

Detection of *Legionella pneumophila* by Strand Displacement Amplification on the BDProbeTec™ ET System

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ABSTRACT

■ *Legionella pneumophila* causes approximately 3-8% of all community-acquired pneumonias. While culture for *Legionella* is regarded as the gold standard, it requires special media and requires 3-5 days to form visible colonies. Serological methods are insensitive and non-specific. A properly designed nucleic acid amplification method would offer speed, specificity and sensitivity. We have developed a method for the detection of *L. pneumophila* using strand displacement amplification and real-time detection targeting the macrophage infectivity potentiator (mip) gene. The assay includes an internal amplification control to validate results, demonstrates compatibility with common DNA extraction kits, has an analytical sensitivity of less than 100 copies/reaction and detects all available ATCC™ serogroups of *L. pneumophila*. The assay does not cross-react with any other members of the genus *Legionella* or other organisms tested. This assay is part of a respiratory panel currently under development for the BDProbeTec™ ET System, including *Mycoplasma pneumoniae* and *Bordetella pertussis* that utilize a universal fluorescence energy transfer detector probe. This assay will provide a rapid and efficient method for detection of *L. pneumophila*.

INTRODUCTION

■ *Legionella pneumophila*, the causative agent of over 90% of all *Legionella* cases, is the etiological agent for the estimated 8,000 to 18,000 cases of pneumonia diagnosed in the U.S. each year¹. With a mortality rate as high as 25-40%, there is a need for a rapid and sensitive assay for the detection of *Legionella* in clinical and environmental specimens and to expedite prescription of antibiotic therapy.

Traditional methods of detection, direct fluorescence antibody (DFA) staining, culture, and/or urine antigen assays are considered the gold standards for diagnosis of *Legionella* infection, however; they are often insensitive, non-specific and cumbersome to perform. Strand Displacement Amplification (SDA) technology offers the potential of combined increased sensitivity and rapid results.

We have developed a BDProbeTec™ ET assay for detection of the macrophage infectivity potentiator (mip) gene of *L. pneumophila*. This assay involves SDA of *L. pneumophila* DNA and real-time fluorescence detection utilizing a universal fluorescence energy transfer (FET) probe. An internal amplification control (IAC) has been developed, that is co-amplified with native target DNA, to identify samples which may contain compounds that may inhibit the SDA reaction (Figure 1). The dual-dye capabilities of the BDProbeTec™ ET System allow detection of both native target and IAC to occur in 60 minutes. Assay workflow follows an easy to use liquid protocol (Figure 2).

The BDProbeTec™ ET *L. pneumophila* assay, in conjunction with other tests currently under development for *Mycoplasma pneumoniae*, *Bordetella pertussis*, and the *Chlamydiaceae* family, has the potential to provide a rapid and specific means for diagnosing much of the 400,000 to 1.6 million cases of pneumonia diagnosed in the U.S each year.

ACKNOWLEDGEMENTS

We would like to thank Gordon Franklin, Mark Hall, Daryl Shank, Christine Keys, Martin Jenkins and Max Kuhn for their contributions to this poster.

¹ http://www.cdc.gov/ncidod/dbmd/diseaseinfo/legionellosis_g.htm

METHODS

DNA TARGET AND INTERNAL AMPLIFICATION CONTROL. A partial clone of the *L. pneumophila* mip gene was used as the target in 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. Dilutions of a 1.0 McFarland standard of 16 different strains of *L. pneumophila*, Diplex SDA was performed at a target level of 250 organisms/reaction (Table 1).

CROSS-REACTIVITY. Diplex SDA reactions were seeded with a variety of organisms at a concentration of ~10⁶ organisms or viral particles/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 *L. pneumophila* assay in both a monoplex and diplex system, 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 *L. pneumophila* assay IAC to detect an inhibited 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 *L. pneumophila* (ATCC 33152). Colony counts for the starting dilution were determined prior to dilution and frozen storage. Two hundred microliters of sputum, seeded and unseeded, was processed using the QIAamp® DNA Blood Mini Kit and the blood and body fluid spin protocol (Qiagen, Inc.) according to the instructions of the manufacturer. Twelve replicates were assayed per spike level in the diplex BDProbeTec™ ET *L. pneumophila* system (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 T3 value of 60. Positive samples have T3 < 60.

Table 1: Strain Specific *L. pneumophila* Specificity

Organism	Subspecies	Serogroup	ATCC #	Mean T3 (n=3)
<i>Legionella pneumophila</i>	<i>pneumophila</i>	1	33152	10.3
<i>Legionella pneumophila</i>	none	1	33153	13.9
<i>Legionella pneumophila</i>	<i>pneumophila</i>	2	33154	12.3
<i>Legionella pneumophila</i>	<i>pneumophila</i>	3	33155	10.2
<i>Legionella pneumophila</i>	<i>fraseri</i>	4	33156	10.8
<i>Legionella pneumophila</i>	<i>fraseri</i>	5	33216	9.9
<i>Legionella pneumophila</i>	<i>pascaliei</i>	5	33735	10.5
<i>Legionella pneumophila</i>	<i>pneumophila</i>	6	33215	12.3
<i>Legionella pneumophila</i>	none	7	33823	11.6
<i>Legionella pneumophila</i>	<i>pneumophila</i>	8	35096	12.7
<i>Legionella pneumophila</i>	<i>pneumophila</i>	9	35289	11.0
<i>Legionella pneumophila</i>	<i>pneumophila</i>	10	43283	12.3
<i>Legionella pneumophila</i>	<i>pneumophila</i>	11	43130	11.1
<i>Legionella pneumophila</i>	<i>pneumophila</i>	12	43290	11.9
<i>Legionella pneumophila</i>	<i>pneumophila</i>	13	43736	16.3
<i>Legionella pneumophila</i>	<i>pneumophila</i>	14	43703	11.8

All serogroups tested were detected at 250 copies/rxn.

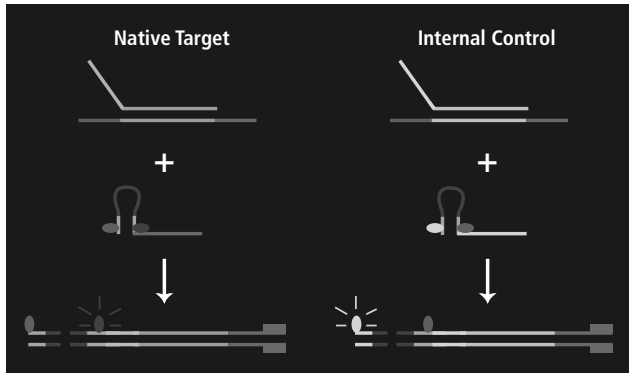
Table 2: Cross-Reactivity. (All organisms tested at ~10⁶ organisms/reaction.)

Legionella species	Non-Legionella species	Non-Legionella species	Non-Legionella species
<i>L. bozemanii</i> ATCC 33217	<i>Acinetobacter calcoaceticus</i> ATCC 13339	<i>Neisseria gonorrhoeae</i> ATCC 19424	Adenovirus-5 ABI Type 5
<i>L. dumoffii</i> ATCC 33279	<i>Actinomyces israelii</i> ATCC 10049	<i>Neisseria meningitidis</i> ATCC 13077	<i>Blastomyces dermatitidis</i> ATCC 4292
<i>L. feeleii</i> ATCC 700514	<i>Aeromonas hydrophila</i> ATCC 7966	<i>Neisseria mucosa</i> ATCC 19696	<i>Chlamydia pneumoniae</i> ABI AR-39
<i>L. longbeachae</i> ATCC 33462	<i>Bordetella bronchiseptica</i> ATCC 10580	<i>Prevotella oralis</i> ATCC 33269	<i>Chlamydia trachomatis</i> ABI LGV2
<i>L. micdadei</i> ATCC 33204	<i>Bordetella parapertussis</i> ATCC 15311	<i>Salmonella choleraesuis</i> serotype <i>enteritidis</i> ATCC 13076	<i>Coccidioides immitis</i> ATCC 7366
<i>L. anisa</i> ATCC 35292	<i>Bordetella pertussis</i> ATCC 9797	<i>Salmonella choleraesuis</i> serotype <i>typhi</i> ATCC 19430	<i>Cryptococcus neoformans</i> ATCC 36556
<i>L. cherrii</i> ATCC 35252	<i>Branhamella catarrhalis</i> ATCC 25285	<i>Serratia marcescens</i> ATCC 8100	Cytomegalovirus-ABI AD-169
<i>L. cincinnatiensis</i> ATCC 43753	<i>Candida albicans</i> ATCC 44808	<i>Staphylococcus aureus</i> , protein A-producing ATCC 12598	Enterovirus (Echovirus) ABI 11
<i>L. erythra</i> ATCC 35303	<i>Citrobacter freundii</i> ATCC 8090	<i>Staphylococcus aureus</i> , non-protein A-producing ATCC 25923	Herpesvirus-1 ABI MacIntyre
<i>L. fairfieldensis</i> ATCC 49588	<i>Corynebacterium diphtheriae</i> ATCC 11913	<i>Staphylococcus epidermidis</i> ATCC E155	<i>Histoplasma capsulatum</i> ATCC 12700
<i>L. gormanii</i> ATCC 33297	<i>Corynebacterium jeikeium</i> ATCC 43734	<i>Stenotrophomonas maltophilia</i> ATCC 13637	Influenza virus A- BDAD PR8
<i>L. hackeliae</i> ATCC 35250	<i>Eikenella corrodens</i> ATCC 23834	<i>Veillonella parvula</i> ATCC 10790	Influenza virus B-BDAD HK/5/72
<i>L. jordanis</i> ATCC 33623	<i>Enterobacter aerogenes</i> ATCC 13048	<i>Streptococcus</i> group B ATCC 12386	<i>Mycoplasma pneumoniae</i> -ABI Haflica
<i>L. maceachernii</i> ATCC 35300	<i>Enterobacter cloacae</i> ATCC 13047	<i>Streptococcus pneumoniae</i> ATCC 6303	Parainfluenza 1 virus-BDAD Sendai
<i>L. oakridgensis</i> ATCC 33761	<i>Enterococcus faecalis</i> ATCC 29212	<i>Streptococcus pyogenes</i> ATCC 19615	Rhinovirus ABI 80-015
<i>L. sainthelensi</i> ATCC 35248	<i>Enterococcus faecium</i> ATCC 19434	<i>Streptococcus mutans</i> ATCC 25175	Resp. Syncytial virus, Long strain ABI 74-093
<i>L. spiritensis</i> ATCC 35249	<i>Escherichia coli</i> ATCC 11775	<i>Porphyromonas asaccharolytica</i> ATCC 25260	
<i>L. worsleiensis</i> ATCC 49508	<i>Fusobacterium nucleatum</i> ATCC 25586	<i>Peptostreptococcus anaerobius</i> ATCC 27337	
	<i>Haemophilus influenzae</i> ATCC 33533		
	<i>Haemophilus parainfluenzae</i> ATCC 7901		
	<i>Klebsiella pneumoniae</i> subsp. <i>ozaenae</i> type 4 ATCC 11296		
	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> 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 *L. pneumophila* Assay

RESULTS

• 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

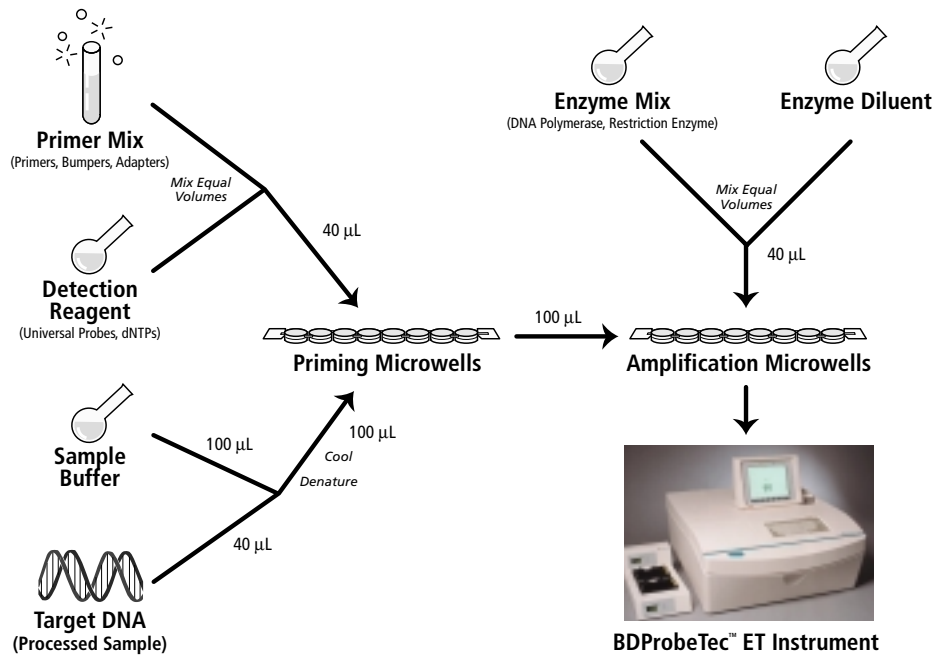
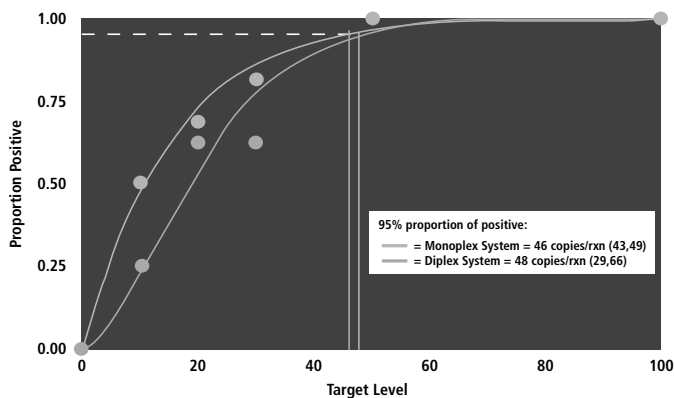
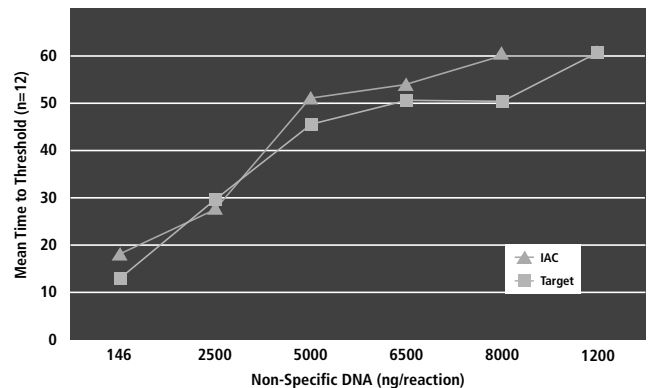


Figure 3. Limit of Detection in Monoplex and Diplex Systems



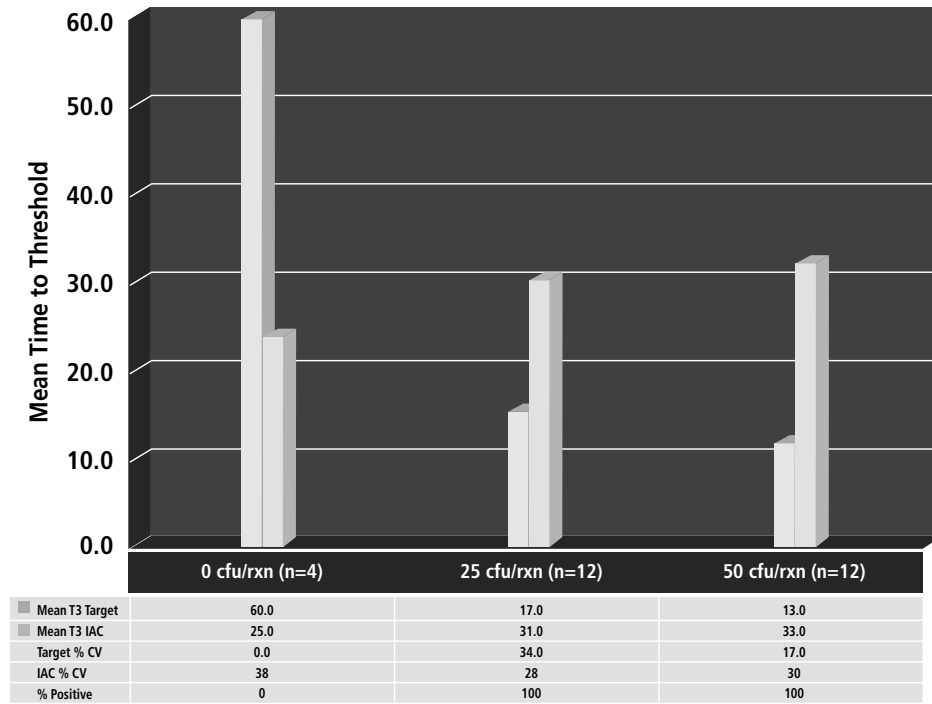
The analytical sensitivity of the BDProbeTec™ ET *L. pneumophila* Assay is not affected upon the addition of the internal amplification control.

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



Legionella target and IAC are uniformly affected by increasing amounts of non-specific DNA.

Figure 5. Detection of *L. pneumophila* from Spiked Sputum Using QIAamp® DNA Mini Kit



The BDProbeTec™ ET *L. pneumophila* Assay and IAC are compatible with QIAamp® extraction technology.

CONCLUSIONS

- A sensitive and specific SDA assay for the detection of *L. pneumophila* DNA has been developed for use with the BDProbeTec™ ET System. The assay may be coupled with the QIAamp® DNA extraction technology.
- An internal amplification control has been developed to verify negative results and identify inhibitory samples.
- The universal detection system offers cost and time saving advantages over other FET-based methods.
- The *L. pneumophila* assay provides a useful adjunct to other BDProbeTec™ assays that are under development for *M. pneumoniae*, *Bordetella pertussis* and the Chlamydiaceae family.