

# Detection of Severe Acute Respiratory Syndrome-Associated Coronavirus (SARS-CoV) by Reverse Transcriptase-Strand Displacement Amplification on the BD ProbeTec™ ET System

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## ABSTRACT

Due to the abrupt emergence of Severe Acute Respiratory Syndrome (SARS) and its pandemic spread, there is a clinical need for a rapid and sensitive tool to aid in the diagnosis of SARS-associated coronavirus (SARS-CoV) infection. To that end, we have developed a novel Strand Displacement Amplification-based method for the detection of SARS-CoV on the BD ProbeTec™ ET System. The assay targets a region within the SARS-CoV nucleocapsid gene and employs real-time detection using a universal fluorescent energy transfer probe. An RNA-based Internal Amplification Control (IAC) is included in each reaction to monitor for inhibition of the assay and verify negative results. Here we report data generated with a semi-optimized assay format that utilizes a combination of dried primers, detector probes and SDA enzymes with liquid reverse transcriptase enzyme and assay controls. The analytical sensitivity of the system was determined using enumerated stocks of SARS-CoV and an encapsidated clone, Ambion® Armored RNA® of the target sequence:

	95% Limit of Detection (copies/reaction)	95% Confidence Intervals (copies/reaction)
SARS-CoV Vietnam Isolate 200300592	81	68, 94
Ambion® Armored RNA®	116	105, 128

The assay does not exhibit cross-reactivity with any of the 3 strains of non-SARS coronavirus tested, nor with any of 51 bacteria and fungi commonly found in the respiratory tract.

We also describe a modified QIAGEN® specimen processing procedure for the recovery of SARS-CoV RNA from stools, viral transport medium, lower respiratory specimens and nasopharyngeal aspirates. Further development of the assay is in progress to convert the system to a completely dried format. Nevertheless, in its current form, the SARS-CoV assay may have potential utility as an aid in the diagnosis of patients presenting with SARS or SARS-like symptoms.

## INTRODUCTION

Between November 2002 and July 2003 a total of 8,427 probable cases of a new infectious disease were reported from 29 countries. Dubbed Severe Acute Respiratory Syndrome, the infection is characterized by non-specific influenza-like symptoms including fever, myalgia, dry non-productive cough, lymphopenia and infiltrate on chest radiography, and has a mortality rate among the elderly of up to 50%. The causative agent of SARS was determined to be a novel coronavirus (SARS-CoV) that is transmitted by droplet infection. Although at present there is no effective antiviral therapy for SARS, the disease was successfully contained thanks to the implementation of strict quarantine measures, enforcement of infection control procedures and the use of a plethora of hastily concocted homebrew diagnostic procedures. Nevertheless, during the brief pandemic, the economic cost associated with the outbreak was immense and reached \$10 billion in China and Canada alone. News of the first suspected case of SARS to occur within the general population this past winter came from China in late December. Definitive diagnosis of the patient was severely delayed due to conflicting data obtained with different diagnostic methods, thereby highlighting the need for properly validated in vitro diagnostic assays for SARS. The conclusions of an international SARS Laboratory Workshop held in October of 2003 include recommendations for standardized laboratory test procedures to assist in diagnosis of the disease, as well as a process of strict quality assurance for laboratory tests and protocols.<sup>1</sup> Here we present preliminary data on a semi-optimized assay for the detection of SARS-CoV in a variety of clinical specimens using the BD ProbeTec™ ET system.

<sup>1</sup>WHO. 2003. Summary of the discussion and recommendations of the SARS laboratory workshop, 22 October.

## METHODS

**n RNA TARGET.** A fragment within the SARS-CoV nucleocapsid gene containing the SDA target region was cloned into the *Escherichia coli* plasmid vector, pUC19. *In vitro* transcripts were prepared from linearized plasmid DNA utilizing the MEGAscript™ High Yield SP6 Transcription Kit (Ambion, Inc.). Analytical quantification of the RNA transcript stocks was performed using the RiboGreen® RNA Quantitation Reagent And Kit (Molecular Probes, Inc.). Transcripts were diluted in carrier RNA for use in assay development.

**n INTERNAL AMPLIFICATION CONTROL.** *In vitro* transcripts for use as an internal amplification control (IAC) were prepared from a mutated clone of the SARS-CoV target sequence. IAC was included in each Reverse Transcriptase-Strand Displacement Amplification (RT-SDA) reaction to verify the efficiency of both reverse transcription and amplification, and to monitor for the presence of potential inhibitors including contaminating RNases.

**n ANALYTICAL SENSITIVITY.** The limit of detection of the SARS-CoV assay was determined by testing different levels of SARS-CoV viral particles, Armored RNA® (Ambion, Inc.), and RNA transcripts (Table 1). For experiments with viral particles and Armored RNA, lysis and purification of nucleic acid was accomplished using a QIAamp Viral RNA Mini Kit (QIAGEN, Inc.), whereas, for *in vitro* transcripts, RNA was added directly to the priming microwells without prior processing. In all cases, 16 replicates were tested at each of six target levels.

**Viral Particles.** SARS-CoV Vietnam Isolate 200300592 was received from the CDC and propagated. The stock was enumerated by electron microscopy (Electron Microscopy Bioservices, LLC).

**Armored RNA.** Particles were quantified by UV spectrophotometer according to the manufacturer's recommended protocol.

**RNA Transcripts.** (Description above: RNA TARGET)

**n SPECIFICITY/ CROSS-REACTIVITY.** Assay specificity was evaluated using 3 strains of non-SARS-related coronavirus (Table 2) and 51 clinically relevant bacteria and fungi (Table 3). Organism cell lysates were boiled, mixed with sample diluent, and added directly to the priming microwells. For organisms with a DNA based genome, the RT step was omitted. Potential cross-reactant bacteria and fungi were tested at levels between  $1.0 \times 10^7$  and  $3.0 \times 10^9$  organisms/mL.\*

**n PROCESSING OF CLINICAL SPECIMENS.** A customized specimen processing procedure based on the QIAamp® MinElute™ Virus Spin Kit (QIAGEN, Inc.) was developed for use with stools, lower respiratory samples, nasopharyngeal (NP) aspirates, as well as throat swabs expressed into viral transport medium (M4, M5 and Copan). Eight clinical samples from each of the following sample types: stool, sputum, NP aspirate, and throat swabs in M4 medium, were seeded with the equivalent of 333 copies/reaction of Armored RNA. Each sample was processed with the QIAamp MinElute Virus Spin Kit and tested with the BD ProbeTec SARS-CoV assay (Figure 3). The assay results are shown in Figure 1.

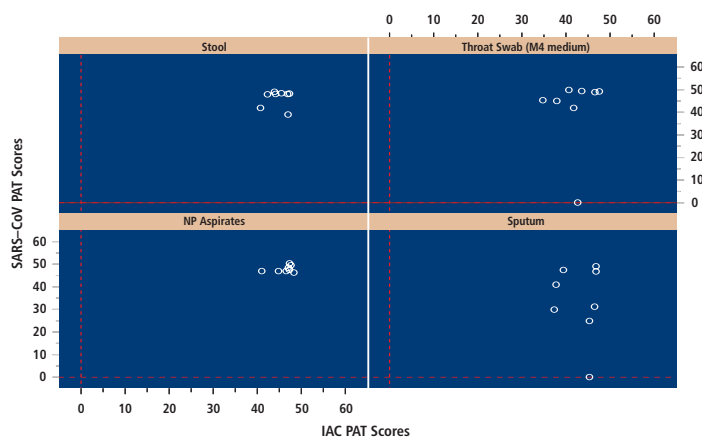
**Lower Respiratory Specimens/Throat swabs expressed into viral transport medium/NP Aspirates.** Add 0.200 mL of sample directly to lysis buffer.

**Stool.** Add 0.500 mL of sample to 2.5 mL of 0.89% Sodium Chloride. Vortex. Centrifuge for 20 minutes at 4000 x g. Filter the supernatant through a 0.22 µm filter. Add 0.200 mL of filtered supernatant to lysis buffer.

**n DATA ANALYSIS.** All experiments were performed on the BD ProbeTec ET System. Data were analyzed using the novel Passes After Threshold (PAT) algorithm (Figure 2) developed for this instrument. The presence or absence of SARS-CoV RNA was determined by calculating the PAT scores for the specimen based on predetermined threshold values. The BD ProbeTec instrument automatically reported results as positive, negative or indeterminate.

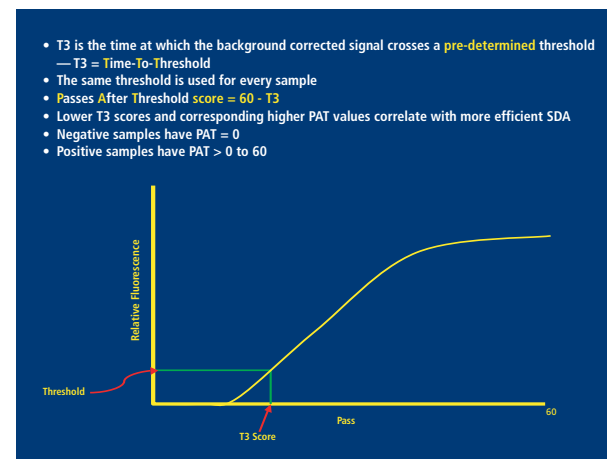
## RESULTS

**Figure 1:** Specimens Processed with the QIAamp MinElute Virus Spin Kit



Dashed lines represent cutoffs

**Figure 2:** BD ProbeTec ET System PAT Algorithm



RESULTS (CONTINUED)

Figure 3: BD ProbeTec ET System SARS-CoV RT-SDA Workflow

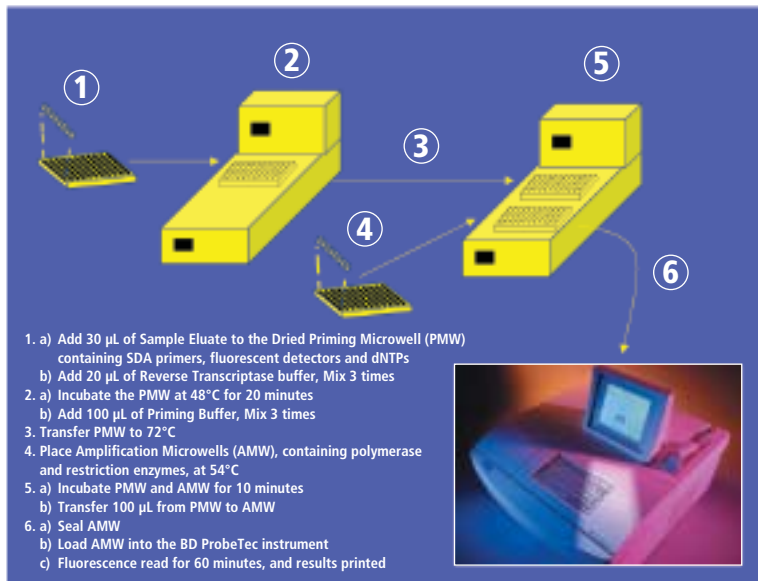


Figure 4: SARS-CoV infected Vero E6 cells

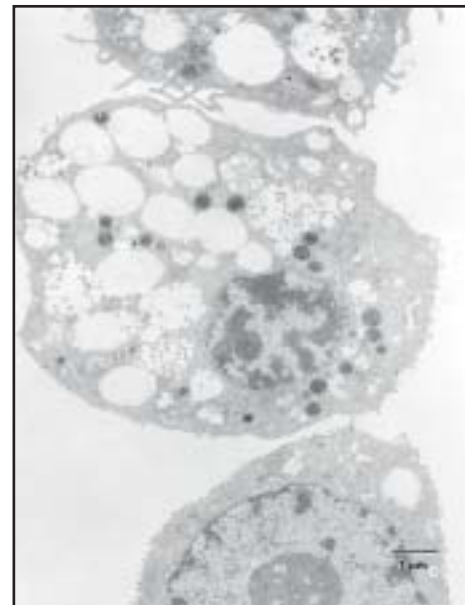


Figure 5: Vesicle containing SARS-CoV

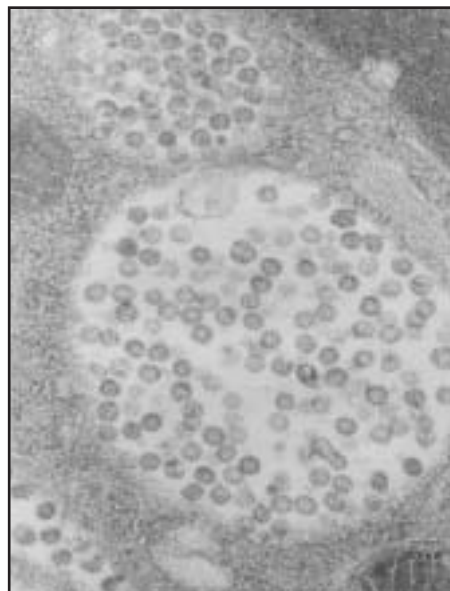


Table 1: Limit of Detection for SARS-CoV Viral Particles, Armored RNA, and RNA Transcripts

	95% Limit of Detection (copies/reaction)	95% Confidence Intervals (copies/reaction)
SARS-CoV Vietnam Isolate 200300592	81	68, 94
Ambion Armored RNA	116	105, 128
SARS-CoV RNA transcripts	167	127, 207

Table 2: SARS-CoV Assay Human Coronavirus Specificity

No.	Isolate	Strain
1	Human Coronavirus (229E)	VRATC0
2	Human Coronavirus (Dallas-1)	ATCC
3	Human Coronavirus (OC-43)	ATCC

\*For these experiments, no IAC was included in the reaction mixture.  
ATCC = American Type Culture Collection.

• None of the human coronavirus strains produced a positive result in the BD ProbeTec ET SARS-CoV Assay

## RESULTS (CONTINUED)

**Table 3:** SARS-CoV Assay Specificity/Cross-Reactivity Panel

No.	Isolate	Strain #	No.	Isolate	Strain #
1	<i>Acinetobacter calcoaceticus</i>	BD 13339	27	<i>Legionella pneumophila</i>	ATCC 33152
2	<i>Actinomyces israelii</i>	ATCC 10049	28	<i>Moraxella catarrhalis</i>	ATCC 25238
3	<i>Aeromonas hydrophila</i>	ATCC 7966	29	<i>Moraxella osloensis</i>	ATCC 19976
4	<i>Alcaligenes faecalis</i>	ATCC 8750	30	<i>Morganella morganii</i>	ATCC 25830
5	<i>Bacteroides fragilis</i>	ATCC 25285	31	<i>Mycobacterium tuberculosis</i>	ATCC 27294
6	<i>Blastomyces dermatitidis</i>	ATCC 4292	32	<i>Mycoplasma pneumoniae</i>	ATCC 29342
7	<i>Bordetella pertussis</i>	ATCC 9797	33	<i>Neisseria meningitidis</i>	ATCC 13077
8	<i>Candida albicans</i>	ATCC 44808	34	<i>Neisseria mucosa</i>	ATCC 19696
9	<i>Chlamydophila pneumoniae</i>	ATCC 53592	35	<i>Peptostreptococcus anaerobius</i>	ATCC 27337
10	<i>Citrobacter freundii</i>	ATCC 8090	36	<i>Plesiomonas shigelloides</i>	ATCC 14029
11	<i>Clostridium perfringens</i>	ATCC 13124	37	<i>Porphyromonas asaccharolytica</i>	ATCC 25260
12	<i>Corynebacterium diphtheriae</i>	ATCC 11913	38	<i>Proteus mirabilis</i>	ATCC 29906
13	<i>Corynebacterium jeikeium</i>	ATCC 43734	39	<i>Providencia stuartii</i>	ATCC 35031
14	<i>Cryptococcus neoformans</i>	ATCC 36556	40	<i>Pseudomonas aeruginosa</i>	ATCC 27853
15	<i>Edwardsiella tarda</i>	ATCC 15469	41	<i>Serratia marcescens</i>	ATCC 8100
16	<i>Eikenella corrodens</i>	ATCC 23834	42	<i>Staphylococcus aureus</i>	ATCC 12598
17	<i>Enterobacter aerogenes</i>	ATCC 13048	43	<i>Staphylococcus epidermidis</i>	ATCC E155
18	<i>Enterococcus faecalis</i>	ATCC 29212	44	<i>Stenotrophomonas maltophilia</i>	ATCC 13637
19	<i>Escherichia coli</i>	ATCC 11775	45	<i>Streptococcus mitis</i>	ATCC 6249
20	<i>Fusobacterium nucleatum</i>	ATCC 25586	46	<i>Streptococcus mutans</i>	ATCC 25175
21	<i>Haemophilus influenzae</i>	ATCC 33533	47	<i>Streptococcus pneumoniae</i>	ATCC 6303
22	<i>Haemophilus parainfluenzae</i>	ATCC 7901	48	<i>Streptococcus pyogenes</i>	ATCC 19615
23	<i>Histoplasma capsulatum</i>	ATCC 12700	49	<i>Veillonella parvula</i>	ATCC 10790
24	<i>Kingella kingae</i>	ATCC 23330	50	<i>Vibrio parahaemolyticus</i>	ATCC 17802
25	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i>	ATCC 13883	51	<i>Yersinia enterocolitica</i>	ATCC 27729
26	<i>Lactobacillus acidophilus</i>	ATCC 4356			

\*For these experiments, no IAC was included in the reaction mixture.

BD = Becton Dickinson

- None of the organisms tested produced a positive result in the BD ProbeTec ET SARS-CoV Assay

## DISCUSSIONS & CONCLUSIONS

n We have developed a novel assay for the detection of SARS-CoV using RT-SDA on the BD ProbeTec ET System.

n The BD ProbeTec ET assay is specific for SARS-CoV and does not cross-react with non-SARS human coronaviruses, or other clinically relevant bacteria or fungi.

n For cultured virus, an Armored RNA clone, and *in vitro* RNA transcripts, the analytical sensitivities of the assay ranged from 81-167 copies/reaction.

n The BD ProbeTec ET SARS-CoV RT-SDA assay was shown to be compatible with a modified QIAamp MinElute Virus Spin Kit protocol for analysis of stool, sputum, NP aspirate and throat swab (M4 medium) specimens.

n The BD ProbeTec ET SARS-CoV Assay is being developed to augment the atypical pneumonia assays for the *Chlamydiaceae* Family, *Legionella pneumophila* and *Mycoplasma pneumoniae* that are also currently under development for this platform. The BD ProbeTec ET *Legionella pneumophila* (LP) Amplified DNA Assay was recently cleared by the US Food and Drug Administration.

### IN PROGRESS:

n Conversion of the SARS-CoV RT-SDA assay to a more streamlined and user-friendly dried microwell format, similar to that employed with the BD ProbeTec ET CT/GC Amplified DNA Assay.

n Field Trials with archived SARS-CoV-positive and -negative clinical specimens in Hong Kong.