Detection of *Mycobacterium tuberculosis* DNA with thermophilic strand displacement amplification and fluorescence polarization

G. Terrance Walker* and C. Preston Linn

Strand displacement amplification (SDA) is an isothermal, in vitro method for diagnostics that amplifies a target DNA sequence by using a restriction enzyme and DNA polymerase. We have combined a new thermophilic form of SDA that involves restriction enzyme BstBI and polymerase exo-Bsa with fluorescence polarization for detection of *Mycobacterium tuberculosis* DNA by using the IS6110 insertion element as the target sequence. A 5'-fluorescein-labeled oligodeoxynucleotide detector probe hybridizes to the amplified product as it rises in concentration during SDA, and the single- to double-stranded conversion is monitored through an increase in fluorescence polarization. The associated change in polarization upon amplification of the target sequence is enhanced by specific polymerase binding to the double-stranded detector probe. Fewer than 10 *M. tuberculosis* genomes can be amplified and detected with an extremely simple protocol that takes only 20 min and uses relatively simple instrumentation and reagents, all of which can be purchased off-the-shelf.

INDEXING TERMS: restriction enzymes • polymerases • nucleic acids • infection • bacteria • tuberculosis

Tuberculosis is one of the most common human infectious diseases, infecting an estimated 1.8 billion worldwide with ~2.7 million deaths per year [1]. The disease is caused by *Mycobacterium tuberculosis*, *M. bovis*, and *M. africanum*. Culture-based diagnosis provides exquisite sensitivity and specificity but requires 1–6 weeks because of the species' slow growth rates. Acid-fast organisms can be quickly identified in stained smears but with low sensitivity (>104 organisms/mL). Consequently, there is an acute need for a rapid and sensitive test, a challenge for which nucleic acid diagnostics is well suited.

Strand displacement amplification (SDA) is an in vitro method for DNA detection that provides 1010-fold amplification of a target DNA sequence in 15 min during constant-temperature incubation at 60 °C [2]. SDA is based on the ability of a restriction enzyme to nick a hemimodified recognition site and the ability of a polymerase to displace a downstream DNA strand during replication. Exponential amplification is achieved through repeated nicking, strand displacement, and priming of displaced strands (Fig. 1A). Despite the complicated appearance of schematic depictions of SDA, the technique is extremely simple to perform: Target DNA is heat-denatured in the presence of all reagents except the restriction enzyme and polymerase. Amplification then proceeds during single-temperature incubation after addition of the enzymes.

Numerous methods have been devised for detecting an amplified target sequence, most involving physical separation of hybridized and unhybridized forms of a labeled detector probe. Fluorescence polarization is a simple technique for monitoring the hybridization of a fluorescently labeled oligonucleotide without separation of single- and double-stranded forms [3]. A single-stranded oligodeoxynucleotide is relatively flexible and tumbles quickly in solution compared with its double-stranded counterpart. Consequently, a fluorescent dye attached to an oligodeoxynucleotide will lead to slower tumbling (longer correlation time) upon hybridization. The average correlation time for a dye population is reflected in the fluorescence polarization value of the sample, independent of the total dye concentration. Therefore, probe hybridization can be detected without removal of excess probe as long as an appreciable percentage of total probe is converted to the double-stranded form.

Previously, we developed assays for detection of *M. tuberculosis* DNA that were based on SDA and fluorescence polarization with either La Jolla Blue [4, 5] or fluorescein [6] as the fluorophore. The assays are based on hybridization of a detector probe to the amplified target sequence that increases in concentration during SDA, allowing real-time detection through the corre-

---

1 Nonstandard abbreviations: SDA, strand displacement amplification; DTT, dithiothreitol; BSA, bovine serum albumin; and NALC, N-acetyl-L-cysteine-sodium hydroxide-sodium citrate.

Becton Dickinson Research Center, P.O. Box 12016, 21 Davis Dr., Research Triangle Park, NC 27709-2016.

*Author for correspondence. Fax 919-549-7572; e-mail walker@bdrc.bd.com.

Received November 16, 1995; accepted May 13, 1996.

Reference: 1996 Cl. Chem. 42:10. 1604-1608
Corresponding increase in polarization [4, 5]. We have now adopted the fluorescein-based assay to a recently developed thermophilic form of SDA that involves the restriction enzyme BsoBI and an exonuclease-deficient polymerase from Bacillus caldotenax (exo<sup>-</sup> Bca) [2].

The current SDA/polarization assay provides very rapid and sensitive detection of <i>M. tuberculosis</i> DNA with a simple protocol: SDA reactions are assembled and incubated 15 min at 60 °C and the fluorescence polarization values are recorded in a commercially available fluorometer especially designed for measuring fluorescence polarization for fluorescein. We can detect a single <i>M. tuberculosis</i> genome by using the IS6110 element as the target sequence [7]. We enhanced <i>M. tuberculosis</i>-specific polarization by post-SDA addition of a DNA polymerase that binds specifically to the double-stranded probe, further slowing its tumbling time in solution.

**Materials and Methods**

All oligodeoxynucleotides were purified by denaturing gel electrophoresis. The 5'-labeled fluorescein oligodeoxynucleotide detector probe (5'-dATCCGTATGGTGATAACGTCCTT-<i>T</i>CA) was prepared by standard procedures with the reagent 6-FAM Amidite (P/N 401527; Applied Biosystems, Foster City, CA). It binds nucleotide positions 985–1010 of the IS6110 element of <i>M. tuberculosis</i> [7], which is contained within the SDA target (is6110 nucleotide positions 970–1025). BsoBI (160 000 kU/L) was purchased from New England Biolabs (Beverly, MA). Exo<sup>-</sup> Bca (22 000 kU/L) was purchased from PanVera (Madison, WI; distributor for Takara). An exonuclease-deficient Klenow fragment of <i>E. coli</i> polymerase I (Exo<sup>+</sup> Klenow) was purchased from US Biochemical (Cleveland, OH) at 5000 kU/L.

Fluorescence polarization values were recorded on an instrument specifically designed for fluorescein (Model FPM 1; Jolley Consulting and Research, Round Lake, IL). Samples were contained in disposable borosilicate glass test tubes (12 × 75 mm; Fisher Scientific, Pittsburgh, PA), and maintained at 37 °C during polarization measurements. Fluorescence polarization is a dimensionless quantity expressed as (<i>I</i>_pers−<i>I</i>_perm)/(<i>I</i>_pers+<i>I</i>_perm) where <i>I</i>_pers is the emission intensity when the emission and excitation polarizers are in parallel orientations, and <i>I</i>_perm is the emission intensity when the two polarizers are perpendicularly oriented. Polarization values are usually expressed as "milli" polarization (mP) units: 1000(<i>I</i>_pers−<i>I</i>_perm)/(<i>I</i>_pers+<i>I</i>_perm).

SDA was performed generally as previously described [2].

**Fig. 1.** SDA cycle with simultaneous hybridization and extension of fluorescently labeled detector probe.

SDA starts with a target-generation step during which target fragments flanked by nickable BsoBI sites are generated from the genomic target sequence. This is immediately followed by the exponential SDA cycle in a single and concerted reaction. Fig. 1 depicts the SDA cycle but omits the target-generation step, which is described elsewhere [2, 9]. (A) The SDA cycle operates with an excess of primers <i>S</i>2 and <i>S</i>3, which bind to opposite strands of the target sequence, flanking the region to be amplified. <i>S</i>1 and <i>S</i>2 contain target binding regions at their 3' ends and a recognition site (6'TCGGG) for the restriction enzyme BsoBI located immediately 5' to the target binding regions (BsoBI recognition sites are designated by raised boxes). During each round of the cycle, the 3' end of <i>S</i>2 binds to the 3' end of the displaced target strand <i>T</i>2, forming a duplex with 5' overhangs. Likewise, <i>S</i>2 binds to <i>T</i>1, the complement of <i>T</i>2. Exo<sup>-</sup> Bca extends the recessed 3' ends of the duplexes with dATP, dTTP, dGTP and dCTPαS, producing hemiphosphorothioate recognition sites that are nicked by BsoBI. These nicking and extension/displacement steps cycle continuously (short upturned arrows) because extension at a nick regenerates a nickable BsoBI recognition site. The strand displaced from the <i>S</i>1-<i>T</i>2 duplex is identical to <i>T</i>1. Likewise, the short displaced strand from the <i>S</i>2-<i>T</i>3 duplex is identical to <i>T</i>2. Consequently, target amplification is exponential because each displaced <i>T</i>2 binds a new <i>S</i>3 primer while each displaced <i>T</i>1 binds a new <i>S</i>2 (long upturned arrows). Sense and antisense strands are differentiated by thick and thin lines, respectively. Intact and nicked BsoBI recognition sequences are depicted by _ and __. The partial BsoBI recognition sequence 6'TCGGG and its complement 5'CCCGA are present at the 5' and 3' ends of displaced strands as represented by ____ and ___ . Additional details of the SDA cycle are reported elsewhere [2, 9, 10]. In the present study, SDA is performed in the presence of fluorescently labeled detector probe (D) that hybridizes downstream of primer <i>S</i>1. This detector probe goes through a series of extension and displacement events as part of a series of side reactions that occur concurrently with the SDA cycle without interference, as shown in (B). The 5'-fluorescein-labeled detector probe D binds a displaced strand downstream from the SDA primer <i>S</i>1 (structure I). This complex is identical to the complex shown at the top left side of the SDA cycle in A. Exo<sup>-</sup> Bca simultaneously extends D, <i>S</i>1, and <i>T</i>2 in this complex (structure II). Extension of <i>S</i>1 displaces the extension product of D, forming structures III and IV. Structure IV in B is identical to the corresponding structure shown on the left side of A, where it continues in the SDA cycle. The displaced extension product of D (structure III) hybridizes to the other SDA primer (<i>S</i>3). Forming structure V. Exo<sup>-</sup> Bca extends structure V, forming structure VI, which undergone linear SDA upon BsoBI nicking of the primer sequence on <i>S</i>3 (structure VII). The strands displaced during linear SDA bind additional detector probes (structure VIII) that are extended by exo<sup>-</sup> Bca (structure IX). Structures I, VI, VII, VIII, and IX all account for forms of double-stranded detector probe upon target-specific SDA. Estimated polarization values are indicated for various forms of the detector probe in the absence and presence of the SDA polymerase exo<sup>-</sup> Bca at 37 °C on the basis of measured values for the detector probe hybridizing to its complement as described in the text.
Each 100-μL SDA sample contained final concentrations of 35 mmol/L K₂HPO₄ (pH 7.5), 3 mmol/L Tris-HCl (pH 7.9), 15 mmol/L NaCl, 0.3 mmol/L dithiothreitol (DTT), 10.5 mmol/L MgCl₂, 1.4 mmol/L each dGTP, dATP, dTTP, and dCTP; (Pharmacia), 0.1 g/L bovine serum albumin (BSA), 500 ng of human placental DNA, 15 mmol/L primer S₁ (\dCGATTCCGCCTCCAGACCTCTGGGTTGTACTGAGATCCCCT; the BsoBI recognition sequence is in bold italics and the IS6110 target binding sequence is underlined), 60 mmol/L primer S₂ (\dACCAGCTATCGATGTTCGACC), 5 mmol/L each primers B₁ (\dCGCTGATACCCGAT) and B₂ (\dTGGACCCGCAAC), 320 U of BsoBI, 8 U of exo⁻ Bca, 5 mmol/L 5'-fluorescein-labeled detector probe, and the indicated amounts of M. tuberculosis DNA.

SDA samples were prepared as follows. We first prepared 70-μL samples with the following volumes of stock solutions: 12.5 μL of distilled water, 10 μL of 350 mmol/L K₂HPO₄ (pH 7.5), 10 μL of 1 g/L BSA, 10 μL of 14 mmol/L each dATP, dTTP, dGTP, and dCTP; 10 μL of 50 mmol/L detector probe, 7.5 μL of 100 mmol/L MgCl₂, and 10 μL of a primer mixture containing 150 mmol/L S₁, 600 mmol/L S₂, 50 mmol/L B₁, and 50 mmol/L B₂. It is important to add the reagents in the indicated order to minimize precipitation when K₂HPO₄ and MgCl₂ are combined at high concentrations. Next, various amounts of target M. tuberculosis DNA were added to each sample in a 10-μL aliquot of 10 mmol/L Tris-HCl, pH 7.9, 10 mmol/L MgCl₂, 50 mmol/L NaCl, and 1 mmol/L DTT that also contained 500 ng of human placental DNA. These 80-μL samples were denatured by heating for 2 min in a boiling water bath, followed by 3 min at 60 °C for primer annealing.

Vendor stock solutions of BsoBI (160 000 kU/L) and exo⁻ Bca (22 000 kU/L) were diluted together to 16 000 kU/L and 400 kU/L, respectively, with 10 mmol/L Tris-HCl (pH 7.9), 10 mmol/L MgCl₂, 50 mmol/L NaCl, and 1 mmol/L DTT. This enzyme mixture was prepared at room temperature immediately before addition (20 μL) to each incomplete 80-μL SDA sample equilibrated at 60 °C. Upon enzyme addition and immediate vortex-mixing, SDA proceeded for 15 min at 60 °C and was then terminated by addition of 6 μL of 0.5 mol/L EDTA. Amplified samples were diluted with 0.9 mL of 55 mmol/L NaCl, 111 mmol/L Tris-HCl (pH 7.5), 0.7 mmol/L K₂HPO₄ (pH 7.4), 1.1 mmol/L EDTA, 0.7 mmol/L β-mercaptoethanol, 27 mg/L BSA, 0.2 mL/L Triton X-100, and 70 mL/L glycerol. Fluorescence polarization was measured after equilibrating 10 min at 37 °C. Then 5 μL of 5000 kU/L exo⁻ Klwo were added and fluorescence polarization values were recorded again at 37 °C.

SDA and polarization detection in the presence of processed spumt was performed with culture-negative respiratory specimens. Sputum samples were processed by an N-acetyl-L-cysteine–sodium hydroxide–sodium citrate (NALC) protocol to generate 1-mL processed samples containing 25 mmol/L K₂HPO₄ (pH 7.5) [8]. Aliquots (10 μL) from the processed samples were added to SDA reactions with provision for the K₂HPO₄ contribution from the processed sample, and subsequent polarization detection was as described above.

Results and Discussion

The associated change in exclusion volume that accompanies hybridization of a fluorescently labeled oligodeoxynucleotide decreases the fluorophore’s motion in a manner that can be detected through an increase in fluorescence polarization, depending on the fluorescent lifetime of the dye. We have designed a polarization detection system in which a fluorescently labeled probe hybridizes to the target sequence during SDA (Fig. 1). The detector probe (D) binds to one of the strands displaced during the SDA cycle at a location immediately downstream from SDA primer S₁ (Fig. 1B, structure I). S₁ and D are extended by polymerase (structure II), resulting in displacement of the probe extension product (structure III) in a manner analogous to the strand displacement reaction intrinsic to SDA (Fig. 1A). (Compared with SDA in the absence of a detector probe [2], we have reduced the concentration of S₁ to help ensure that it is not extended before hybridization of the downstream detector probe, which is present at only 5 mmol/L.)

The displaced, single-stranded probe extension product (structure III) binds the other SDA primer (S₂), forming a complex (structure V) that is extended by polymerase (structure VI). This double-stranded complex (structure VI) provides a template for linear SDA in which BsoBI nicking of its recognition site on S₂ (structure VII) and polymerase extension/displacement at the nick produce single strands, to which additional detector probes bind (structure VIII) and are extended (structure IX). Structures I, VI, VII, VIII, and IX all account for hybridized forms of the detector probe that are detectable through high polarization values.

The polarization change can be enhanced by including a protein that binds specifically to the double-stranded form and further reduces the dye’s movement in solution. Previously, we developed a system that involved a genetically engineered EoRI restriction endonuclease [EoRI(ghn11)], which lacks cleavage activity but retains binding affinity for the double-stranded EoRI recognition sequence [6]. During SDA, in a target-specific manner, a single-stranded probe containing an EoRI recognition sequence at its 5' end was converted to a fully double-stranded form that bound EoRI(ghn11), thereby enhancing the conformation-associated change in polarization. However, polarization enhancement by EoRI(ghn11) was diminished by competitive binding to the human DNA that was added to mimic clinical specimen conditions. That study also included an earlier form of SDA that operated at 40 °C and produced high amounts of background amplification products that also competed for EoRI(ghn11) binding.

The present report reduces the problem with background DNA in two ways. First, we have adopted a new form of SDA that produces less background amplification because it operates at a more stringent hybridization temperature (60 °C) and requires only 15 min. Second, we found that DNA polymerases specifically enhance polarization of the hybridized detector probe even in the presence of 10 μg of human DNA. For example, hybridization of the detector probe to its oligodeoxynucleotide complement at 37 °C produces an increase in polarization from 55 to 70 mP, whereas the corresponding change is from 55 to 125 mP for 0.5 pmol of detector probe in
the presence of 8 U of exo" Bca. Polymerases probably maintain specificity for the hybridized detector probe in the presence of high-molecular-mass human DNA because the ends of the detector probe helix resemble replication sites. We suspect many other types of double-strand-specific proteins will produce comparable polarization enhancement, but exo" Bca is attractive because it is the polymerase used in the current thermophilic SDA system [2].

We applied fluorescence polarization detection to the thermophilic SDA system previously developed for M. tuberculosis [2]. Samples (100 μL) containing different amounts of M. tuberculosis DNA underwent SDA in the presence of detector probe. After 15 min of SDA at 60 °C, the samples were diluted to 1 mL and fluorescence polarization values were recorded at 37 °C on a commercial instrument designed for fluorescence polarization measurements of fluorescein. Dilution of the sample before polarization measurement is necessary because 1 mL is the instrument's minimum sample volume.

The detector probe exhibits target-dependent polarization (Fig. 2). Samples containing higher M. tuberculosis concentrations exhibit higher polarization, whereas the zero M. tuberculosis sample exhibits a value corresponding to single-stranded probe. The sample containing 10 M. tuberculosis genome is clearly detectable over the zero M. tuberculosis sample, with the 1-genome sample barely above background. Polarization enhancement by the SDA polymerase is directly observed for the higher M. tuberculosis samples, for which polarization approaches 125 mP, the value corresponding to complete hybridization under SDA conditions containing exo" Bca. Thermophilic SDA with polarization enhancement by the SDA polymerase has improved the detection sensitivity at low target concentrations by ~10-fold compared with the previous system that operated at 40 °C and had EcoRI(ghn111) as the enhancer [6]. Additionally, the time required for SDA and polarization detection has been shortened from 3 h to 20 min.

After the initial measurement of polarization values, we added 25 U of exo" Klenow and remeasured polarization to see if additional polymerase would further enhance detection. Even higher polarization values were observed for the amplified samples containing M. tuberculosis DNA but not for the zero M. tuberculosis sample, further improving detection sensitivity. (Exo" Klenow was a rather arbitrary choice of a polymerase for polarization enhancement that was not related to its previous use as the polymerase in SDA [9–12].) The polarization increase upon addition of exo" Klenow indicates that 8 U of exo" Bca were not saturating for enhancing polarization after SDA. We tried using more exo" Bca during SDA, but higher polymerase concentrations decreased amplification; the optimal exo" Bca concentration is a balance between SDA efficiency and polarization enhancement.

Upon hybridization of an oligodeoxynucleotide, we observe a polarization increase that is further enhanced by double-strand-specific protein binding. An obvious interpretation is to attribute the polarization changes to slower molecular tumbling (longer correlation times) due to increases in exclusion volumes. However, evidence suggests that the polarization changes may also reflect hindered motion of the fluorophore in the double-strand and protein-bound states. For example, previous studies indicated that polarization enhancement by the protein EcoRI requires proximity between the dye and protein and an optimal tether length attachment of the dye to the oligodeoxynucleotide [13]. In that study, dynamic polarization measurements supported a hindered rotation model for the fluorophore. These observations raise the possibility that direct contacts between the dye and the double-stranded helix/protein may be partially responsible for polarization increases. At this point, therefore, we do not know whether our polarization measurements reflect changes in tumbling times and (or) direct fluorescein contacts. Nevertheless, polarization accurately reports hybridization of the detector probe during SDA.

We have not rigorously determined the precision, accuracy, or statistically defined detection limit of the assay. The major source of error is undoubtedly not the polarization measurement but rather the SDA step, with variability associated with amplification efficiency and to some extent the level of background reactions that diminish protein enhancement of polarization. Polarization values decrease slightly if SDA is extended past 15 min because the target amplification plateaus but background amplification continues (data not shown). Our sensitivity in a clean system with highly purified M. tuberculosis DNA is consistently between 10 and 1 M. tuberculosis genomes, which corresponds to 100–10 copies of IS6110 target sequence.

Two obvious concerns surround potential clinical applications of this technology. One is SDA compatibility with tuberculosis respiratory specimens. Although previous studies demonstrated M. tuberculosis detection in sputum specimens with an earlier version of SDA [8, 14, 15], corresponding results have not been published for the current thermophilic SDA protocol. The second concern is that clinical specimens may interfere with polarization measurements either by introducing background
fluorescence or increasing viscosity, which would affect the tumbling time of the detector probe. As a preliminary glimpse, we supplemented SDA samples with sputum processed by a NALC protocol [8] and observed no effect on either polarization or total fluorescence intensity. In addition, positive SDA/polarization results were obtained for processed sputum samples supplemented with $10^4$ M. tuberculosis genomes (data not shown).

In summary, we have combined SDA with fluorescence polarization with a fluorescein-labeled detector probe. Probe hybridization to the amplified target occurs simultaneously with SDA, as indicated by an increase in polarization. Extremely sensitive detection of M. tuberculosis DNA was achieved with a simple protocol after only 15 min of SDA. We were able to enhance the sensitivity of the system by including a protein that binds specifically to the double-stranded detector probe. Future development will combine thermophilic SDA with simultaneous polarization measurement in a closed-tube, homogeneous format that guards against false positives due to accidental contamination with previous amplification products because the reactions are never opened after SDA.

We thank Cathy Spargo for teaching us the thermophilic SDA protocol and Jim Nadeau for helpful discussions. We thank Gerald A. Denys, Methodist Hospital of Indiana, for supplying the NALC-processed sputum specimens and Deborah Howard for additional processing for SDA.

References


