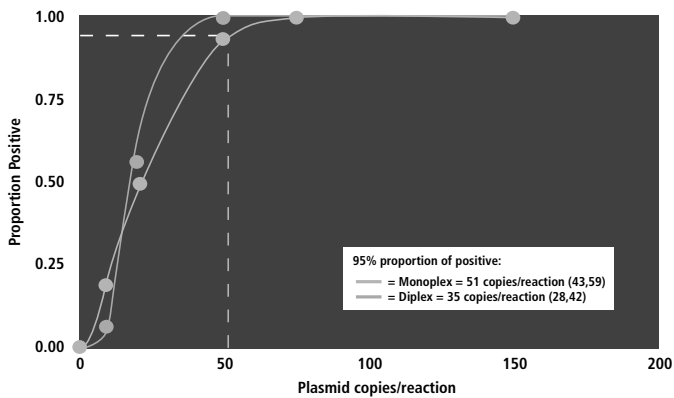
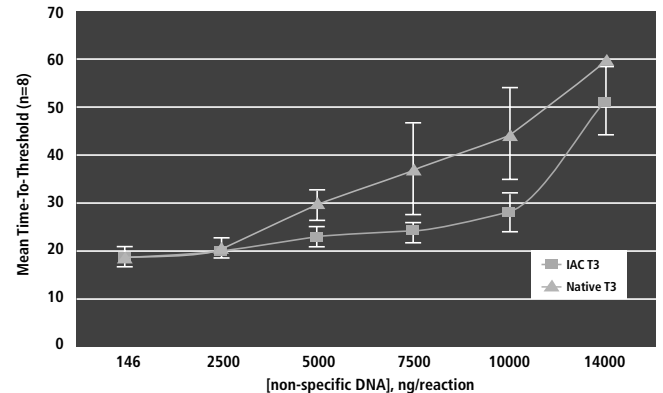


Figure 3. Limit of Detection for the *M. pneumoniae* SDA Assay



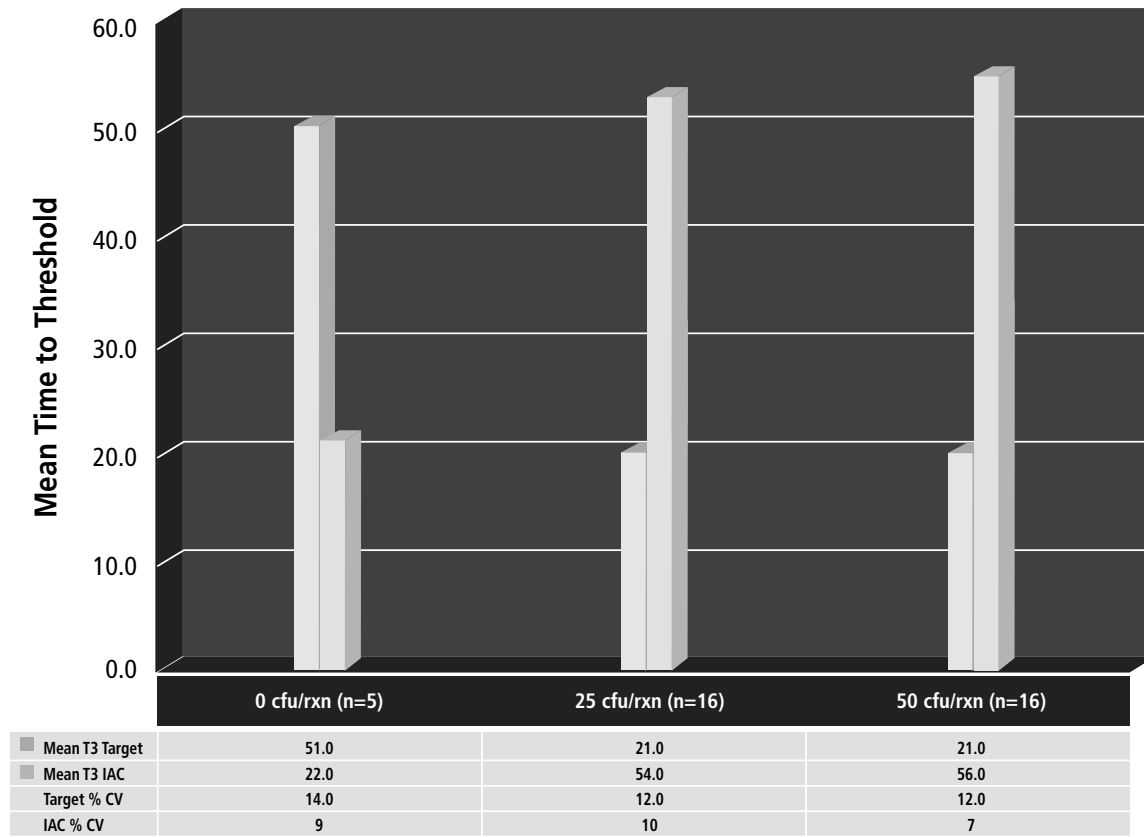
Incorporation of the IAC does not adversely affect the Limit of Detection.

Figure 4. Internal Amplification Control Effectiveness (SDA Response to Non-Specific DNA)



As non-specific DNA level is increased, Time-To-Threshold increases similarly for both native and IAC targets.

Figure 5. Detection of *M. pneumoniae* in Spiked Sputum Using the QIAamp® DNA Mini Kit



The BDProbeTec™ ET *M. pneumoniae* assay is compatible with QIAamp® extraction technology.

CONCLUSIONS

- A sensitive and specific SDA assay has been developed for the detection of *M. pneumoniae* DNA.
- The assay is compatible with the QIAamp® DNA extraction technology.
- This assay incorporates an internal amplification control that validates negative results and identifies specimens that may contain inhibitors of the SDA reaction.
- The BDProbeTec™ ET *M. pneumoniae* assay does not cross-react with other bacteria, viruses or fungi likely to be encountered in respiratory specimens.
- As part of a respiratory panel currently under development, including assays for *Legionella pneumophila*, *Bordetella pertussis* and the *Chlamydiaceae* family, the *M. pneumoniae* assay has the potential to expand the capabilities of the BDProbeTec™ ET System.