

Detection of *Chlamydia pneumoniae* and Other Organisms of the *Chlamydiaceae* Family by Strand Displacement Amplification on the BD ProbeTec™ ET System *

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ABSTRACT

Three species in the *Chlamydiaceae* family: *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*), *Chlamydia trachomatis* and *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) cause disease in humans including respiratory infection (pneumonia), trachoma, and sexually transmitted infections of the reproductive organs. Pneumonia can be caused by *C. pneumoniae* and *C. psittaci* (psittacosis) in adults, and by *C. trachomatis* in newborn infants. *C. pneumoniae* has also been associated with chronic diseases such as atherosclerosis and asthma. Currently, commercially available molecular methods are only directed towards the detection of *C. trachomatis* in urine or on genital swabs and no such assays exist for the detection of other pathogens or potential pathogens within the *Chlamydiaceae* family. We have developed a novel Strand Displacement Amplification-based method for the detection of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* that involves real-time detection with a universal fluorescent energy transfer probe on the BD ProbeTec™ ET System. The target for the assay, which includes an internal amplification control to validate negative results, is the ribonuclease P RNA gene. Here we report that the system has an analytical sensitivity of approximately 100 genomic equivalents for each of the three species: *C. pneumoniae*, *C. trachomatis* and *C. psittaci*. Preliminary data demonstrate that the assay is specific for members of the *Chlamydiaceae* family and does not exhibit cross-reaction with any of 105 bacteria, viruses and fungi commonly found in the respiratory or genital tracts. The system is compatible with common DNA extraction kits and is able to detect <1000 elementary bodies of *C. pneumoniae* per milliliter of spiked sputum. The *Chlamydia* genus assay is a useful addition to the atypical pneumonia panel being developed for the BD ProbeTec™ ET System that includes tests for *Legionella pneumophila* and *Mycoplasma pneumoniae*.

INTRODUCTION

Three species in the family *Chlamydiaceae*: *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*), *Chlamydia trachomatis* and *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) cause human disease including trachoma, respiratory infection (pneumonia), and sexually transmitted infections of the reproductive organs such as urethritis, cervicitis, pelvic inflammatory disease, and epididymitis. Pneumonia can be caused by *C. pneumoniae* and *C. psittaci* (psittacosis) in adults, and by *C. trachomatis* in newborn infants. There is also evidence linking *C. pneumoniae* with atherosclerotic vascular disease and several species within the *Chlamydiaceae* are known to cause disease in animals. There is, therefore, a significant clinical need to detect all the pathogens or potential pathogens within the *Chlamydiaceae* family in a wide range of sample types.

Traditional methods for laboratory diagnosis of *Chlamydiaceae* infection include culture and serology. However, these techniques are seldom attempted because they are complicated, slow and generally available only in reference laboratories. Furthermore, serological results are retrospective in nature and often useful only for epidemiologic purposes. Nucleic acid amplification is, therefore, an attractive technology for the rapid, sensitive and specific detection of pathogens within the *Chlamydiaceae* family. Currently, commercially available molecular methods are only directed towards the detection of *C. trachomatis* in urine or on urogenital swabs and no such assays exist for the detection of other pathogens within the *Chlamydiaceae*. To address this, we have developed a novel Strand Displacement Amplification (SDA)-based method for the detection of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* that involves real-time detection with a universal fluorescent energy transfer probe on the BD ProbeTec™ ET System (Figure 1a). The assay amplifies a conserved region within the ribonuclease P RNA (*rnpB*) gene and incorporates an internal amplification control (IAC) that is co-amplified with native target DNA to identify processed samples that contain inhibitors of the SDA reaction (Figure 1b). The dual-dye capabilities of the BD ProbeTec™ ET System allow for the detection of both native target and IAC to occur within 60 minutes using an easy to follow SDA protocol (Figure 2).

The *Chlamydia* genus assay comprises part of an atypical pneumonia panel currently under development for the BD ProbeTec™ ET System that also includes assays for *Legionella pneumophila* and *Mycoplasma pneumoniae*. Here we demonstrate the sensitivity and specificity of the *Chlamydia* genus assay system and its application to the detection of *C. trachomatis* and *C. pneumoniae* in spiked sputum specimens.

MATERIALS AND METHODS

DNA TARGETS AND INTERNAL AMPLIFICATION CONTROL. Fragments of the ribonuclease P RNA gene from *C. pneumoniae*, *C. trachomatis* and *C. psittaci* were cloned into a pUC19 vector and used as targets in assay development. The internal amplification control (IAC) was generated by site-directed mutagenesis of the *C. pneumoniae* target clone. Analytical quantification of plasmid stocks was performed using the Picogreen® assay (Molecular Probe, Inc.). Duplex SDA reactions contained 100 copies of the IAC plasmid.

DATA ANALYSIS. All experiments were performed using the BD ProbeTec™ ET System. Data were analyzed using the Time-to-Threshold (T3) algorithm developed for this instrument (Figure 3). Negative samples never achieve the threshold value and are assigned a T3 value of 60. Positive samples have a T3 < 60.

Table 1. *Chlamydia* Genus Assay Specificity

Species	Serovar/Strain	Strain #	Target Level
<i>C. pneumoniae</i>	AR-39	ATCC ^a 53592	500 EB/reaction
<i>C. pneumoniae</i>	TW-183	ATCC VR-2282	500 EB/reaction
<i>C. pneumoniae</i>	CM-1	ATCC VR-1360	2.5 TCID [*] /reaction
<i>C. pneumoniae</i>	2043	ATCC VR-1355	2.5 TCID/reaction
<i>C. pneumoniae</i>	CDC/CWL-029	ATCC VR-1310	2.5 TCID/reaction
<i>C. pneumoniae</i>	2023	ATCC VR-1356	2.5 TCID/reaction
<i>C. trachomatis</i>	A	ATCC VR-571	500 EB/reaction
<i>C. trachomatis</i>	B	ATCC VR-573	500 EB/reaction
<i>C. trachomatis</i>	Ba	ATCC VR-347	500 EB/reaction
<i>C. trachomatis</i>	C	ATCC VR-572	500 EB/reaction
<i>C. trachomatis</i>	D	ATCC VR-885	500 EB/reaction
<i>C. trachomatis</i>	E	ATCC VR-348	500 EB/reaction
<i>C. trachomatis</i>	F	ATCC VR-346	500 EB/reaction
<i>C. trachomatis</i>	G	ATCC VR-878	500 EB/reaction
<i>C. trachomatis</i>	H	ATCC VR-879	500 EB/reaction
<i>C. trachomatis</i>	I	ATCC VR-880	500 EB/reaction
<i>C. trachomatis</i>	J	ATCC VR-886	500 EB/reaction
<i>C. trachomatis</i>	K	ATCC VR-887	500 EB/reaction
<i>C. trachomatis</i>	L1	ATCC VR-901B	500 EB/reaction
<i>C. trachomatis</i>	L2	ATCC VR-902B	500 EB/reaction
<i>C. trachomatis</i>	L3	ATCC VR-903B	500 EB/reaction
<i>C. psittaci</i>	Cal-10	BD ^b 86-31306	500 EB/reaction
<i>C. psittaci</i>	Borg	ABI ^c 4094-032895	500 copies/reaction

SPECIFICITY. Primer specificity was evaluated using six strains of *C. pneumoniae*, 15 strains of *C. trachomatis*, and two strains of *C. psittaci* (Table 1).

CROSS-REACTIVITY. Cell lysates and genomic DNA from 105 organisms were tested in monoplex SDA reactions (Table 2). Parallel assays were performed with the addition of 250 copies of the *C. pneumoniae* *rnpB* gene target clone to ensure that negative results were not due to suppression of amplification.

LIMIT OF DETECTION. To determine the analytical sensitivity of the *Chlamydia* genus assay, duplex SDA reactions were performed on dilutions of the cloned target nucleic acid sequences for *C. pneumoniae*, *C. trachomatis* and *C. psittaci*. Sixteen replicates were tested at each target level (Figure 4).

SPUTUM SAMPLE PROCESSING. Compatibility of Qiagen DNA extraction technology in conjunction with SDA was demonstrated by spiking a sputum pool with varying concentrations of *C. pneumoniae* and *C. trachomatis*. The parental stocks of *C. pneumoniae* (AR39 ATCC 53592) and *C. trachomatis* (L2, ATCC VR-902B) were quantitated by counting DFA-stained elementary bodies using a fluorescent microscope. Seven hundred and fifty microliters of NALC-treated sputum, was processed using the QIAamp® DNA Blood Mini Kit (Qiagen, Inc.). Organisms were seeded into the sputum prior to NALC treatment. Five replicate SDA reactions were performed on each sputum sample using the duplex BD ProbeTec™ ET *Chlamydia* genus system (Figure 5). Sample processing methodology was validated by analysis of 109 sputum samples spiked with *C. pneumoniae* or *C. trachomatis* (Figure 6a, b, c).

All strains of *C. pneumoniae*, *C. trachomatis* and *C. psittaci* were detected at the above target levels.

^a American Type Culture Collection

^b Becton Dickinson

^c Advanced Biotechnologies, Inc.

* Tissue culture infective dose

Table 2. *Chlamydia* Genus Assay Cross-Reactivity Panel of 105 Bacteria, Viruses and Fungi (All tested at ~10⁶ organisms/reaction, unless otherwise noted)

Organism Strain #	Organism Strain #	Organism Strain #	Organism Strain #
<i>Acinetobacter calcoaceticus</i> ATCC ^a 13339	<i>Enterobacter cloacae</i> ATCC 13047	<i>Legionella pneumophila</i> ATCC 33152	<i>Propionibacterium acnes</i> ATCC 6919
<i>Acinetobacter lwoffii</i> ATCC 19001	<i>Enterococcus faecalis</i> ATCC 29212	<i>Listeria monocytogenes</i> ATCC 7644	<i>Proteus mirabilis</i> ATCC 29906
<i>Actinomyces israelii</i> ATCC10049	<i>Enterococcus faecium</i> ATCC 19434	<i>Mobiluncus mulerieris</i> ATCC 35243	<i>Providencia stuartii</i> ATCC 35031
Adenovirus-5 ABI ^a 74-070*	Enterovirus (Echovirus-11) ABI 74-084*	<i>Moraxella lucanata</i> ATCC 17967	<i>Pseudomonas aeruginosa</i> ATCC 27853
<i>Aeromonas hydrophila</i> ATCC 7966	Epstein-Barr Virus Sigma 104H0854	<i>Moraxella osloensis</i> ATCC 19976	Resp. Syncytial virus, Long strain ABI 74-093
<i>Alcaligenes faecalis</i> ATCC 8750	<i>Escherichia coli</i> ATCC 11775	<i>Morganella morganii</i> ATCC 25830	Rhinovirus ABI 80-015*
<i>Bacillus subtilis</i> ATCC 12100	<i>Flavobacterium meningosepticum</i> ATCC 13253	<i>Mycobacterium avium</i> ATCC 25291	<i>Salmonella choleraesuis</i> serotype enteritidis ATCC 13076
<i>Bacteroides fragilis</i> ATCC 25285	<i>Fusobacterium nucleatum</i> ATCC 25586	<i>Mycobacterium goodii</i> ATCC 14470	<i>Salmonella choleraesuis</i> serotype typhi ATCC 19430
<i>Blastomyces dermatitidis</i> ATCC 4292	<i>Gardnerella vaginalis</i> ATCC 14018	<i>Mycobacterium intracellulare</i> ATCC 13950	<i>Salmonella minnesota</i> ATCC 9700
<i>Bordetella bronchiseptica</i> ATCC 10580	<i>Gemella haemolysans</i> ATCC 10379	<i>Mycobacterium smegmatis</i> ATCC 19420	<i>Salmonella typhimurium</i> ATCC 13311
<i>Bordetella parapertussis</i> ATCC 15311	Group B <i>Streptococcus</i> ATCC 12386	<i>Mycobacterium tuberculosis</i> ATCC 27294	<i>Serratia marcescens</i> ATCC 8100
<i>Bordetella pertussis</i> ATCC 9797	<i>Haemophilus influenzae</i> ATCC 33533	<i>Mycoplasma genitalium</i> ATCC 33530*	<i>Staphylococcus aureus</i> , protein A-producing ATCC 12598
<i>Branhamella catarrhalis</i> ATCC 25285	<i>Haemophilus parainfluenzae</i> ATCC 7901	<i>Mycoplasma hominis</i> ATCC 23114*	<i>Staphylococcus aureus</i> , non-protein A-producing ATCC 25923
<i>Candida albicans</i> ATCC 44808	Herpes Simplex Virus, type II ABI 4079-022895	<i>Mycoplasma pneumoniae</i> ATCC 63-030*	<i>Staphylococcus epidermidis</i> ATCC E155
<i>Candida glabrata</i> ATCC 90030	Herpes Simplex Virus, type I ABI 68-097*	<i>Neisseria cinerea</i> ATCC 14685	<i>Stenotrophomonas maltophilia</i> ATCC 13637
<i>Candida tropicalis</i> ATCC 750	<i>Histoplasma capsulatum</i> ATCC 12700	<i>Neisseria gonorrhoeae</i> ATCC 19424	<i>Streptococcus mitis</i> ATCC 6249
<i>Citrobacter freundii</i> ATCC 8090	HIV-1 ABI 4314-042198	<i>Neisseria meningitidis</i> ATCC 13077	<i>Streptococcus mutans</i> ATCC 25175
<i>Clostridium perfringens</i> ATCC 13124	HPV type 16 ATCC 45113D	<i>Neisseria mucosa</i> ATCC 19696	<i>Streptococcus pneumoniae</i> ATCC 6303
<i>Coccidioides immitis</i> ATCC 7366	HPV type 18 ATCC 45152D	<i>Neisseria polysaccharea</i> ATCC 43768	<i>Streptococcus pyogenes</i> ATCC 19615
<i>Corynebacterium diphtheriae</i> ATCC 11913	Influenza virus A (PR8) BDc 940422*	Parainfluenza I virus (Sendai) BD 951010*	<i>Streptomyces griseus</i> ATCC 10137
<i>Corynebacterium jeikeium</i> ATCC 43734	Influenza virus B (HK/5/72) BD 4356*	<i>Peptostreptococcus anaerobius</i> ATCC 27337	<i>Trichomonas vaginalis</i> ATCC 30001
<i>Corynebacterium renale</i> ATCC 19412	<i>Kingella kingae</i> ATCC 23330	<i>Peptostreptococcus asaccharolyticus</i> ATCC 29743	<i>Ureaplasma urealyticum</i> ATCC 27618
<i>Cryptococcus neoformans</i> ATCC 36556	<i>Klebsiella pneumoniae</i> ssp. ozaenae, type 4 ATCC 11296	<i>Peptostreptococcus productus</i> ATCC 27340	<i>Vibrio parahaemolyticus</i> ATCC 17802
Cytomegalovirus (AD-169) ABI 68-125*	<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i> ATCC 13883	<i>Plesiomonas shigelloides</i> ATCC 14029	<i>Yersinia enterocolitica</i> ATCC 27729
<i>Edwardsiella tarda</i> ATCC 15469	<i>Lactobacillus acidophilus</i> ATCC 4356	<i>Porphyromonas asaccharolyticus</i> ATCC 25260	
<i>Eikenella corrodens</i> ATCC 23834	<i>Lactobacillus brevis</i> ATCC 14869	<i>Prevotella melaninogenica</i> ATCC 25845	
<i>Enterobacter aerogenes</i> ATCC 13048	<i>Legionella micdadei</i> ATCC 33204	<i>Prevotella oralis</i> ATCC 33269	

None of the organisms tested cross-reacted in the BD ProbeTec™ ET *Chlamydia* Genus Assay

^a American Type Culture Collection

^b Advanced Biotechnologies, Inc.

^c Becton Dickinson

* < 10⁶ organisms/reaction

RESULTS

Figure 1a. Detection of SDA Using Universal Probes

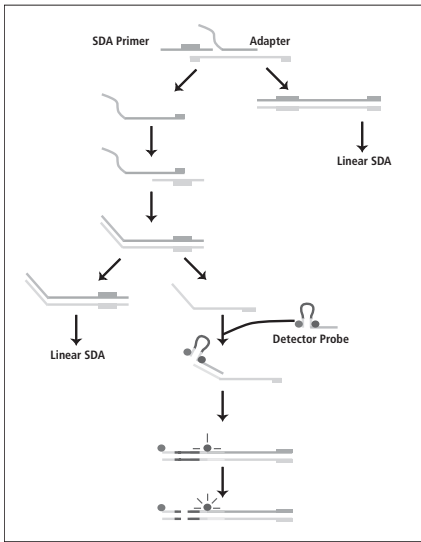


Figure 1b. Diplex Universal Detection

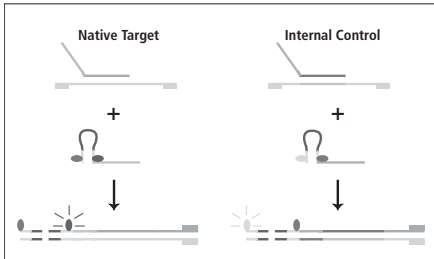
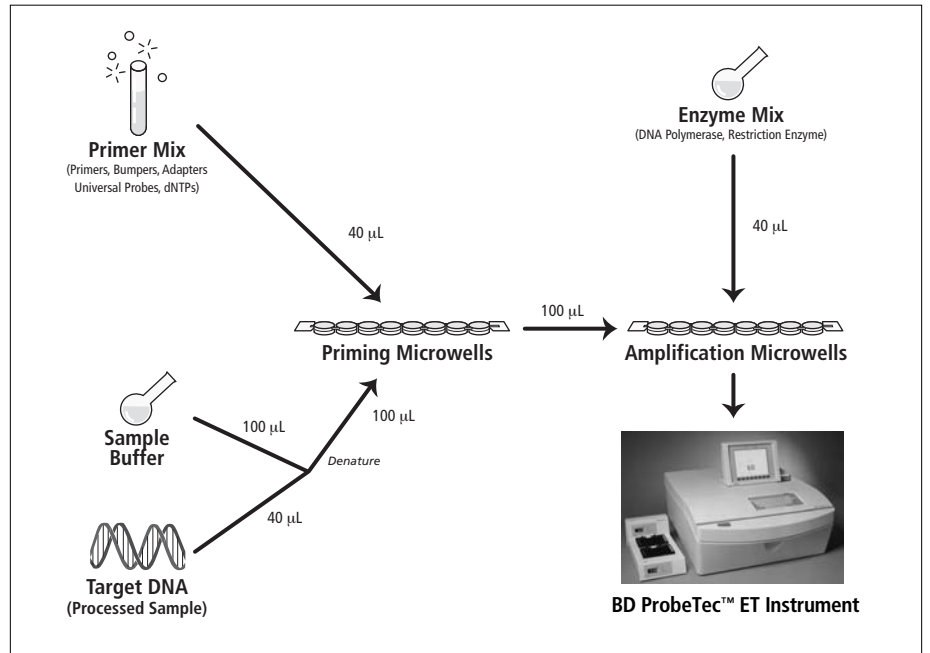


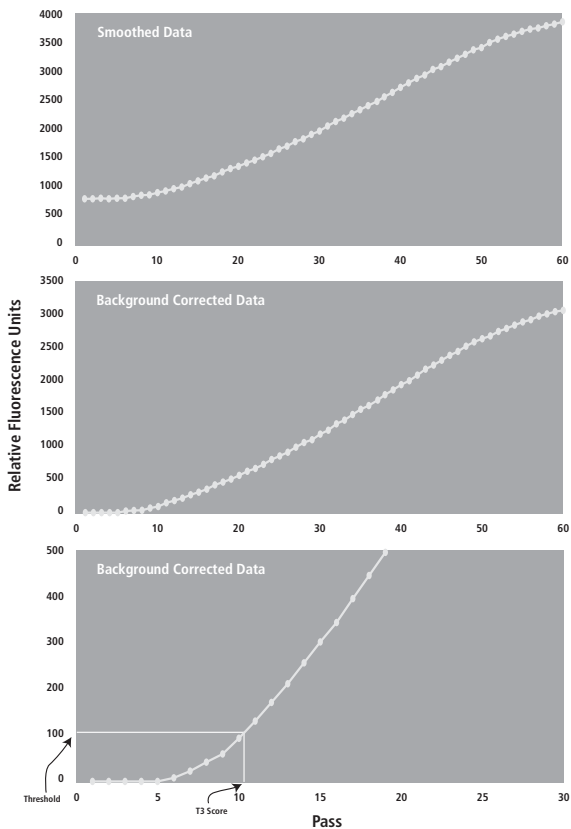
Figure 2. BD ProbeTec™ ET Liquid SDA Workflow



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 3. BD ProbeTec™ ET Data Analysis: The T3 Algorithm



Data is collected over 60 passes and smoothed to remove instrument noise. Signals obtained from the first few passes are averaged and the mean is subtracted from the readings obtained throughout the run. The background corrected data is analyzed in the T3 calculation

T3 = Time-To-Threshold

T3 is the time at which the background corrected signal crosses a **pre-determined** threshold

The same threshold is used for every sample.

Lower T3 scores correlate with more efficient amplification. Negative samples never achieve the threshold value and are assigned T3 values of 60. Positive samples have T3 < 60.

Figure 4. Limit of Detection for Diplex SDA of Cloned Target DNA from *C. pneumoniae*, *C. trachomatis* and *C. psittaci*

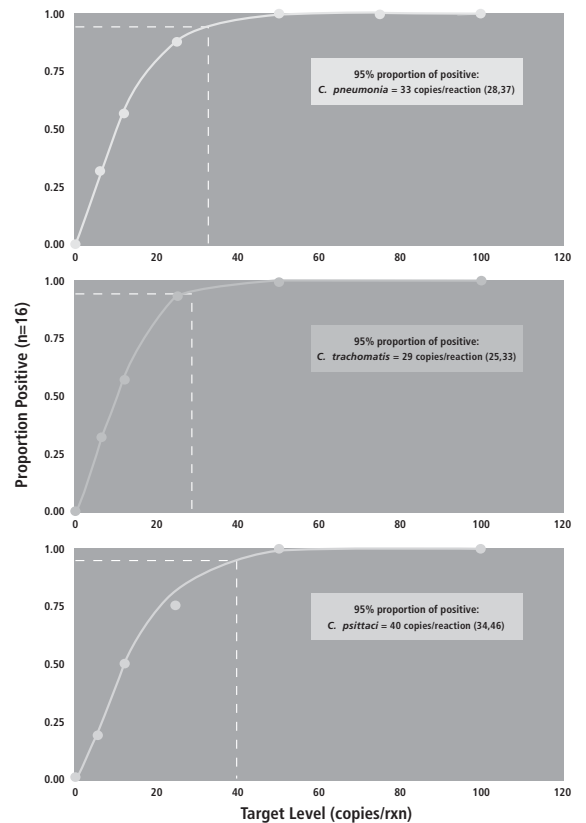
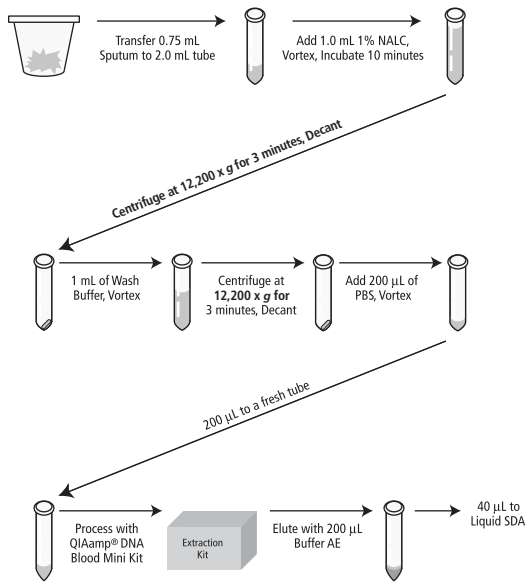


Figure 5. Sample Processing Workflow



CONCLUSIONS

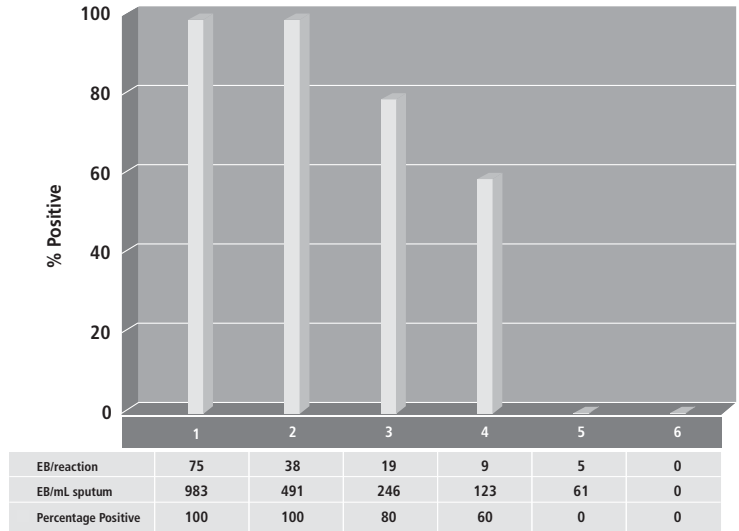
- We have developed a sensitive and specific assay for the detection of *C. pneumoniae* and other organisms of the *Chlamydiaceae* family using strand displacement amplification on the BD ProbeTec™ ET System.
- Analytical sensitivity (genomic equivalents per reaction):
 - C. pneumoniae* 33 (28, 37)
 - C. trachomatis* 29 (25, 33)
 - C. psittaci* 40 (34, 46)
- An internal amplification control is used to validate negative results and identify processed specimens that may contain inhibitors of the SDA reaction.
- The assay can detect < 1000 elementary bodies of *C. trachomatis* or *C. pneumoniae* per milliliter of spiked sputum using a commercial extraction method.
- The *Chlamydia* genus assay is a useful addition to the atypical pneumonia panel being developed for the BD ProbeTec™ ET System that includes tests for *Legionella pneumophila* and *Mycoplasma pneumoniae*.
- Conversion of the *Chlamydia* genus assay to a dried microwell format as used in other BD ProbeTec™ ET Systems is currently in progress.

ACKNOWLEDGEMENTS

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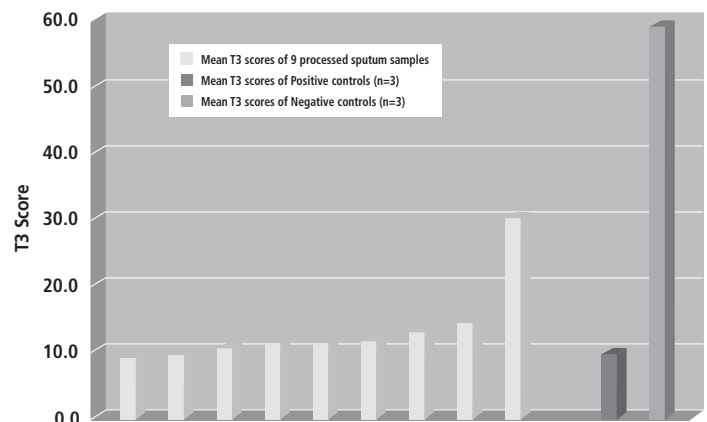
Figure 6. Sample Processing Validation

6a. *C. pneumoniae* EB Titration



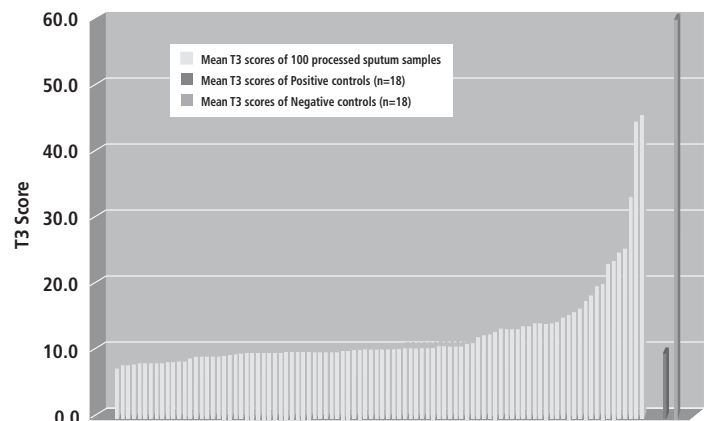
10 replicate SDA reactions were run at each spike level. All reactions containing ≥ 38 EB were positive.

6b. Detection of *C. pneumoniae* in spiked sputum



Each yellow bar represents a mean T3 score of 5 SDA assay replicates obtained from 0.75 mL of sputum spiked with 1875 EBs of *C. pneumoniae* (2500 EB/mL). All 9 specimens yielded positive results with T3 scores < 60. Negative controls all yielded T3 scores of 60.

6c. Detection of *C. trachomatis* in spiked sputum



Each bar represents a mean T3 score of 5 SDA assay replicates obtained from 0.75 mL of sputum spiked with 688 EBs of *C. trachomatis* (917 EB/mL). All 100 specimens yielded positive results with T3 scores < 60. Negative controls all yielded T3 scores of 60.