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DNA detection by strand displacement amplification and fluorescence polarization with signal enhancement using a DNA binding protein

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ABSTRACT

Strand displacement amplification (SDA) is an isothermal *in vitro* method of amplifying a DNA sequence prior to its detection. We have combined SDA with fluorescence polarization detection. A 5′-fluorescein-labelled oligodeoxynucleotide detector probe hybridizes to the amplification product that rises in concentration during SDA and the single- to double-strand conversion is monitored through an increase in fluorescence polarization. Detection sensitivity can be enhanced by using a detector probe containing an *Eco*RI recognition sequence at its 5′-end that is not homologous to the target sequence. During SDA the probe is converted to a fully double-stranded form that specifically binds a genetically modified form of the endonuclease *Eco*RI which lacks cleavage activity but retains binding specificity. We have applied this SDA detection system to a target sequence specific for *Mycobacterium tuberculosis*.

INTRODUCTION

In its simplest sense nucleic acid probe technology is based upon hybridization of a labelled nucleic acid probe to a target sequence. Numerous methods have been devised for detecting this hybridization event, most involving physical separation of hybridized and unhybridized forms of the probe. Fluorescence polarization (FP) is a simple technique for monitoring hybridization of a fluorescently labelled oligonucleotide without separation of single- and double-stranded forms (1). A single-stranded oligonucleotide is relatively flexible and tumbles quickly in solution compared with its double-stranded counterpart. Consequently, a fluorescent dye attached to an oligonucleotide will experience slower tumbling (longer correlation time) upon hybridization, although the dye movement may not completely reflect that of the oligonucleotide, depending upon the nature of the linkage between the two bodies. The average correlation time for a dye population is reflected in the FP value of the sample, independent of the total dye concentration. Therefore, hybridization of an oligonucleotide probe can be detected without removal of excess probe as long as an appreciable percentage of the total probe is converted to the double-stranded form.

Although FP provides a simple and accurate means of DNA detection, the associated change in FP values is not extremely large. For example, hybridization of a fluorescein-labelled oligodeoxynucleotide is typically accompanied by an FP change from ~45 to ~65 millipolarization units (mP). Although this change can be adequate for detecting the presence of a target sequence, a 20 mP change does not allow one to estimate the concentration of hybridized target over a significant range. For example, if a 100 µl sample contains 2 nM detector probe (~10^{11} molecules), then a 20 mP change allows measurement of target levels between 10^{11} and 10^{10} molecules if FP values are accurate to within ~2 mP. On the other hand, if the associated change in FP is 200 mP, then the range of detectable target is extended to between 10^{11} and 10^9 molecules. Expanding the dynamic range obviously improves detection sensitivity. The change in FP for hybridization of a detector probe can be enhanced by including a protein that binds specifically to the double-stranded form, thereby increasing its molecular weight and correlation time (2–4). We have enhanced the FP change by including the endonuclease *Eco*RI or a genetically modified form [*Eco*RI(Gln111)], the latter of which binds specifically to the *Eco*RI recognition site but does not cleave it (10). *Eco*RI or *Eco*RI(Gln111) binding to a double-stranded, fluorescein-labelled detector probe containing an *Eco*RI recognition site increases the FP change for the single- to double-stranded conversion by ~6-fold, providing a convenient means of improving DNA detection.

We have combined FP detection with an *in vitro* nucleic acid amplification technique known as strand displacement amplification (SDA), which provides 10^8-fold amplification of a target DNA sequence during constant temperature incubation (5–8). SDA is based upon the ability of a restriction enzyme to nick a hemi-modified recognition site and the ability of a polymerase to displace a downstream DNA strand during replication. The method consists of a target generation process (Fig. 1A) that makes copies of the target sequence flanked by nickable restriction sites followed by exponential amplification of these modified target sequences (Fig. 1B) through repeated nicking, strand displacement and priming of displaced strands. Despite the complicated appearance of Figure 1, the experimental protocol of SDA is simple. Target DNA is heat denatured in the presence of all reagents except the restriction enzyme and polymerase, the

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sample is cooled to ~40°C, the enzymes are added and the sample is incubated at ~40°C.

We have modified the original SDA protocol to include a 5′-fluorescein-labelled detector probe that is converted from a single- to double-stranded form in a target-dependent manner during SDA. FP values are then recorded to detect target-dependent conversion of the probe using the sequence-specific protein *Eco*RI(Gln111) to enhance the signal.

MATERIALS AND METHODS

All oligodeoxynucleotides were synthesized on an Applied Biosystems Inc. instrument (Model 380B) and purified by denaturing gel electrophoresis. 5′-Fluorescein-labelled oligodeoxynucleotides were prepared by standard procedures using the reagent 6-FAM Amidite from Applied Biosystems Inc. (P/N 401527) according to the product insert protocols. Two different 5′-fluorescein detector probes were used. Probe D_1 (5′-dATCCGTATGGTGGATAACGTCTTTCA) binds nucleotide positions 985–1010 of the IS6110 element of *Mycobacterium tuberculosis* (9), which is contained within the sequence being amplified by SDA (IS6110 nucleotide positions 972–1023). The other detector probe, D_2, (5′-dGGAATTCATCCGTATGGTGG-ATAACGTCTTTCA) is identical to D_1 but has an additional sequence (5′-dGGAATTC) at its 5′-end which contains the *Eco*RI recognition sequence.

FP values were recorded on an FPM 1 instrument from Jolley Consulting and Research, Inc. (Round Lake, IL), an instrument specifically designed for fluorescein. Excitation and emission wavelengths are fixed through interference filters appropriate for fluorescein. Samples were contained in disposable borosilicate glass test tubes (catalog no.14-962-10B; Fisher) and maintained at 37°C during FP measurement. FP is a dimensionless quantity that is expressed as

$$FP = (I_{par} - I_{perp})/(I_{par} + I_{perp}),$$

where I_{par} and I_{perp} represent emission intensity when the emission polarizer is in the parallel and perpendicular position respectively in relation to the excitation polarizer. FP values are usually expressed in terms of millipolarization units (mP)

$$FP\ (mP) = 1000(I_{par} - I_{perp})/(I_{par} + I_{perp}).$$

Initial experiments designed to measure the FP change associated with hybridization of the 5′-fluorescein-labelled oligodeoxynucleotides (Fig. 3) were performed as follows. Samples (100 µl) containing 10 nM 5′-fluorescein-labelled detector probe with or without an equivalent amount of complement oligodeoxynucleotide were prepared in 4 mM TAE, 50 mM NaCl, pH 7.8. Hybridization occurred over 30 min at 37°C. The samples were then diluted to 1 ml using 55 mM NaCl, 111 mM Tris–HCl, pH 7.5, 0.7 mM K_2HPO_4, pH 7.4, 1.1 mM EDTA, 0.7 mM β-mercaptoethanol, 27 µg/ml bovine serum albumin (BSA), 0.02% Triton X-100, 7% (v/v) glycerol. The 1 ml samples were equilibrated for 15 min at 37°C and FP values were recorded at 37°C. Then 5 µl 100 000 U/ml *Eco*RI (New England BioLabs) or 5 µl 1.6 µM (as protein dimer) *Eco*RI(Gln111) (10) were added to the samples and FP readings were recorded at 37°C after the samples were incubated for 2 h at 37°C.

Experiments involving SDA and subsequent addition of *Eco*RI or *Eco*RI(Gln111) (Fig. 4) were performed as follows. SDA reactions were performed on samples containing *M.tuberculosis*

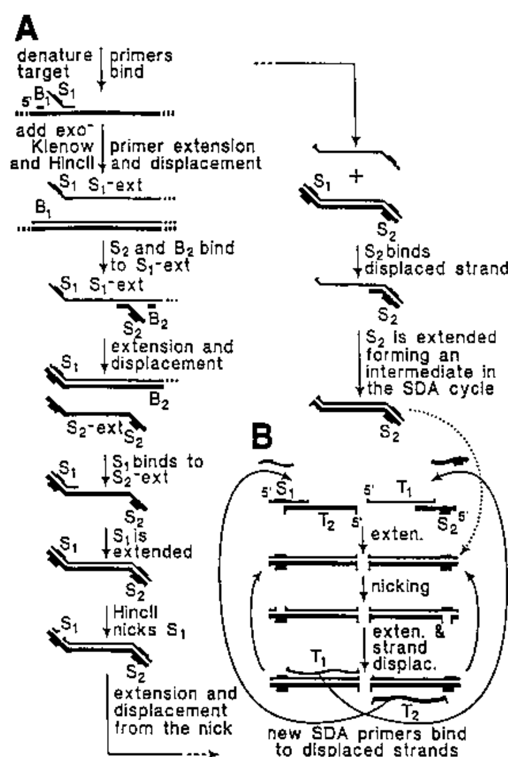

Figure 1. Schematic representation of SDA. SDA is performed using an excess of four primers (B_1, B_2, S_1 and S_2). S_1 and S_2 contain target binding regions at their 3′-ends and a recognition site (5′-GTTGAC) for the restriction enzyme *Hinc*II located immediately 5′ of target binding regions (*Hinc*II recognition sites are designated by the raised boxes). S_1 and S_2 bind to opposite strands of the target sequence, flanking the region to be amplified. B_1 and B_2 are simply target binding sequences (containing no *Hinc*II recognition sites) and bind at positions 5′ to S_1 and S_2. (**A**) Starting at the top of the figure, the target DNA is heat denatured. S_1 and B_1 then hybridize to one strand of the target upon lowering the temperature to 41°C. (Only one of the two target strands is shown. A corresponding series of reactions orginate on the other strand.) *Hinc*II and exo⁻ Klenow (an exonuclease-deficient form of DNA polymerase I from *E.coli*) are then added to the sample. At this point the remaining steps proceed as a single cascade. Exo⁻ Klenow, which is present in large molar excess over the number of target strands, simultaneously extends S_1 and B_1 using dGTP, dCTP, dUTP and dATPαS. As S_1 is extended the extension product (S_1-ext) is displaced through extension of B_1. S_1-ext serves as the target for binding of S_2 and B_2. Simultaneous extension of S_2 and B_2 results in displacement of an S_2 extension product (S_2-ext). An S_1 primer binds to S_2-ext and is extended, forming a double-stranded structure with a hemiphosphorothioate *Hinc*II site at each end. *Hinc*II nicks the unmodified strand of the hemiphosphorothioate site on S_1, leaving intact the thio-modified complementary strand of the *Hinc*II site. [*Hinc*II nicking can also occur at the hemiphosphorothioate site on the opposite end of the fragment (not shown).] Exo⁻ Klenow then extends the 3′-end at the nick and displaces the downstream strand. An S_2 primer binds to the displaced stand and is extended, forming an intermediate in the SDA cycle, shown in (B) by the dashed arrow. (**B**) The SDA cycle is where exponential amplification occurs. During each round of the cycle the 3′-end of S_1 binds to the 3′-end of the displaced target strand T_2, forming a duplex with 5′-overhangs. Likewise, S_2 binds to T_1, the complement of T_2. Exo⁻ Klenow extends the recessed 3′-ends of the duplexes, producing hemiphosphorothioate recognition sites that are nicked by *Hinc*II. These nicking and extension/displacement steps cycle continuously (short upturned arrows), because extension at a nick regenerates a nickable *Hinc*II recognition site. The strand displaced from the $S_1 \cdot T_2$ duplex is identical to T_1. Likewise, the displaced strand from the $S_2 \cdot T_1$ duplex is identical to T_2. Consequently, target amplification is exponential, because each displaced T_2 binds a new S_1 primer while each displaced T_1 binds a new S_2 (long upturned arrows). Sense and antisense strands are differentiated by thin and thick lines. Intact and nicked *Hinc*II recognition sequences are depicted by _■■_ and _■ ■_. The partial *Hinc*II recognition sequence 5′-GAC and its complement 5′-GTC are present at the 5′- and 3′-ends of displaced strands, as represented by ■_ and _■. Additional details are reported elsewhere (5,6).

target DNA generally as previously described (5–7). Each 100 µl sample contained 50 mM K_2HPO_4, pH 7.6, 7 mM $MgCl_2$, 0.5 mM dUTP, 0.2 mM each dGTP, dCTP and dATPαS (Pharmacia), 16% (v/v) glycerol, 0.1 mg/ml BSA, 100 ng human placental DNA, 50 nM primer S_1 (5′-dGCATTATAGTACCTGTCT*GTTGAC*ACT-GAGATCCCCT; *Hin*cII recognition sequence italicized), 300 nM primer S_2 (5′-dTTGAATAGTCGGTTACTT*GTTGAC*GGCGTA-CTCGACC; *Hin*cII II recognition sequence italicized), 25 nM each primers B_1 (5′-dCGCTGAACCGGAT) and B_2 (5′-dTG-GACCCGCCAAC), 300 U *Hin*cII (New England Biolabs), 1 U exo^- Klenow (United States Biochemical), 10 nM 5′-fluorescein-labelled detector probe D_2 and the indicated amounts of *M.tuberculosis* target DNA. For each sample all reagents except *Hin*cII and exo^- Klenow were assembled in a microcentrifuge tube and the sample was heated in a boiling water bath for 2 min and then equilibrated at 41°C in a water bath. Then 4 µl 75 U/µl *Hin*cII and 2 µl 0.5 U/µl exo^- Klenow were added in a single aliquot. SDA proceeded for 3 h at 41°C and was terminated by addition of EDTA to 10 mM. Samples were then diluted with 0.9 ml 55 mM NaCl, 111 mM Tris–HCl, pH 7.5, 0.7 mM K_2HPO_4, pH 7.4, 1.1 mM EDTA, 0.7 mM β-mercaptoethanol, 27 µg/ml BSA, 0.02% Triton X-100, 7% glycerol. The 1 ml samples were equilibrated at 37°C and FP values were recorded at 37°C. Then 5 µl 100 000 U/ml *Eco*RI (New England BioLabs) or 5 µl 1.6 µM *Eco*RI(Gln111) were added, the samples were incubated for 3.5 h at 37°C and FP readings were again recorded.

RESULTS

The associated change in exclusion volume that accompanies a single- to double-strand conversion of a fluorescently labelled oligodeoxynucleotide probe results in an increase in correlation time (slower tumbling in solution) that is detectable by FP. Correlation times can be increased further through protein binding to the double-stranded probe. We have designed an FP detection system in which a fluorescently labelled, single-stranded oligodeoxynucleotide is converted to a double-stranded form in a target-dependent manner during SDA (Fig. 2). We have used two types of detector probes (D_1 and D_2). D_1 consists simply of a target binding sequence. D_2 is identical to D_1 except that it contains an *Eco*RI recognition sequence at its 5′-end. Single- to double-strand conversion of a detector probe during SDA is first described for D_1. As shown in Figure 2A, D_1 binds to one of the strands displaced during the SDA cycle at a location immediately downstream of SDA primer S_1 (structure I). S_1 and D_1 are then extended by polymerase, resulting in displacement of the probe extension product (structure II) in a manner analogous to the strand displacement reaction intrinsic to SDA (Fig. 1). The displaced single-stranded probe extension product (structure III) binds the other SDA primer (S_2), forming a complex (structure IV) which becomes fully double-stranded through polymerase extension (structure V). This double-stranded complex (structure V) provides a template for linear SDA in which the *Hin*cII site on S_2 is nicked and polymerase extension/displacement at the nick produces single-stranded strands to which additional D_1 probes bind (structure VI) and upon which they are extended (structure VII). Structures I, II, V, VI and VII all account for double-stranded forms of the D_1 detector probe that are detectable by an increase in FP value.

D_2 likewise binds to a displaced target strand immediately downstream of S_1 (structure I, Fig. 2B) and undergoes a

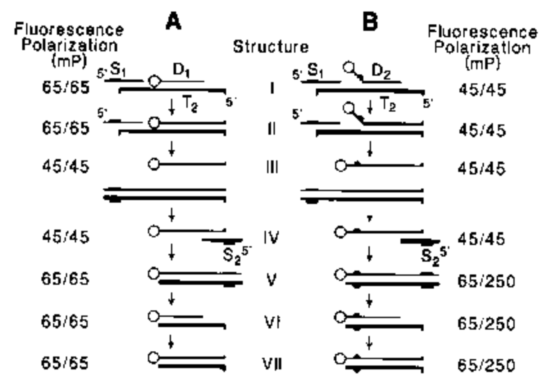

Figure 2. Single- to double-stranded conversion of the fluorescently labelled detector probe during SDA. This series of hybridization, extension and displacement steps occurs concurrently with SDA. Approximate FP values are indicated for various forms of the detector probes in the absence and presence of the protein enhancer *Eco*RI (Gln111) respectively. (**A**) The 5′-fluorescein-labelled detector probe (D_1) binds a displaced strand downstream from the SDA primer S_1 (structure I). This complex is identical to the complex shown at the top left side of the SDA cycle (Fig. 1B), where it is depicted without the detector probe. Exo^- Klenow simultaneously extends D_1 and S_1 along the displaced strand (structure II). Extension of S_1 displaces the extension product of D_1 (structure III), which then binds the other SDA primer (S_2), forming structure IV. This complex is then extended by exo^- Klenow, forming a complex (structure V) which undergoes linear SDA upon nicking of the primer sequence on S_2 by *Hin*cII and strand displacement by exo^- Klenow. The strands displaced during this linear SDA bind additional detector probes (structure VI), which are extended by exo^- Klenow (structure VII). Structures I, II, V, VI and VII all account for forms of double-stranded detector probe upon target-specific SDA. (**B**) An analogous series of steps occurs with the 5′-fluorescein detector probe D_2, which is identical to D_1 except that it also contains an *Eco*RI recognition sequence at its 5′-end that is not homologous to the target sequence being amplified. In the case of D_2, structures V–VII account for double-stranded sources of D_2 that are detectable through an increase in FP values and can bind *Eco*RI or *Eco*RI(Gln111). Sense and antisense strands are differentiated by thin and thick lines. *Hin*cII recognition sequences are depicted by _■■_. The partial *Hin*cII recognition sequence 5′-GAC and its complement 5′-GTC are present at the 5′- and 3′-ends of displaced strands, as represented by ■_ and _■. The *Eco*RI recognition sequence is depicted by ___.

subsequent cascade of extension and displacement steps that are completely analogous to those of D_1. In the case of D_2, however, only structures V–VII account for double-stranded sources that can be detected by FP (Fig. 2B). Likewise, these are the double-stranded structures that bind *Eco*RI and *Eco*RI(Gln111). Structures I-IV for D_2 have single-stranded 5′-ends that do not bind the proteins or exhibit higher FP values.

The entire process described in Figure 2 occurs simultaneously with the SDA cycle depicted in Figure 1B. Although inclusion of additional primers tends to increase background reactions in some amplification systems (e.g. PCR), the fluorescent probe does not increase SDA background reactions, because any undesired mispriming between the probe and an SDA primer (B_1, B_2, S_1 or S_2) does not generate a product that can be exponentially amplified, since the fluorescent probe does not contain a *Hin*cII site. SDA requires participation of two *Hin*cII site-containing primers to achieve exponential amplification (8). This is in contrast to PCR, where any oligonucleotide containing an extendable 3′-end can serve as an amplification primer.

Before combining FP detection with SDA we performed a preliminary experiment where we measured FP changes for D_1 and D_2 hybridizing to their complementary oligodeoxynucleotides in

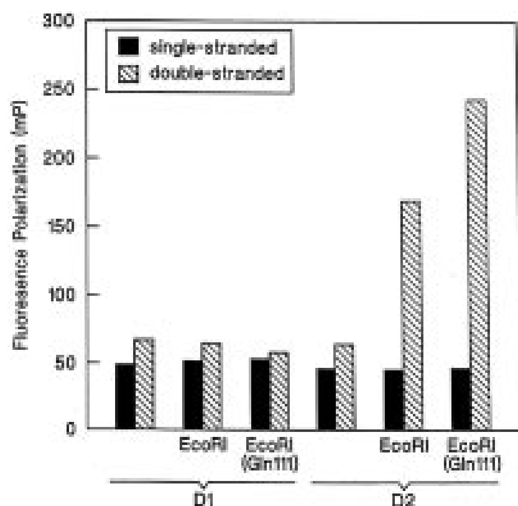

Figure 3. FP values as a function of probe conformation and protein binding. Polarization values were recorded for single- and double-stranded forms of the two detector probes in the presence or absence of either *Eco*RI or *Eco*RI(Gln111). Detector probe D_1 lacks an *Eco*RI recognition site, while detector D_2 contains an *Eco*RI site at its 5′-end.

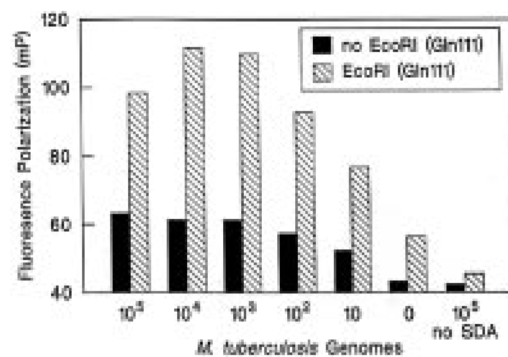

Figure 4. SDA and FP detection of *M.tuberculosis* DNA. SDA was performed in the presence of detector probe D_2 on samples containing the indicated amounts of target *M.tuberculosis* DNA. After SDA FP was measured before and after addition of *Eco*RI(Gln111) as indicated. The negative control sample contained dATP instead of dATPαS, which disables SDA but allows background hybridization or extension of the detector probe by exo⁻ Klenow.

the absence and presence of either *Eco*RI or *Eco*RI(Gln111). Oligodeoxynucleotides were hybridized in a Mg^{2+}-free solution designed to support *Eco*RI and *Eco*RI(Gln111) binding but not cleavage by *Eco*RI. Preliminary FP values were then recorded at 37°C before addition of *Eco*RI or *Eco*RI(Gln111). Subsequently *Eco*RI or *Eco*RI(Gln111) was added to a final protein dimer concentration that was four times that of the oligodeoxynucleotides, to ensure stoichiometric binding, and FP values were again recorded at 37°C.

FP values increase from ~50 to ~65 mP upon hybridization of the detector probes to their complements in the absence of protein (Fig. 3). *Eco*RI and *Eco*RI(Gln111) increase the FP values for double-stranded D_2, but not its single-stranded form. In contrast, protein binding does not affect FP values for either D_1 conformation, because it lacks an *Eco*RI site. *Eco*RI(Gln111) produces a larger increase in FP for double-stranded D_2 compared with *Eco*RI, perhaps due to its higher binding affinity (10).

Next we applied FP detection to an SDA system previously developed for *M.tuberculosis* DNA (5,7). Samples containing different amounts of *M.tuberculosis* DNA underwent SDA in the presence of D_2. Following SDA FP values were determined before and after addition of *Eco*RI(Gln111) (Fig. 4). Before addition of *Eco*RI(Gln111) D_2 exhibits a target-dependent increase in FP value. Samples containing *M.tuberculosis* target DNA exhibit higher FP values than the samples lacking *M.tuberculosis* DNA, even down to a level of 10 *M.tuberculosis* genomes. Over a range of higher *M.tuberculosis* levels (10^3–10^5 genomes) FP values reach a maximum value (~62 mP) consistent with 100% double-stranded detector probe (Fig. 3), due to complete conversion (probe saturation). Probe saturation occurs between 10^2 and 10^3 genomes, which corresponds to 10^3 and 10^4 copies of the IS6110 target sequence (~10 copies per genome). Since there are ~10^{12} detector probes per SDA reaction, the observed amplification factor is between 10^8- and 10^9-fold, which is consistent with previous reports for this SDA system using ^{32}P detector probes with gel electrophoresis analysis (5). Nearly identical results are obtained with detector D_1 in the absence of *Eco*RI(Gln111) (data not shown).

The sample lacking *M.tuberculosis* DNA should exhibit FP values identical to unhybridized detector probe. Higher FP values for the no *M.tuberculosis* DNA can arise primarily from two sources. First, the sample may be accidently contaminated with minute quantities of *M.tuberculosis* DNA or SDA products (amplicons) carried over from previous reactions. Low level contamination of negative samples with a few target molecules is a critical concern with amplification techniques like SDA, since they provide detection of a few target molecules. Alternatively, the detector probe in the no *M.tuberculosis* DNA sample could be converted to a double-stranded form either directly through background hybridization, perhaps to the 100 ng human DNA that was present in all the samples, or through some polymerase-mediated activity, such as extension of a transiently formed hairpin conformation by the detector probe. A control sample was included to elucidate possible sources of background signal in no *M.tuberculosis* DNA sample. The control sample contained dATP instead of dATPαS during SDA. Substitution with dATP still enables any non-specific extension of the detector probe by exo⁻ Klenow, but it disables the SDA mechanism by allowing double-stranded cleavage of *Hin*cII sites. The FP value for this control sample indicates that non-specific hybridization of the detector probe is extremely low, since it is very close to the single-stranded value in Figure 3. Likewise, the SDA sample lacking *M.tuberculosis* target DNA exhibits a signal that is also representative of single-stranded detector probe in the absence of *Eco*RI(Gln111), suggesting that amplicon contamination was extremely low for this sample.

After the initial recording of FP values *Eco*RI(Gln111) was added to each sample and FP values were again recorded at 37°C. *Eco*RI(Gln111) significantly increased the FP values for SDA samples containing target *M. tuberculosis* DNA, expanding the difference between them and the no *M.tuberculosis* DNA and control samples. However, the SDA samples containing *M.tuberculosis* DNA did not achieve the same level of polarization enhancement observed in the simpler hybridization experiment (Fig. 3), even for the SDA samples reaching probe saturation

(10^3–10^5 genomes). In Figure 3 we observed an ~180 mP increase upon addition of *Eco*RI(Gln111) to the double-stranded oligodeoxynucleotide, whereas the largest polarization increases were only 35–51 mP for the SDA experiment in Figure 4. We suspect this is due to *Eco*RI(Gln111) binding to the non-target DNA in the SDA samples, which is a combination of the added human DNA (100 ng) and background products generated during SDA. Protein partitioning between the detector probe and other DNA is probably also responsible for the extended time necessary to achieve binding equilibrium for these SDA samples. Unlike the simpler experiment in Figure 3, where polarization readings stabilized within a few minutes after addition of *Eco*RI(Gln111), the SDA samples required 3.5 h to reach binding equilibrium. Also in contrast to the simpler experiment using the detector probes and their oligodeoxynucleotide complements (Fig. 3), *Eco*RI did not increase FP values for SDA reactions performed with detector probe D_2 (data not shown), again presumably due to its lower binding affinity (10) and partitioning between the detector probe and other DNA.

This background DNA issue may also be responsible for the larger error in polarization values for the samples containing *Eco*RI(Gln111) in Figure 4. For example, we expected the 10^3–10^5 genome samples to exhibit the same polarization value in the presence of *Eco*RI(Gln111) as observed in its absence. Comparable values were also expected for the no *M.tuberculosis* DNA sample and the 'no SDA' sample. Perhaps varying amounts of non-target DNA in these samples is responsible for the higher variability. We did not attempt to increase association kinetics and achieve binding saturation by adding more protein, because higher protein levels approach a practical limitation. This variability issue will have to be resolved before widespread application of the technique can be undertaken. We expect a new form of SDA (13), which operates at higher temperatures and produces less background amplification, will go a long way in this regard.

DISCUSSION

We have combined SDA with FP detection using a fluorescein-labelled detector probe. Samples containing as few as 10 *M.tuberculosis* genomes were detectable using a very simple protocol. Probe hybridization to the amplified target occurs simultaneously with SDA, as indicated by subsequent FP measurement. We were able to enhance the sensitivity of the system by including *Eco*RI(Gln111), which binds specifically to the double-stranded detector probe.

We are currently working toward simultaneous SDA and FP detection in a homogeneous closed-tube format. Such a system offers many advantages over current post-amplification methods. A homogeneous, simultaneous method is not only faster and simpler because it involves fewer sample manipulations, but it also guards against the false positive problem associated with accidental contamination with previous amplification products (amplicons), because there is no need to open samples after amplification. Another advantage of a simultaneous format is that it provides a baseline measurement at the start of SDA (time zero), thereby obviating extensive control samples to account for background fluorescence. Since the only critical variable changing during simultaneous SDA and detection is the concentration of the amplified product, it is the change in polarization that is important, not the initial or final values.

Previously we discovered that the *Eco*RI recognition site must be very close to the fluorescein label at the 5′-end of the oligodeoxynucleotide for the protein to increase polarization of the double-stranded form (2). The detector probe in the current study contains a recognition site just 1 nt from the 5′-fluorescein. Positioning the recognition site 5 nt from the 5′-fluorescein abolishes the effect of *Eco*RI binding (2). This suggests that polarization enhancement by the protein may not in fact derive from an increase in exclusion volume, but rather from direct contact between protein and dye. Fluorescein binding to the protein may restrict its motion. Alternatively, direct fluorescein–protein contact may not be responsible, but close proximity between dye and protein may be necessary due to helical flexibility. In the case of the oligodeoxynucleotide with the recognition sequence 5 nt from the 5′-fluorescein (2) restricted motion at the recognition site may not translate to the 5′-fluorescein due to flexibility in the intervening helix. Regardless of the explanation, the observations urge caution when it comes to monitoring protein binding to oligonucleotides using FP.

We suspect that the greater ability of *Eco*RI(Gln111) (versus *Eco*RI) to increase polarization is related to its higher binding affinity (10). Unlike the catalytic situation of DNA cleavage in the presence of Mg^{2+}, the current FP system requires stoichiometric protein binding. The ability of a restriction enzyme to bind specifically to its double-stranded recognition site is related to its absolute and relative affinities for the recognition site and random sequence DNA. In comparison with *Eco*RI, the respective *Eco*RI(Gln111) binding affinities are ~1000- and 100-fold greater for the recognition site and random sequence DNA, so *Eco*RI(Gln111) generally binds tighter and is ~10-fold more specific for the recognition sequence (10). *Eco*RI(Gln111) is probably more effective at enhancing polarization because of its higher specificity and absolute affinity for the detector probe recognition site, which is located just 1 nt from the end of the helix, an unfavourable location considering that the footprint of *Eco*RI extends beyond the recognition site (11) and the fact that *Eco*RI uses flanking sequences to scan for the site in a one-dimensional manner (12). [We assume *Eco*RI(Gln111) has similar traits.] In comparison with samples containing just oligodeoxynucleotides (Fig. 3), slower protein binding was observed under SDA conditions, which includes 100 ng human DNA. This probably reflects partitioning between the oligodeoxynucleotide probe and random sequence DNA.

We have developed a FP detection system that should be generally applicable to a range of nucleic acid probe assays that include other amplification strategies, such as PCR. In addition, a number of restriction enzymes are probably applicable. We have tested a few other restriction enzymes and found that although they enhance polarization of a double-stranded oligodeoxynucleotide in a clean system as does *Eco*RI, they too are ineffective in the presence of background DNA (data not shown). We have not tried rare cutting restriction enzymes (e.g. *Not*I) in the hope that they would be more specific, as suggested by one of the manuscript's reviewers. As previously mentioned, we expect that a new form of SDA (13), which operates at higher temperatures and produces less background amplification, will improve the specificity of protein binding to the hybridized detector probe. For all restriction enzymes tested to date we placed the recognition site at the 5′-end of the oligodeoxynucleotide, very near the fluorophore, so we do not know if this is a necessary conditions for all restriction enzymes, as is the case for *Eco*RI (2).

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