Strand Displacement Amplification and Homogeneous Real-Time Detection Incorporated in a Second-Generation DNA Probe System, BDProbeTecET

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Background: Amplified DNA probes provide powerful tools for the detection of infectious diseases, cancer, and genetic diseases. Commercially available amplification systems suffer from low throughput and require decontamination schemes, significant hands-on time, and specially trained laboratory staff. Our objective was to develop a DNA probe system to overcome these limitations.

Methods: We developed a DNA probe system, the BDProbeTecET, based on simultaneous strand displacement amplification and real-time fluorescence detection. The system uses sealed microwells to minimize the release of amplicons to the environment. To avoid the need for specially trained labor, the system uses a simple workflow with predispensed reagent devices; a programmable, expandable-spacing pipettor; and the 96-microwell format. Amplification and detection time was 1 h, with potential throughput up to 564 patient results per shift. We tested 122 total patient specimens obtained from a family practice clinic with the BD ProbeTecET and the Abbott LCx® amplified system for the detection of Chlamydia trachomatis and Neisseria gonorrhoeae.

Results: Based on reportable results, the BDProbeTecET results for both organisms were 100% sensitive and 100% specific relative to the LCx.

Conclusions: The BDProbeTecET is an easy-to-use, high-throughput, closed amplification system for the detection of nucleic acid from C. trachomatis and N. gonorrhoeae and other organisms.

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The amplified detection of nucleic acids has been achieved using a variety of techniques, including strand displacement amplification (SDA),† PCR, ligase chain reaction, nucleic acid sequence-based amplification, and transcription-mediated amplification (1–5). Each of these amplification methods uses different approaches to achieve the amplification of low copies of nucleic acid to amounts that can subsequently be detected. Detection methods are typically performed after the amplification step and include such approaches as colorimetric detection, chemiluminescence, and gel electrophoresis detection (6, 7). The shortcomings of these systems include low throughput, significant hands-on time, complex workflow, and the potential for amplicon contamination. These shortcomings have generally limited the adoption of amplified probe methods for routine use. Furthermore, to avoid false-positive results, the generation of amplicons during the amplification process requires separate amplification and detection areas, and sometimes multiple rooms or chemical or enzymatic schemes to reduce their concentrations (8, 9). Although semi-automated systems

† Nonstandard abbreviations: SDA, strand displacement amplification; ET, energy transfer; CT, Chlamydia trachomatis; GC, Neisseria gonorrhoeae; EB, elementary body; and AC, amplification control.
have been introduced, throughput, hands-on time, and other shortcomings still exist.

For amplified DNA probe technology to become more routine and to be adopted into clinical laboratory settings having fewer skilled technologists, there is a need for simpler, higher throughput and more user-friendly systems. Recently, methods using real-time detection of amplified nucleic acids have been described (5, 10, 11). These methods represent the future in molecular diagnostics and may soon allow laboratories to attain these goals. However, none of these methods has been adapted into commercially available user-friendly systems for diagnostic settings.

We describe the first of these second-generation systems, the BDProbeTecET. This system is based on the simultaneous amplification of nucleic acids by SDA and real-time detection using fluorescence energy transfer (ET). When the BDProbeTecET system is applied to the detection of Cilumavia trachonatis (CT) or Neisseria gonorrhoeae (GC), as few as 10–15 GC cells or CT elementary bodies (EBs) can be detected reliably in 1 h on the instrument. The system configuration and workflow permits a throughput of up to 564 patient results per shift.

**Materials and Methods**

**ASSAY CONSUMABLES AND BUFFERS**

The consumables used in the assay include two sets of microwells. The microwells are provided as strips of eight, which can be broken into individual tests if needed. The microwells are color-coded on the basis of assay type. For each test, two microwells are needed. One microwell (Priming Microwell) contains dried SDA primers, one of the four nucleotides, and fluorescence oligonucleotide probe. A second microwell (Amplification Microwell) contains the remaining dried SDA reagents, including SDA enzymes. Processed specimen is used to rehydrate these reagents. All CT and GC microwells and sample diluents were provided by Becton Dickinson Microbiology Systems ( Sparks, MD).

**TARGET REGIONS, PRIMERS, AND PROBES**

SDA has been previously described (1–5), and its principle is summarized in Figs. 1 and 2, which are discussed further under Results. Detection utilizes fluorescence ET as described in Fig. 3A and as discussed with the data under Results. For the amplification and detection of CT, the multicopy cryptic plasmid (12) was chosen as a target region. The region being amplified spans the following sequence: 5'-CAGGATTTGCTGACACAGCTCCGACAGCTCAGCCTCCGATGATGGC-GAATATCCTTACAGCTTCCAAATATCTATCAGGATAGAACACCTTCTCAG-3'. The SDA amplification primer and bumper primer pairs (target-binding region underlined, BsoBI restriction site bold and italicized) are as follows: 5'-ACC CCA TCG AAT GCA TGT TCT GGG GAG ACT CT- TAAA GAT A-3' and 5'-CAT TGG TIG ATG AAT TAT T-3'; 5'-CGA TTC GCCTCC AGA CT CTC GGG ACA AAA TCA ACA CCT G-3' and 5'-CAG CAA ATA ATC CTT GG-3'. The detector probe utilized for real-time detection is 5'-(Fam)-TAG CAC CCG AG TCTG (Rox)-CGC AGC CAA AAT GAC AGC TTC TGA TGG AA-3'.

For the amplification and detection of GC, a region within the multicopy plasmid gene-inverting protein homologue (13) was chosen. The target region spans the following sequence: 5'-CGCAAATCATCAAGCCCATGAAATAACGCTTGAAGTTTAAAGGAGAAAATAAAGGACGAGCGGAGAAAGCTAATGCAAGGAAE- GCCGTAGACCGTCTTTGA-3'. The SDA amplification primer and bumper primer pairs (target-binding region underlined, BsoBI restriction site bold and italicized) are as follows: 5'-CGA TTC CCG GCC AGT CCT CTC GGG AAC ACG TIG AAC TIT T-3' and 5'-CCG AAA TCA TCA AAG-3'; 5'-ACC GCA TCG AAT GCA TGT CTC GGG TCC TIT CAG CTA TGA GGC-3' and 5'-TCA AGA CGG TCC TTC ACG-3'. The detector probe utilized for real-time detection is 5'-(Fam)-TAG CAC CCG AGT CCT (Rox)-TTC TCC GTC TGC TCT TTT ATC TTC TC-3'.

**SYSTEM HARDWARE COMPONENTS**

The standard system hardware components (Fig. 4, discussed further below) include a programmable, expandable-spacing pipettor, a priming and warming heater, and the BDProbeTecET fluorescent reader. For the detection of CT and GC, an additional lysing heater and lysing rack is included for specimen processing. The expandable spacing pipettor is capable of transferring samples from eight specimen tubes into microwells in a standard 8 × 12 array. The pipettor is programmable and is capable of dispensing and mixing volumes up to 1200 μL. Acrosol-resistant tips (Matrix Technologies) were used for all transfers.

The lysing rack and lysing heater are used to heat lysed specimen for CT or CT/GC testing. The lysing rack holds 96 specimen tubes and can be placed directly into the lysing heater. After the lysing step, the rack is removed to allow the samples to cool. One ergonomic feature of the rack is that it permits caps on the tubes to be removed with one hand. The priming and warming heater contains two fixed-temperature stations capable of maintaining two distinct temperatures. Each station holds one metal microwell tray. One station, maintained at a setpoint of 72.5 °C, is used in the priming step. The second station, maintained at a setpoint of 54 °C, is used to prewarm the amplification mixture before the tray is placed into the instrument. Guide pins on this block aid in the orientation of an adhesive sealer card, which is placed onto the prewarmed microwell tray before transfer of the tray into the BDProbeTecET instrument.

The BDProbeTecET instrument is a fluorescent reader capable of maintaining constant temperature (52.5 °C), monitoring real-time fluorescence, and reporting results through an algorithm. It consists of a heated stage capable of holding one 96-microwell tray at a time. Microwell
trays are scanned once per minute, using fluorescent excitation and detection through the bottom of the well. Trays are moved in one dimension on fixed rails over the optical station. The optical station consists of an optical bundle with eight branches, which permits an electronically multiplexed interrogation of eight microwells, thus eliminating the need for two-dimensional motion of the tray. Emitted light passes through a custom optical band-pass filter, is detected by a photomultiplier tube, and is analyzed by software.

URINE SAMPLE COLLECTION, TRANSPORT, AND PROCESSING (FIG. 5)

Urine specimens are collected in sterile, plastic, preservative-free specimen collection cups. A urine processing pouch (Becton Dickinson Microbiology Systems) is added to the sample cup, and the sample cup is capped. The urine processing pouch contains a proprietary material capable of removing amplification inhibitors and stabilizing urine specimens containing CT stored up to 6 days or GC up to 4 days at 18–30 °C, or 6 days at 2–8 °C for specimens containing CT or GC (manuscript in preparation). At the testing site, 4 mL of urine is removed and transferred into a 4-mL tube. After centrifugation at 2000g for 30 min, the supernatant is decanted. Sample diluent (2 mL) is added, the capped sample is vortex-mixed and placed into the lysing rack. The rack is placed into the lysing heater (114 °C) for 30 min. Samples are then removed from the heater and cooled for 15 min at room temperature before use.

SWAB SAMPLE COLLECTION, TRANSPORT, AND PROCESSING (FIG. 5)

Male urethral specimens are collected using rayon swabs (MiniTip CULTURETTE™ DIRECT; Becton Dickinson). For the collection of female endocervical specimens, a cleaning swab is used first to remove mucus. The endocervical specimen is then collected with a polyurethane-tipped swab (CULTURETTE DIRECT; Becton Dickinson). Both male and female swab specimen types can be transported without preservative or liquid additive to the testing laboratory at 2–30 °C for up to 6 days. At the testing laboratory, the swabs are expressed into tubes supplied prefilled with 2 mL of sample diluent. The swabs

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**Fig. 1. Mechanism of SDA.**
are discarded, and the expressed sample fluid is heat-lysed and cooled as described above for urine specimens.

**ASSAY PROCEDURE**

For each tray of specimens assayed, one positive control and one negative control are included in the microwell tray set up and are tested like samples. Their positions are determined by the user and appear on the plate layout report generated by the instrument during the login of specimens. A separate microwell for each control and specimen is used for an amplification control (AC). The AC well contains an amplifiable DNA sequence and actin flag inhibitory specimens. Thus, for a CT test, a 96-well plate will contain by one positive control (which also acts as a control for CT and GC primers and reagents), one negative control, and up to 46 samples. Each of the 48 CT wells has a corresponding AC well. For specimens being tested for both CT and GC, one plate contains one positive control, one negative run control, and up to 30 samples. Each of the 30 samples and two controls requires three microwells—one for CT, one for GC, and one for AC. Once the layout of the microwells is determined, the test is begun.

Primed microwells are placed into their respective microwell trays. Samples are lysed and then cooled at room temperature. For a CT/GC test, 600 μL of lysed and cooled specimen is aspirated from eight specimen tubes simultaneously. The pipettor tip spacing plunger is adjusted to collapse the spacing of the tips, and 150 μL is dispensed into each of three CT, GC, and AC priming wells. The remaining liquid and tips are discarded. This step continues until all specimens are dispensed into the priming tray.

The priming tray is covered and incubated at room temperature for at least 20 min, but for convenience in performing multiple runs throughout a shift, trays may incubate at room temperature for up to 6 h.

After incubation, the cover is removed from the priming microwell tray. The amplification microwells are placed in the amplification tray. The priming and amplification trays are placed into their stations on the priming and warming heater for 10 min. At the end of 10 min, 100 μL is transferred from each microwell column in the priming tray into the corresponding amplification microwell column in the warming station. The pipettor is programmed to dispense 100 μL and mix 50 μL of sample in the amplification wells three times to hydrate and mix the sample and reagents. Tips are discarded, and the same steps repeated until all samples and controls have been transferred.

After the samples are transferred, a rigid self-adhering amplification sealer is applied to permanently seal the microwells in the warming station. Paper backing is removed from the adhesive side of the sealer and the sealer is applied smoothly to the top of the microwells. Guide pins on the warming station of the priming and

![Diagram](attachment:probe_conversion.png)

Fig. 2. Detector probe conversion during SDA.

warming heater facilitate the alignment of the sealer onto the microwell tray.

The sealed amplification tray is placed into the BDPProbeTecET instrument. Amplification, fluorescence detection, and data analysis occur in the instrument. Results are printed after the 1-h amplification and detection are complete. After the completion of the assay, the sealed microwells are lifted from the trays and discarded into a sealable plastic pouch, which provides a second layer of containment. The metal tray is rinsed with water and dried before reuse.

**REAL-TIME DETECTION KINETIC PLOTS**

Various concentrations of CT (serovar LGVII, ATCC VR 902B) EBs or GC cells (ATCC 19424) were added into sample diluent and tested according to the assay procedure described above. Strains were obtained from American Type Culture Collection.

**CLINICAL STUDIES**

Swab and/or urine specimens were collected from consenting symptomatic and asymptomatic patients at a community family practice clinic. For endocervical and penile urethral specimens, two swabs were collected, one
for testing with the Abbott LCx® and one for testing with the BDProbeTecET.

Urine specimens were divided and assayed on both the Abbott LCx and the BDProbeTecET. The results shown are the initial test results without discrepant resolution.

Results

To best understand the basis of the real-time detection system, it is important to highlight the amplification and detection mechanisms. Fig. 1 shows the two discrete phases, Target Generation and Exponential Target Amplification, in the mechanism of SDA. For the purpose of simplicity, the process is shown for only one strand of a double-stranded target, with the understanding that this process occurs on both strands and yields exponential amplification. For the Target Generation Phase, a double-stranded DNA target (1) is denatured and allowed to hybridize to two primers, B1 and S1 (2). The B1 is a bumper primer, whereas the S1 primer contains the single-stranded restriction enzyme sequence 5' to a target-binding region of the primer. In the presence of the Bst DNA polymerase and dNTP mixture, simultaneous extension products of B1 (3) and S1 are generated (4). This process displaces the S1 products, which may then be hybridized to the opposite strand primers, B2 and S2 (5). The simultaneous extension of both primers produces species 6. Species 6 is capable of carrying out the Exponential Target Amplification Phase. Species 6 is a substrate for the BsoBI enzyme in the mixture, which recognizes the appended double-stranded BsoBI site. This BsoBI site contains a thioated dCTP nucleotide incorporated during the Target Amplification Phase, which makes the site refractory to double-stranded cleavage by the enzyme. Instead, the strand lacking this modified JC within the restriction site is nicked by the BsoBI (7). The Bst DNA polymerase next binds to this nick and begins the synthesis of a new strand while simultaneously displacing the downstream strand (8–10). This step recreates the double-stranded species 7, and the iterative nicking and displacement process repeats. The displaced strands are capable of binding to opposite strand primers, which produces exponential amplification at 52.5 °C. These single-stranded products also bind detector probe for real-time detection.

Present in the SDA reaction is a single-stranded DNA probe containing fluorescein and rhodamine labels (see Materials and Methods). The region between these labels includes a stem-loop structure. The loop comprises a recognition sequence for the BsoBI enzyme. This probe also contains a target-specific sequence 3' to the rhodamine label. Before specific target amplification by SDA, the fluorescein and rhodamine labels are proximal to each other such that any excitation of the fluorescein leads to transfer of the emitted energy to the rhodamine label. The net effect is that very little emission from excited fluorescein is detected. After SDA, the probe is converted to a double-stranded species, which is cleaved by the BsoBI restriction enzyme. This cleavage causes the physical

![SDA process diagram](https://via.placeholder.com/150)

Cleared duplex yields fluorescent signal

**Fig. 3.** Mechanism of fluorescence ET produced from a single-stranded probe during SDA (A) and real-time detection of lysed CT EBs and GC cells (B).
separation of the fluorescein and rhodamine labels such that no ET from the excited fluorescein to rhodamine can occur. The net effect is that emission is detected from an excited fluorescein label, and this emission is indicative of specific amplification attributable to the presence of the target sequence.

The specific steps for the conversion of this fluorescent single-stranded probe are shown in Fig. 2. In step 1, a displaced strand (thick line), the probe containing fluorescein (○) and rhodamine (●), and an amplification primer hybridize. The simultaneous extension by the DNA polymerase of both the amplification primer and probe leads to displacement of an extended probe (2). In step 3, the extended probe binds the opposite strand primer and is extended (4). This extension creates a double-stranded BsoBI site, which is flanked by both the fluorescein and rhodamine labels. This extension step creates a BsoBI site that lacks thioated dCTP incorporation at the nucleotide position of BsoBI cleavage. As a consequence, binding of BsoBI at the site causes double-stranded cleavage instead of nicking (5); the two labels thus are physically separated, and emission of fluorescein is detected. These steps occur simultaneously during the SDA process. This detection process is distinguished from Taqman in several ways, including the fact that Taqman uses PCR, thus exploiting the 5'-3' exonuclease activity of the Taq polymerase, whereas SDA uses exonuclease-free polymerase, and that the Taqman method requires thermocycling for the formation of fluorescent product.

An overview of the fluorescence ET detection process that occurs during SDA is presented in Fig. 3A. As seen in the kinetic plots of Fig. 3B, the formation of these fluorescent products is both continuous and rapid. For both CT and GC, the detection of small numbers of cells or EBs occurs within 1 h. The dose–response seen in Fig. 3B allows for the potential application of target quantification on this system.

The components of the system instrumentation and the workflow are shown in Figs. 4 and 5. The workflow is designed to allow for multiple analytical runs within a single shift and optimal time to first results. For example, 96 specimens and controls can be lysed at one time. This provides sufficient specimens for two cycles of CT assays, or three cycles of CT/GC assays. Lysed specimens may cool from 15 min to 6 h. Once specimens are dispensed into the priming microwells, they may be incubated up to 6 h. Thus, specimens sufficient for additional analyses may be lysed, primed, and staged for the priming and warming, and detection steps. With six analytical runs achievable in a single shift, up to 180 CT/GC combination tests or up to 276 CT assays can be performed. This AC feature can be deselected by the user, if desired, thus

![Urine/Swab Sample Preparation](image1)
![Primming and Warming](image2)
![Amplification and Detection](image3)
![Lysing Heater and Sample Rack](image4)
![BDProbeTecET Reader](image5)

Fig. 4. Hardware components of the BDProbeTecET system.
allowing 94 wells for CT detection per analytical run. For other analytes under development, the instrument is capable of monitoring two amplification reactions in one well, allowing for multiplex test results, such as an analyte and internal control. In both of these cases, a throughput of up to 564 patient results can be achieved per shift.

The detection of CT and GC from clinical samples is shown in Tables 1 and 2. The algorithm resident in the instrument reports results as positive, negative, indeterminate (inhibited AC), or equivocal on the basis of presellected cutoff values established using clinical specimens. The reportable results shown in Tables 1 and 2 do not include indeterminate (two urine specimens each in the CT assay) or equivocal specimens (two swabs in the CT assay and one in the GC assay), which must be retested. The Abbott LCx does not have an AC, and thus cannot report indeterminate results. Because indeterminate and equivocal results are not reportable results, they are not included into calculations on sensitivity or specificity. The sensitivity and specificity for reportable results using the BDProbeTecET CT and GC assays relative to the Abbott LCx are 100%.

**Table 1. Detection of CT in clinical samples.**

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<thead>
<tr>
<th>BDProbeTecET</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
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<tr>
<td>Total</td>
<td>10</td>
<td>47</td>
</tr>
</tbody>
</table>

**Table 2. Detection of GC in clinical samples.**

<table>
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<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
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<td>0</td>
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<tr>
<td>Negative</td>
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<tr>
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<td>1</td>
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</table>

**Discussion**

Although a limited number of specimens were tested, the data show that excellent performance can be achieved with the system when patient swab and urine specimens are used. Complete characterization of system performance, including clinical trial data, will be published separately (manuscript in preparation). Separate internal studies (data not shown) of interfering substances show that the presence of up to 2% blood (swabs) does not affect results. One of the reasons for this is that the effect of such potentially interfering substances is mitigated by the real-time data acquisition and metrics applied by the algorithm. For example, the real-time detection permits subtraction of signals resulting from fluorescent substances. This is a distinct advantage in a clinical setting, where such substances may be encountered in specimens.

Contamination with amplicons or target DNA is also minimized with the system design. The controlled dispense and aspirate programs on the pipettor prevent splattering in the transfer steps. Aerosol-resistant tips prevent sample-to-sample carryover. Because no amplification occurs in the priming wells, no amplicon contamination is possible at this stage. The sealed microwells and
subsequent disposal into sealed plastic pouches provides two levels of containment from amplicon release. Thus overall, the system approach has been to “design out” cross-over and contamination potential. Internal studies on cross-contamination, where high-positive and low-positive tubes are interspersed, demonstrate 0.1% (1 of 960) cross-contamination and validate that this design out approach has met with success.

In conclusion, the BDProbeTecET system, which is based on real-time fluorescent detection and SDA, provides a new technology for the clinical setting. The system has superior throughput and time to first results, simple workflow, and minimal risk for release of amplicon contamination.

We thank Judith Lovchik of the University of Maryland, Baltimore, MD, for testing the BDProbeTecET and Abbott LCx systems on clinical specimens. We also thank Neil Jensen for providing the kinetic plots and Francois Guillot for workflow graphics. The fluorescent probe design and real-time probe conversion reactions depicted in Fig. 2 were conceived by J.G. Nadeau, J.B. Fitzner, J.L. Schramm, and C.P. Linn [European patent application (1998). Publication no. 0881302].

References


