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Strand displacement amplification—an isothermal, *in vitro* DNA amplification technique

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ABSTRACT

Strand Displacement Amplification (SDA) is an isothermal, *in vitro* nucleic acid amplification technique based upon the ability of HincII to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of exonuclease deficient klenow (exo⁻ klenow) to extend the 3′-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as target for an antisense reaction and vice versa. In the original design (G. T. Walker, M. C. Little, J. G. Nadeau and D. D. Shank (1992) *Proc. Natl. Acad. Sci* 89, 392–396), the target DNA sample is first cleaved with a restriction enzyme(s) in order to generate a double-stranded target fragment with defined 5′- and 3′-ends that can then undergo SDA. Although effective, target generation by restriction enzyme cleavage presents a number of practical limitations. We report a new target generation scheme that eliminates the requirement for restriction enzyme cleavage of the target sample prior to amplification. The method exploits the strand displacement activity of exo⁻ klenow to generate target DNA copies with defined 5′- and 3′-ends. The new target generation process occurs at a single temperature (after initial heat denaturation of the double-stranded DNA). The target copies generated by this process are then amplified directly by SDA. The new protocol improves overall amplification efficiency. Amplification efficiency is also enhanced by improved reaction conditions that reduce nonspecific binding of SDA primers. Greater than 10^7-fold amplification of a genomic sequence from *Mycobacterium tuberculosis* is achieved in 2 hours at 37°C even in the presence of as much as 10 μg of human DNA per 50 μL reaction. The new target generation scheme can also be applied to techniques separate from SDA as a means of conveniently producing double-stranded fragments with 5′- and 3′-sequences modified as desired.

INTRODUCTION

Strand Displacement Amplification (SDA) is an isothermal, *in vitro* method of amplifying DNA. The original SDA design provides powerful amplification compatible with diagnostic nucleic acid assays (1). However, it requires restriction enzyme cleavage of the DNA sample prior to amplification in order to generate an amplifiable target fragment with defined 5′- and 3′-ends. The target restriction step not only complicates the experimental protocol, but it also limits the choice of target DNA sequences because the sequence must be flanked by convenient enzyme restriction sites. In addition, the target DNA typically must be double-stranded for restriction enzyme cleavage. We have simplified the SDA protocol by replacing the target restriction step with a novel method of generating amplifiable fragments. SDA reactions can now be performed in three simple steps: 1) the target DNA sample is heat denatured in the presence of primers and other reagents, 2) HincII and exo⁻ klenow are added, and 3) the sample is incubated at 37°C.

As in the original report on SDA (1), we applied this improved version to genomic DNA samples from *Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis. Enhanced amplification was achieved by virtue of the new target generation scheme and improved reaction conditions which lower background reactions that attentuate target amplification.

The new target generation scheme is a novel means of producing a double-stranded fragment with 5′- and 3′-ends modified as desired. We propose a few general applications for the method.

MATERIALS AND METHODS

Materials

The large fragment of *E. coli* DNA polymerase I (klenow) was purchased from Boehringer Mannheim (sequencing grade). Exonuclease deficient large fragment of *E. coli* DNA polymerase I (exo⁻ klenow) (2) was purchased from United States Biochemical. HincII was purchased from New England Biolabs at a concentration of 75 units/μL. Ultra pure human placental DNA (Sigma) was phenol/chloroform extracted and ethanol precipitated to remove Na_2EDTA. Deoxynucleoside

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Table I. Amplification of *M. tb* genomic DNA

Initial *M. tb* genome copies (molecules)	Amplified Product (molecules)	Amplification Factor*	Human DNA (μg)
500000	$>1\times10^{12}$	$>2\times10^{5}$	0.5
50000	$>1\times10^{12}$	$>2\times10^{6}$	0.5
5000	7×10^{11}	1×10^{7}	0.5
500	2×10^{11}	4×10^{7}	0.5
50	2×10^{10}	4×10^{7}	0.5
zero+	4×10^{8}		0.5
100	4×10^{11}	4×10^{8}	0.1
100	7×10^{10}	7×10^{7}	1
100	2×10^{10}	2×10^{7}	10

*Amplification factors were calculated considering there are ~10 copies of the target IS6110 sequence per *M. tb* genome (1).
+Signal due to accidental contamination with the equivalent of ~1 *M. tb* genome copy.

5'-triphosphates (dGTP, dCTP, TTP) and 2'-deoxyadenosine 5'-O-(1-thiotriphosphate) (dATPS) were purchased from Pharmacia. Oligodeoxynucleotides were synthesized on an Applied Biosystems, Inc. 380B instrument and purified by denaturing polyacrylamide gel electrophoresis. *M. tb* cultures were generously provided by Salmon Siddiqi, Becton Dickinson Diagnostic Instrument Systems, Sparks MD. Daryl Shank (Becton Dickinson Research Center) kindly prepared genomic DNA from *M. tb* as previously described (1). Aerosol Resistant Tips (ART) (Continental Laboratory Products) were used to reduce contamination of SDA reactions with previously amplified products.

SDA Reactions

Genomic *M. tb* DNA was serially diluted in 50 mM K_iPO_4, pH 7.4 containing 0.01 or 0.1 μg/μL human placental DNA. SDA was performed in 50 μL reactions containing varying amounts of *M. tb* DNA, 0.1 – 10 μg human placental DNA as indicated, 150 units HincII, 5 units exo$^-$ klenow, 1 mM each dGTP, dCTP, TTP and dATPS, 50 mM K_iPO_4, pH 7.4, 6 mM $MgAcetate_2$, 3% (v/v) 1-methyl 2-pyrrolidinone (NMP) (Sigma), 3% glycerol (from stock enzyme solutions), 500 nM SDA primers S_1 and S_2 and 50 nM primers B_1 and B_2. Prior to addition of HincII and exo$^-$ klenow, reaction samples were heated 4 minutes at 95°C to denature target DNA followed by 1 minute at 37°C to anneal primers. Upon addition of HincII and exo$^-$ klenow, amplification proceeded 2 hours at 37°C and was then terminated by heating 2 minutes at 95°C.

Amplified products were detected using a oligodeoxynucleotide detector probe (Figure 3 and Table I) or SDA primers (Figure 4) which were 5'-^{32}P-labelled using T4 polynucleotide kinase in a 50 μL reaction containing 6.9 mM Tricine (pH 7.6), 50 mM TRIS-HCl (pH 8), 10 mM $MgCl_2$ 5 mM DTT, 2.3 μM γ-^{32}P-ATP (3000 Ci/mmol, 10 mCi/mL), 50 units kinase (New England Biolabs) and either 1 μM oligodeoxynucleotide detector probe or 10 μM SDA primer. ^{32}P-labelling was carried out for 1 hour at 37°C and terminated by heating 2 minutes at 95°C. For Figure 3, aliquots (2 μL) of the ^{32}P-detector probe kinase mix were added to 10 μL aliquots from SDA reactions, and the mixture was heated 2 minutes at 95°C and then 2 minutes at 37°C. The 5'-^{32}P-detector probe was extended to a diagnostic length upon addition of 2 μL of 1 unit/μL klenow and incubation

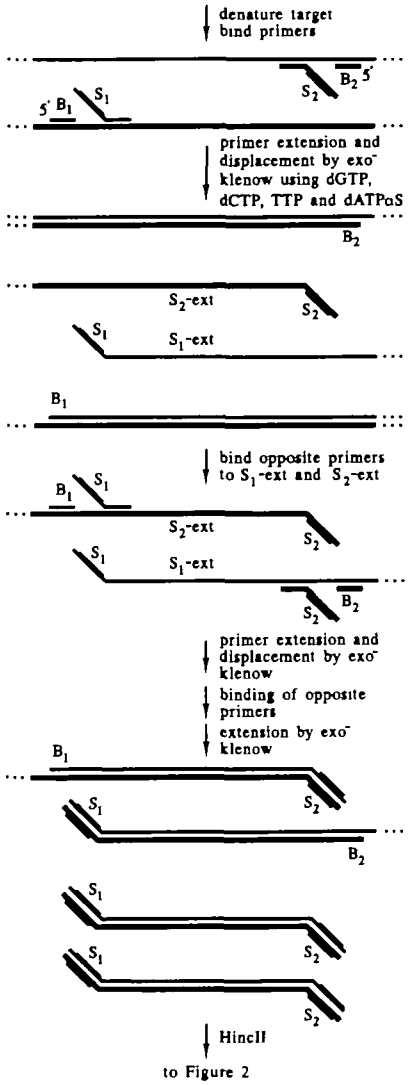


Figure 1. Target generation scheme for SDA. This figure depicts the initial steps in an SDA reaction which transform the original target sequence into the amplification cycle depicted in Figure 2. A target DNA sample is heat denatured. Four primers (B_1, B_2, S_1 and S_2), present in excess, bind the target strands at positions flanking the sequence to be amplified. Primers S_1 and S_2 have HincII recognition sequences (3'GTTGAC) located 5' to the target complementary sequences. The four primers are simultaneously extended by exo$^-$ klenow using dGTP, dCTP, TTP and dATPS. Extension of B_1 displaces the S_1 primer extension product, S_1-ext. Likewise, extension of B_2 displaces S_2-ext. B_2 and S_2 bind to displaced S_1-ext. B_1 and S_1 bind to displaced S_2-ext. Extension and displacement reactions on templates S_1-ext and S_2-ext produce two fragments with a hemiphosphorothioate HincII at each end and two longer fragments with a hemiphosphorothioate *HincII site* at just one end. *HincII nicking* and exo$^-$ klenow extension/displacement reactions initiate at these four fragments, automatically entering the SDA reaction cycle depicted in Figure 2. Sense and antisense DNA strands are differentiated by thin and thick lines. HincII recognition sequences are depicted by (▬).

at 37°C for 10 minutes. For Figure 4, 5'-^{32}P-labelled SDA primers were used directly in amplification reactions. Samples were mixed with an equal volume of 50% (w/v) urea, 20 mM Na_2EDTA, 0.5×TBE (3), 0.05% bromophenol blue/xylene cylanol and analyzed by denaturing gel electrophoresis (10% acrylamide:bis (18:1), 50% (w/v) urea, 0.5×TBE). X-ray film (Kodak X-OMAT AR) was exposed for 30 minutes at room

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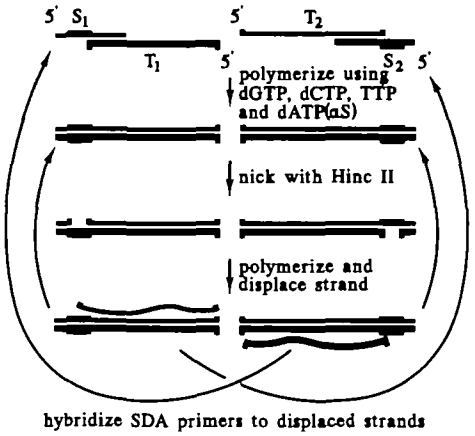


Figure 2. The SDA reaction cycle. These reaction steps continuously cycle during the course of amplification. Present in excess are two SDA primers (S_1 and S_2). The 3'-end of S_1 binds to the 3'-end of the displaced target strand T_1, forming a duplex with 5'-overhangs. Likewise, S_2 binds T_2. The 5'-overhangs of S_1 and S_2 contain the Hinc II recognition sequence (^{5}'GTTGAC). Exo$^-$ klenow extends the 3'-ends of the duplexes using dGTP, dCTP, TTP and dATPS, which produces hemiphosphorothioate recognition sites on $S_1 \cdot T_1$ and $S_2 \cdot T_2$. HincII nicks the unmodified primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. Exo$^-$ klenow extends the 3'-end at the nick on $S_1 \cdot T_1$ and displaces the downstream strand that is equivalent to T_2. Likewise, extension at the nick on $S_2 \cdot T_2$ results in displacement of T_1. Nicking and polymerization/displacement steps cycle continuously on $S_1 \cdot T_1$ and $S_2 \cdot T_2$ because extension at a nick regenerates a nickable HincII recognition site. Target amplification is exponential because strands displaced from $S_1 \cdot T_1$ serve as target for S_2 while strands displaced from $S_2 \cdot T_2$ serve as target for S_1. Sense and antisense DNA strands are differentiated by thin and thick lines. Intact and nicked HincII recognition sequences are depicted by ▬▬ and ▬▮ ▮▬, respectively. The partial HincII recognition sequence ^{5}'GAC and its complement ^{5}'GTC are present at the 5'- and 3'-ends of displaced strands as represented by ▮▬ and ▬▮.

temperature using intensifying screens. ^{32}P-labelled gel electrophoresis bands were excised and quantified by liquid scintillation counting using appropriate gel electrophoresis background controls. The number of amplified product molecules (Table I) was calculated based upon the specific activity of the 5'-^{32}P-detector probe which was measured from aliquots purified by gel electrophoresis. Amplification factors were calculated considering there are ~10 copies of the IS6110 target sequence per *M. tb* genome (1).

RESULTS

A limitation of the original SDA design is the requirement for restriction enzyme cleavage of the target DNA sample prior to amplification (1). The restriction step is necessary to provide target fragments with defined 5'- and 3'-ends. Subsequent binding of the SDA primers to the 3'-ends of these target fragments and extension by exo$^-$ klenow create hemiphosphorothioate HincII recognition sites from which amplification initiates.

We have developed a new method of target generation which eliminates the need for restriction enzyme cleavage of the target prior to SDA (Figure 1). The target DNA sample is heat denatured in the presence of an excess of 4 primers. Two primers (S_1 and S_2) are typical SDA primers, with target binding regions at their 3'-ends and HincII recognition sequences (^{5}'GTTGAC) located 5' to the target complementary sequences. The other two primers (B_1 and B_2) consist simply of target binding regions. S_1 and S_2 bind to opposite strands of the target at positions flanking the sequence to be amplified. B_1 and B_2 bind upstream of S_1 and S_2, respectively. Exo$^-$ klenow (present in excess over the number of target sequences) simultaneously extends all 4 primers using dGTP, dCTP, TTP and dATPαS. Extension of S_1 and S_2 forms two products, S_1-ext and S_2-ext. Extension of B_1 and B_2 results in displacement of S_1-ext and S_2-ext from the original target templates. Displaced and single-stranded S_1-ext then serves as target for primers S_2 and B_2. Likewise, displaced S_2-ext is target for S_1 and B_1. All four primers are extended on templates S_1-ext and S_2-ext. Combined extension and displacement steps produce 2 double-stranded fragments with hemiphosphorothioate HincII recognition sites at each end and two longer double-stranded fragments with a hemiphosophorothioate HincII site at only one end. In the presence of HincII (which can be added at the top of Figure 1 along with exo$^-$ klenow), the four target fragments at the bottom of Figure 1 automatically enter the strand displacement amplification cycle (Figure 2), where HincII nicks the hemiphosphorothioate recognition site, and exo$^-$ klenow extends the 3'-end at the nick while displacing the downstream strand that in turn serves as target for the opposite SDA primer. These steps, repeated continuously over the course of the reaction, produce exponential growth in the number of target sequences. 10^7-Fold amplfication (Table I) theoretically derives from ~23 repetitions or cycles of the steps in Figure 2 ($2^{23} \approx 10^7$). Each displaced strand in Figure 2 contains the sequence ^{5}'GAC at the 5'-end and the complement ^{5}'GTC at the 3'-end. ^{5}'GAC is the portion of the HincII recognition sequence located 3' to the nick site.

The transition between Figures 1 and 2 may become clearer with a detailed account for the last double-stranded fragment at the bottom of Figure 1. If HincII nicks the hemiphosphorothioate site on the left end of the fragment containing the S_1 primer sequence, exo$^-$ klenow initiates replication at the nick and displaces the downstream strand containing ^{5}'GAC at the 5'-end and the full complement of the primer S_2 sequence at the 3'-end. S_2 binds to this displaced strand, and exo$^-$ klenow extends S_2 forming the double-stranded fragment depicted as the product of the step labelled 'polymerize using dGTP, dCTP, TTP and dATP(αS)' on the right side of Figure 2. Similar reactions occur with the entire set of fragments depicted at the bottom of Figure 1.

Duplexes containing two HincII sites in Figure 1 may also undergo more complex reactions in which the two restriction sites are nicked simultaneously. Following dissociation of HincII from the nicked sites, strand extension/displacement may proceed from one or both sites. Although we have not yet determined the relative importance of these different pathways, all such processes ultimately lead to amplifiable species found in Figure 2.

Amplification of *M. tb* DNA

A set of samples containing varying amounts of target genomic *M. tb* DNA and the indicated amounts of human DNA was subjected to SDA. The two SDA primers (S_1 and S_2) contain target binding regions at their 3'-ends that are complementary to opposite strands of the IS6110 sequence of *M. tb* (4) at nucleotide positions 972–984 and 1011–1023 {S_1 = ^{5}'dTTGAATAGTCGGTTACTT*GTTGAC*GGCGTACTCGACC, S_2 = ^{5}'dTTGAAGTAACCGACTATT*GTTGAC*ACTGAGATCCCCT}. (HincII recognition sites are italicized). The two outside primers (B_1 and B_2) bind to opposite strands at nucleotide positions 954–966 and 1032–1044 {B_1 = ^{5}'dTGGACCCGCCAAC, B_2 = ^{5}'dCGCTGAACCGGAT}. After the SDA

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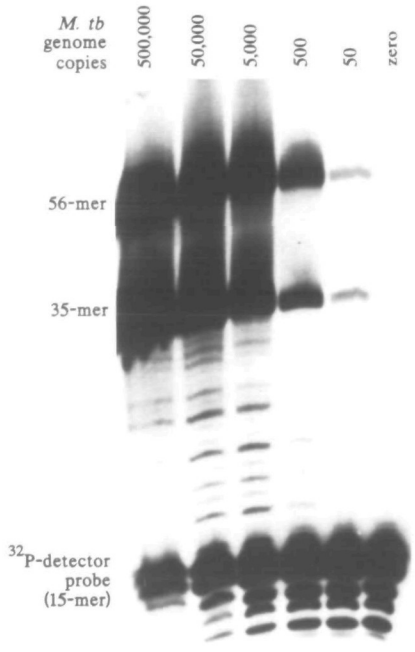


Figure 3. Denaturing gel electrophoresis analysis of SDA reactions using a 5′-^{32}P-labelled detector probe. SDA products were detected by extension of a 5′-^{32}P-labelled probe (15-mer) to a length of 35 or 56 nucleotides. Aliquots (10 μL) from each 50 μL SDA reaction were analyzed. The number of *M. tb* genome copies present in each 50 μL reaction is shown above each lane. Each sample contained 0.5 μg of human DNA. Longer autoradiography exposures reveal target specific bands in the zero target lane. Band intensities are nearly constant for samples containing >5000 intial targets due to the limited amount of ^{32}P-labelled detector probe. Unextended ^{32}P-labelled detector probe appears as multiple bands due to degradation by the 3′-5′ exonuclease activity of klenow used in the detection step. DNA polymerase extension reactions of the 5′-^{32}P-probe were not allowed to proceed below 37°C in order to minimize background detection reactions.

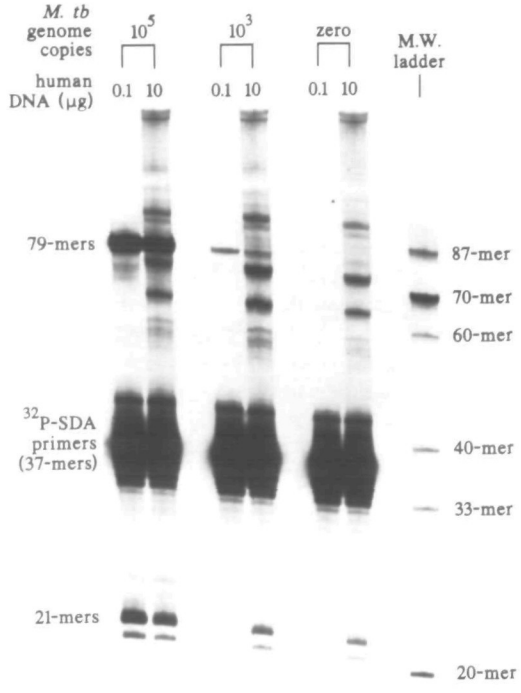


Figure 4. Denaturing gel electrophoresis analysis of SDA reactions using 5′-^{32}P-labelled SDA primers. SDA reactions were performed using 5′-^{32}P-labelled SDA primers (S_1 and S_2) in the presence of 0.1 or 10 μg of human DNA. Aliquots (10 μL) from each 50 μL SDA reaction were analyzed. The number of *M. tb* genome copies present in each 50 μL reaction is shown above each lane. Unreacted 5′-^{32}P-labelled SDA primers are 37-mers. Target specific 5′-^{32}P-labelled amplification products are a set of 21- and 79-mers. Background reaction products appear as a high molecular weight smear and more distinct bands over 55- to 110-mers. The 21-mer bands also derive in part from background reactions (see text). Reactions were not allowed to proceed below 37°C in order to minimize background reactions.

reaction is terminated, all species shown in Figure 2 are present and detectable. In the present study, amplified target fragments were detected by DNA polymerase extension of a 5′-^{32}P-labelled probe (5′-^{32}P-dCGTTATCCACCATAC) (IS6110 nucleotide positions 992−1006) that is complementary to an internal segment of the target sequence. The 5′-^{32}P-labelled detector probe is extended to a length of either 35 or 56 nucleotides when hybridized to one or the other of the following two strands produced during SDA: 5′d*GAC*ACTGAGATCCC-CTATCCGTATGGTGGATAACGTCTTTCAGGTCGAGT-ACGCC*GTC* and 5′dTTGAATAGTCGGTTACTT*GTTGAC*A-CTGAGATCCCCTATCCGTATGGTGGATAACGTCTTTC-AGGTCGAGTACGCC*GTC*. (Partial and complete HincII recognition sequences are italicized). The first strand is one of the strands displaced during SDA and corresponds to IS6110 nucleotide positions 972−1023 with 5′GAC and 5′GTC appended to the 5′- and 3′-ends. The second longer strand is the same strand before nicking by HincII and as such has a 5′-end identical to primer S_1.

Results from amplification of genomic *M. tb* DNA are shown in Figure 3 and Table I. The amount of amplified product is dependent upon the number of initial targets. (However, levels of amplified product are nearly constant for >5000 initial *M. tb* genome copies due to the limited amount of the ^{32}P-detector probe used.) Longer autoradiography exposures reveal target specific ^{32}P-bands in the zero target lane of Figure 3; we estimate each sample is contaminated with the equivalent of ~1 genome copy of *M. tb*. Although this contamination problem hinders accurate determination of sensitivity, SDA enables ^{32}P-detection of as few as 10−50 initial target DNA molecules (1−5 genome copies containing ~10 copies of the target IS6110 sequence) (Table I and unpublished observations).

Background Amplification

As previously discussed (1), background amplification reactions compete with target specific amplification and are more prevalent at higher non-target (human) DNA concentrations. The sensitivity of SDA therefore depends upon the amount of non-target DNA present (Table I). Background reactions presumably begin with extension of primers spuriously bound to non-target sequences. In processes similar to those depicted in Figure 1, these background extension products are then displaced and subsequently hybridized to a second SDA primer, which is in turn extended and displaced. In background reactions, these initial priming events will generally involve imperfectly paired sequences and therefore should occur far less efficiently than the carefully designed priming/extension reactions leading to bona-fide target generation. However, once a non-target sequence is attached to two primer-derived nickable HincII sites (see bottom of Figure 1), it will undergo amplification as effectively as the genuine target sequence.

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To examine the background reactions, SDA was performed using 5′-^{32}P-labelled primers (S_1 and S_2) at various target levels and in the presence of either 0.1 or 10 μg of human DNA (Figure 4). Excess unreacted 5′-^{32}P-labelled SDA primers are 37-mers. Target specific SDA products appear as two ^{32}P-21-mers and a set of four ^{32}P-79-mers. The 21-mers are the nucleotide sequences located 5′ to the HincII nicking sites on the SDA primers (the short strands indicated in Figure 2 above the step labelled 'polymerize and displace strand'). The set of four 79-mers are the unnicked amplification strands shown in Figure 2 above the step labelled 'nick with HincII'. In Figure 4, background amplification products appear only in lanes for reactions containing high (10 μg) human DNA, where they run as a high molecular weight smear and more distinct bands ranging in size from 55−110 nucleotides. The ~43-mer bands, which are present in all lanes in Figure 4, appear in the absence of HincII and as such are not SDA background reaction products (e.g., 'primer-dimers'). (The 43-mer probably represents exo$^-$ klenow extension of a minor hairpin conformation of primer S_1.) The 21-mer bands in Figure 4 also arise from background reactions as well as from target amplification because they derive from the target-independent sequences at the 5′-ends of the SDA primers; notice that 21-mers are apparent in the zero target sample with 10 μg of human DNA although no target specific 79-mers are discernible.

The apparent lack of background products in the low (0.1 μg) human DNA lanes (Figure 4) supports the hypothesis that background reactions arise from spurious amplification of human DNA sequences. Background amplification predominates in reaction mixtures containing high human DNA and low numbers of initial target sequences ($< 10^4$ genome copies). Under these conditions, target amplification proceeds efficiently for only ~2 hours before high levels of background product accumulate and dramatically reduce target amplification by competing for limiting components like HincII. The more target copies initially present, the more target-specific amplification that can occur before essential components are depleted. Consequently, in reactions containing low initial target numbers, the final level of amplified target depends on the initial copy number (1). At very high initial target levels ($> 10^6$ *M. tb* genome copies), however, background reactions are attenuated by target amplification, which predominates under these conditions, and the final level of amplified target is independent of the number of targets initially present (data not shown).

DISCUSSION

We have streamlined the original SDA experimental protocol by eliminating the need to cleave a target DNA sample by a restriction enzyme(s) prior to amplification (1). The new target generation scheme uses 4 primers in a concerted series of DNA polymerase extension and displacement reactions. Although the overall reaction mechanism of SDA appears complex as depicted in Figures 1 and 2, individual reaction steps are invisible to the experimenter. In fact, the experimental protocol consists simply of 1) heat denaturing a target DNA sample in the presence of primers and other reagents 2) addition of HincII and exo$^-$ klenow, and 3) incubation at 37°C.

This new target generation scheme offers a number of advantages over the previous method of generating target fragments by restriction enzyme cleavage (1). Amplification is enhanced because a larger number of hemiphosphorothioate HincII sites is generated from each original double-stranded target. The previous method of target generation produces two double-stranded fragments each with a hemiphosphorothioate HincII site at one end (1), whereas the current method produces four double-stranded fragments with a total of six hemiphosphorothioate HincII sites (Figure 1). In addition, target sequences can now be chosen without regard for convenient flanking restriction sites. Furthermore, SDA can now be applied to either double- or single-stranded target DNA target samples. Previously, targets generally had to be double-stranded for target generation by restriction enzyme cleavage. SDA is now compatible with sample preparation methods which render target genomic DNA single-stranded prior to amplification.

Primers B_1 and B_2 in Figure 1 can also be typical SDA primers containing target binding regions at their 3′-ends and HincII recognition sites located toward their 5′-ends. The result is an enhancement of amplification from additional nicking and extension/displacement reactions at these outside hemiphosphorothioate HincII sites (data not shown). Displaced strands from these outside sites serve as target for both opposite primers.

Performance of SDA with just one SDA primer (S_1 or S_2) generates single-stranded displaced strands in a linear amplification mode. In a related sense, SDA could be performed with an excess of one SDA primer over the other (e.g., $[S_1] >> [S_2]$) which should produce exponential amplification of a target with a predominance of one amplified strand over the other in a manner analogous to 'asymmetric' PCR. In either case, the resultant single-stranded displaced strands could serve as either detector probes (e.g., Southern blots) or templates for DNA sequence analysis.

The ability of SDA to amplify low target numbers at 37°C is quite remarkable when compared with PCR, which achieves exquisite sensitivity only when non-specific priming reactions are diminished by stringent (high temperature) reaction conditions (5). Although the absence of temperature cycling in SDA reduces the number of recurrent opportunities for errant hybridization and primer extension, considerable non-specific priming surely occurs at the 37°C reaction temperature. However, initiation of SDA for any sequence (target or background) must take place via multiple steps similar to those shown in Figure 1. The efficiency of this concerted series of events will be greatly diminished if the primer extension and strand displacement steps must wait for the adventitious, nonspecific hybridization of primers, as will generally be the case for background reactions. Once non-target amplicons are generated through this inefficient process, however, they will be amplified as readily as bona-fide target sequences. Consequently, significant accumulation of background product can result if non-target DNA is present in large excess over specific target sequences. To reduce the potential for these background reactions, it is important to maintain the highest level of stringency compatible with enzyme stability. Accordingly, SDA reactions are not allowed to proceed at temperatures less than 37°C, and we increased stringency by adding organic co-solvents to the reaction. With these modifications to our original reaction conditions (1), we can now amplify specific target sequences $> 10^7$-fold even in the presence of as much as 10 μg of human DNA per 50 μL reaction.

SDA and PCR are affected by common experimental parameters (e.g., target length, target dG·dC content, primer sequences, and organic solvents such as 1-methyl 2-pyrrolidinone, glycerol and formamide). Because it operates under conditions

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of low stringency (37°C) through a strand displacement mechanism, SDA is especially sensitive to experimental parameters. For example, SDA amplification factors decrease ~ 10-fold for each 50 nucleotide increase in target length, over a range of 50 to 200 nucleotides (unpublished observations); this may be related to the non-processive nature of the polymerase. In addition, the best choice for an organic solvent and its optimal concentration appear to depend on the particular sequence to be amplified (unpublished observations); a literature survey suggests this is also true for PCR. As with PCR, care must be taken to choose primer sequences that do not support 'primer-dimer' amplification reactions, which can arise in SDA reactions by the hybridiztion of primer sequences 3′ to their HincII recognition sites.

The scheme in Figure 1 represents a general and convenient method of producing double-stranded fragments with defined 5′- and 3′-ends as illustrated by the following potential applications: 1) The HincII recognition sequences in S_1 and S_2 could be substituted by any restriction sites in order to produce fragments for ligation into a cloning vector. (The scheme in Figure 1 can be performed with dATP instead of dATPS.) 2) The HincII recognition sequences in S_1 and S_2 could be substituted by RNA polymerase promoters for production of transcription templates. 3) The technique could be applied to synthesis of double-stranded cDNA using a 5′-3′ exonuclease deficient reverse transcriptase possessing strand displacement ability (does not require a reverse transcriptase with RNase H activity).

Various isothermal DNA amplification techniques require formation of a double-stranded DNA fragment containing terminal sequences specific for the given amplification technique. For example, the self-sustained sequence replication (3SR) system produces a double-stranded DNA fragment with a bacteriophage RNA polymerase promoter at one or both ends (6). Such a fragment can be generated from target DNA through 2 cycles of PCR using primers containing promoter sequences at their 5′-ends. Alternatively, one can first cleave target DNA with a restriction enzyme(s), creating a fragment with defined 5′- and 3′-ends to which 3SR primers can bind after denaturation in a manner analogous to the original target generation scheme of SDA (1). (It should be noted that 3SR can directly amplify an RNA target sequence without these target generation steps.) Alternatively, the initial steps for 3SR amplification of target DNA could proceed according to the target generation scheme in Figure 1 by simply substituting the HincII sites in S_1 and S_2 with promoter sequences. This target generation scheme could also be applied to amplification by Q-beta technology involving formation of 'target-dependent replicatable RNAs' (7) or to amplification techniques using terminal origins of replication for the bacteriophage Phi 29 (8,9).

The scheme in Figure 1 illustrates the possibility of performing PCR reactions with multiple primers and a DNA polymerase lacking 5′-3′ exonuclease activity and possessing strand displacement ability. The procedure would simply consist of repeated cycles of the steps in Figure 1 using 'nested' primer sets and an intervening heat denaturation step between cycles. Although a possible increase in background amplification must be considered, the use of multiple primer sets should enhance PCR especially during early cycles. For example, two nested primer sets (analogous to B_1, B_2, S_1 and S_2) should produce 6-fold amplification for the first cycle, although the amplification factor converges toward a value of 2-fold at later cycles as the shorter product prevails. In addition to exo$^-$ klenow, the thermophilic large fragment of DNA polymerase I from *Bacillus stearothermophilus* (Bio-Rad) strand displaces and lacks 5′-3′ exonuclease activity.

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