Empirical Aspects of Strand Displacement Amplification

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Strand displacement amplification (SDA) is an isothermal, in vitro DNA amplification technique that is based on the ability of a restriction enzyme to nick the unmodified strand of a hemiphosphorothioate form of its recognition site and the ability of a DNA polymerase to initiate replication at the nick and displace the downstream nontemplate strand. Primers containing recognition sites for the nicking restriction enzyme bind to opposite strands of target DNA at positions flanking the sequence to be amplified. The target fragment is exponentially amplified by coupling sense and antisense reactions in which strands displaced from the sense reaction serve as a target for the antisense reaction and vice versa. A detailed mechanistic description of SDA using the restriction enzyme *HincII* and an exonuclease-deficient form of the Klenow fragment of *Escherichia coli* DNA polymerase I (exo⁻ Klenow) is presented in Figure 1 and discussed elsewhere.^(1,2) Although the series of events depicted in Figure 1 may appear complicated, SDA operates by a very simple protocol: Target DNA is heat-denatured in the presence of all reagents except exo⁻ Klenow and *HincII*. Amplification then proceeds at ~37°C after cooling and addition of the enzymes.

Past publications on SDA^(1,2) presented data for a target sequence from *Mycobacterium tuberculosis*. Because DNA from *M. tuberculosis* is not readily available to most researchers, this review presents amplification of pBR322 so that the experiments can be repeated easily in any laboratory. The purpose of this review is to discuss empirical aspects of SDA using the pBR322 data as a guide for development of other SDA target systems.

AMPLIFICATION OF pBR322

Primer pairs were designed to amplify four regions of pBR322, ranging in length from 52 to 198 bp (Fig. 1; Tables 1 and 2). We chose target sequences that are not present in popular cloning vectors (e.g., pUC19) to alleviate the problem of contaminating target DNA in the laboratory and enzyme preparations. *S*₁ was paired with either *S*_{2a}, *S*_{2b}, *S*_{2c}, or *S*_{2d} so that the four target sequences would have one end in common. Single versions of *B*₁ and *B*₂ were used with all four target fragments by designing them to bind at target locations 5' to those of *S*₁ and *S*_{2d}. SDA reaction conditions were optimized for each target sequence with regard to primer sequences, organic solvents, and enzyme concentrations.

A series of amplification reactions was performed with varying amounts of target DNA from pBR322. In the case of all four target sequences, the level of amplified product is directly related to the initial amount of target (Fig. 2). This dose-response is a result of competition between target-specific and background amplification.^(1,2) Regardless of initial target level, amplification proceeds until total amplified products (both target-specific and background) reach a level at which further exponential amplification is attenuated because *HincII* is no longer in excess.⁽³⁾ Consequently, each sample in Figure 2 for a given target length exhibits a constant amplification factor independent of initial target concentrations. Background amplification increases with increasing amounts of nontarget DNA (human DNA in the present study), which means amplification factors decrease with increasing amounts of nontarget DNA.²

Samples containing no input target DNA exhibit target-specific bands in Figure 2 as a result of accidental contamination with ~10 amplicons (amplification product molecules from previous reactions), a common problem with sensitive amplification techniques. Target-specific products were not detected in a sample that was heat-inactivated immediately after addition of *HincII* and exo⁻ Klenow.

Amplification factors decrease dramatically with increasing target length (Table 2). The strand displacement ability of exo⁻ Klenow is expected to decrease with progressively longer targets. However, amplification factors decrease between 5- and 100-fold for each 50-bp increase in target length. Obviously, sequence, and not just length, is important. Within these target fragments, there may be local sequences that attenuate strand displacement replication. For example, Kong et al.⁽⁴⁾ reported that a DNA polymerase from *Thermococcus litoralis* halts replication at fairly specific sequence positions under strand displacement conditions. Alternatively, polymerases can switch from replicating the template strand to replicating the displaced strand,⁽⁵⁾ which produces a specific stop when the polymerase reaches the 5' end of the displaced strand. We did not search for potential replication termination sites in these pBR322 sequences.

In addition, primer pairs can have short regions of homology that may allow one primer to be extended on the other, forming a primer-dimer. If this background priming occurs between *S*₁ and *S*₂ at positions 3' to the *HincII* site of each primer, a primer-dimer will be formed that is exponentially amplified

FIGURE 1 Schematic representation of SDA. The target DNA sample is heat-denatured in the presence of an excess of four primers (B₁, B₂, S₁, and S₂). S₁ and S₂ contain target-binding regions at their 3' ends. Each also has a recognition site (5'-GTTGAC) for the restriction enzyme *HincII* located immediately 5' to its target-binding region. S₁ and S₂ bind to opposite strands of the target sequence flanking the region to be amplified. B₁ and B₂ consist simply of target-binding sequences and bind at positions 5' to those of S₁ and S₂. After the primers anneal at 37–40°C, *HincII* is added along with an exonuclease-deficient form of the Klenow fragment of *E. coli* DNA polymerase I (exo⁻ Klenow). Exo⁻ Klenow, which is present in excess over the number of target sequences, simultaneously extends all four primers using dGTP, dCTP, TTP, and dATPaS. A cascade of steps follows. S₁ and S₂ are extended and then displaced during extension of B₁ and B₂. The displaced extension products of S₁ and S₂ (S₁-ext and S₂-ext) serve as targets for the opposite primers. Further rounds of extension and displacement produce two fragments with a hemiphosphorothioate *HincII* site 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 reaction cycle at the bottom. During each cycle, the 3' end of S₁ binds to the 3' end of the displaced target strand T₁, forming a duplex with 5' overhangs. Likewise, S₂ binds to T₂. Exo⁻ Klenow extends the 3' ends of the duplexes producing hemiphosphorothioate recognition sites on S₁·T₁ and S₂·T₂. *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 S₁·T₁ and displaces the downstream strand that is equivalent to T₂. Likewise, extension at the nick on S₂·T₂ results in displacement of T₁. Nicking and extension/displacement steps cycle continuously on S₁·T₁ and S₂·T₂ because extension at a nick regenerates a nickable *HincII* recognition site. Target amplification is exponential because strands displaced from S₁·T₁ serve as target for S₂ whereas strands displaced from S₂·T₂ serve as target for S₁. Sense and antisense strands are differentiated by thin and thick lines. Intact and nicked *HincII* recognition sequences are depicted by and . 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 .

because it contains a nickable *HincII* site at each end. These primer-dimers amplify very efficiently owing to their short length, thereby attenuating target-spe-

cific amplification, especially for longer target sequences. Some of the S₁ and S_{2n} pairs in Table 2 may form primer-dimers more readily, which may also contribute

to the lack of a smooth trend in the amplification factors as a function of target length.

Primer-dimers other than that be-

TABLE 1 SDA Primer Sequences for Amplification of pBR322

Primer	Sequence ^a	pBR322 hybridization (nucleotide positions)
S ₁	5'-dTACAATAGTCCCAATCTTGTTGACGTTCCAGTAACC	1801–1813
S _{2a}	5'-dTACAAGTAACCGACATTTGTTGACGGATGCTCACGATA	1852–1839
S _{2b}	5'-dTACAAGTAACCGACTATTGTTGACGGAATTTCTGTTCA ^b	1896–1883
S _{2c}	5'-dTGAAGTAACCGACTATTGTTGACGGCCATGTTAAGG	1950–1938
S _{2d}	5'-dTGAAGTAACCGACTATTGTTGACTCGTTGAGTTTCT	1998–1985
B ₁	5'-dCCAGTTGTTTACCCCT	1781–1795
B ₂	5'-dGCCTGTTTCATCCG	2018–2006

^a*HincII* recognition sites are underscored.^bSee text for alternative sequence.

tween S₁ and S₂ are not efficiently amplified. For example, a primer–dimer between S₁ and B₁ contains only a single *HincII* site and, as such, is amplified in only a linear mode instead of exponentially. Furthermore, although a primer–dimer between S₁ and S₁ (and likewise between S₂ and S₂) contains a nickable *HincII* site at each end, it is not expected to be exponentially amplified in an efficient manner because each displaced strand can form a hairpin conformation that hinders its capture by a new S₁ primer during each cycle of SDA (Fig. 1, bottom).

Inclusion of an organic solvent enhances amplification efficiency. Dimethyl sulfoxide (DMSO) is the optimal choice of organic solvent for all four pBR322 target sequences, although nearly equivalent results were obtained with slightly higher glycerol concentrations. Interestingly, the optimal DMSO concentration increases with increasing target length (Table 2). DMSO is expected to affect primer hybridization, strand displacement efficiency, and general enzyme activity. There is no clear trend in calculated melting temperatures (T_m)⁽⁶⁾ for S_{2a}–S_{2d}, suggesting that opti-

mal DMSO concentrations are not exclusively related to primer hybridization stability. The DMSO trend may reflect offsetting factors affecting strand displacement activity and general enzymatic activity; for example, *exo*[−] Klenow may strand-displace more efficiently at higher DMSO concentrations, but the activity of *HincII* may decrease at higher DMSO concentrations. Consequently, amplification of the longer targets may benefit from enhanced strand displacement even though *HincII* activity is reduced.

The optimal *exo*[−] Klenow concentration for each of the target sequences increases with increasing target length (Table 2). Klenow is not highly processive, replicating only ~8 nucleotides per initiating event.⁽⁷⁾ When an *exo*[−] Klenow molecule dissociates from a template midstream during replication, another polymerase must bind quickly before the partially displaced downstream strand reanneals to the template. Therefore, it is not surprising that higher *exo*[−] Klenow concentrations favor strand displacement replication of longer targets. An extremely simple model predicts that an infinitely high *exo*[−] Klenow concen-

tration should be optimum regardless of target fragment length. However, *exo*[−] Klenow concentrations higher than those indicated in Table 2 decrease amplification, probably because they promote mispriming and subsequent background amplification that competes with target amplification. Very high levels of *exo*[−] Klenow may also inhibit amplification by binding nonspecifically to target DNA.

All of the pBR322 amplification reactions were performed with 150 units of *HincII*. Higher levels of *HincII* were not pursued because of the inhibitory cost of the reagent. In the case of the 52-mer target, amplification factors decrease only 15% and 57% when the *HincII* level is reduced to 100 and 50 units, respectively for a 2-hr reaction using 3.5 units of *exo*[−] Klenow (data not shown). No attempt was made to reoptimize the *exo*[−] Klenow concentration with lower *HincII* levels. Obviously, reasonable SDA results can be obtained with substantially lower *HincII* concentrations.

GENERAL EMPIRICAL ASPECTS OF SDA

Primer and Target Sequences

The sequences of S₁ and S₂ dramatically affect amplification efficiency. S₁ and S₂ usually have 11- to 14-nucleotide target-binding regions with calculated T_m of 40–50°C for 0.5 μM primer in 100 mM NaCl.⁽⁶⁾ This length ensures stringency at 37–42°C in the presence of organic solvents. *Exo*[−] Klenow does not extend efficiently off primers with shorter target-binding regions. Longer target-binding regions on S₁ and S₂ promote background amplification owing to mispriming on non-target DNA under the relatively low stringency conditions of SDA (37°C). As mentioned previously, the 3'-ends of S₁ and S₂ must not bind, forming primer–dimers, because they are amplified efficiently at the cost of target amplification.

The 5' sequences of S₁ and S₂ are not critical to performance. They simply must be long enough and thermodynamically stable enough to provide a priming site for *exo*[−] Klenow after nicking by *HincII*. However, it is important that the 5' sequences do not fortuitously promote background hybridization that competes with target-specific annealing. Historically, we either chose 5' se-

TABLE 2 SDA Conditions for Amplification of pBR322

Primers ^a	Percent DMSO (vol/vol) ^b	<i>exo</i> [−] Klenow (units)	Target length (base pairs) ^c	Amplification factor	Duration of SDA (hr)
S ₁ and S _{2a}	6	3.5	52	4.5 × 10 ⁸ -fold	2
S ₁ and S _{2a}	6	3.5	52	8.1 × 10 ⁸ -fold	3
S ₁ and S _{2b}	10	6	96	1.4 × 10 ⁷ -fold	3
S ₁ and S _{2c}	12	8	150	2.9 × 10 ⁶ -fold	3
S ₁ and S _{2d}	15	10	198	3.0 × 10 ⁴ -fold	3

^aAll reactions also contained B₁ and B₂.^bEach reaction also contained 3% (vol/vol) glycerol contributed by the enzyme stock solutions.^cDistance between target binding regions of S₁ and the indicated version of S₂.

FIGURE 2 SDA of four pBR322 target sequences. The length of each pBR322 target fragment is indicated above each set of reactions. The initial number of pBR322 molecules present in each aliquot analyzed by gel electrophoresis is indicated above each lane. Reactions were performed generally as described previously⁽²⁾ and contained 150 units of *HincII* (New England Biolabs), 1 mM each dGTP, dCTP, TTP, and dATP α S, 50 mM K₂PO₄ (pH 7.4), 6 mM MgCl₂, 3% (vol/vol) glycerol (from stock enzyme solutions), 0.1 mg/ml of BSA (acetylated, New England Biolabs), 50 ng of human placental DNA, 500 nM each S₁ and S₂, 50 nM each B₁ and B₂, and the indicated amounts of DMSO (Table 2), *exo*⁻ Klenow (Table 2), and *EcoRI*-digested pBR322. (A 1- μ g/50 μ l stock solution of pBR322 was linearized with *EcoRI* to facilitate primer binding.) Samples were heat-denatured for 2 min at 95°C, and *HincII* and *exo*⁻ Klenow were added after the samples were incubated for ~2 min at 37°C. After amplification for 3 hr at 37°C, samples were heat-inactivated for 2 min at 95°C. (Δ) A “no-amplification” sample that was treated for 2 min at 95°C immediately after addition of *exo*⁻ Klenow and *HincII*. An aliquot (5 μ l) from each reaction was mixed with 7 μ l of 50 mM K₂PO₄ (pH 7.4); 1 mM each dGTP, dCTP, TTP, and dATP α S; 0.1 mg/ml of BSA; 6 mM MgCl₂; 0.3 μ M 5'-³²P-probe (5'-dGTTACTGATGATGAAC); 50 mM Tris (pH 8); and 10 mM MgCl₂. [The ³²P-probe (1 μ M) was labeled with 50 μ l of 50 mM Tris (pH 8), 10 mM MgCl₂, 50 units of T4 polynucleotide kinase (New England Biolabs), and 350 μ Ci of [γ -³²P]ATP (New England Nuclear) and was used without subsequent purification.] The ³²P-probe anneals to amplified strands containing the S₁ sequence at their 5' ends (pBR322 nucleotide positions 1835–1820). Samples were then heated for 2 min at 95°C followed by 2 min at 37°C. An aliquot of 2 μ l of 0.5 U/ μ l of Klenow (Boehringer Mannheim, sequencing grade) was added, and the samples were incubated for 10 min at 37°C. Samples were mixed with 14 μ l of 50% (wt/vol) urea, 20 mM Na₂EDTA, and 0.05% bromophenol blue/xylene cyanol, and were analyzed by 8% denaturing gel electrophoresis (Acryl-a-Mix 8 from Promega). X-ray film (Fuji RX Film) was exposed for the indicated times using intensifying screens. Exposures >2 min were performed at –70°C. Amplification factors (Table 2) were estimated as described previously.⁽²⁾ Regardless of which version of S_{2n} is paired with S₁, two ³²P-labeled extension products are produced, (indicated by arrows). A 38-mer product (lower band) results from extension of the ³²P-labeled detector probe on a strand containing a nicked *HincII* site, whereas the upper 58-mer band corresponds to a target strand with an unnicked *HincII* site (target strand with a full-length S₁ sequence at its 5' end).

quences that did not form obvious inter- or intraprimer secondary structures or we designed S₁ and S₂ to anneal specifically to one another at their 5'-ends.^(1,2) S' Homology was designed to block potentially damaging secondary structures, although it does not necessarily protect S₁ and S₂ from pernicious hairpin or duplex formation.

The sequences of B₁ and B₂ are not critical to amplification efficiency. However, they must not anneal to any of the other four primers (B₁, B₂, S₁, or S₂) in a manner that attenuates target-specific hybridization.

The target sequence must not naturally contain recognition sites for the nicking restriction enzyme used in SDA. Fortunately, *HincII* appears to retain specificity for its exact recognition sequence, even under SDA conditions containing organic solvents that are known to promote cleavage at sequences closely

resembling recognition sites (“star” sites). (These four pBR322 target sequences contain a number of potential star *HincII* sites.) Therefore, only exact *HincII* recognition sites in the target sequence are of concern. However, it is important to search for all four *HincII* sites (5'-GTPyPuAC).

Primer Concentrations

S₁ and S₂ concentrations of 500 nM ensure target binding on a second time scale. Lower concentrations render target hybridization rate limiting during each SDA cycle (Fig. 1, bottom). Higher concentrations needlessly increase the potential for background amplification. B₁ and B₂ are typically used at lower concentrations (50 nM) to ensure that they do not anneal and extend before S₁ and S₂. During the entire SDA incubation pe-

riod, B₁ and B₂ extend only twice at the beginning.

Organic Solvents

Organic solvents generally destabilize nucleic acid hybridization. Inclusion of organic solvents enhances amplification, probably because it raises the stringency of primer hybridization and facilitates strand displacement. Favorable results are usually obtained with 2–5% 1-methyl 2-pyrrolidinone, 5–20% glycerol or 5–20% DMSO. Adequate results can generally be obtained with either of the three organic solvents over a broad concentration range. However, the optimal choice of organic solvent is target dependent.

Enzyme Sources and Concentrations

Exo⁻ Klenow is commercially available only from U.S. Biochemical at stock con-

centrations of 5–10 U/ μ l. We purchase *HincII* at high concentration (50–75 U/ μ l) from New England Biolabs and Life Technologies, Inc. *HindII*, which is identical to *HincII* except from a different strain of *Haemophilus influenzae*, is available from Boehringer Mannheim at >40 U/ μ l. The unit activity of these enzymes may vary between lots, so a quick concentration optimization experiment should be performed with each new lot.

The ratio of exo^- Klenow to *HincII* is important in addition to the absolute concentration of each. After *HincII* dissociates from a nicked site, exo^- Klenow should extend the nick; *HincII* binding to the nicked site would be unproductive. Six units of exo^- Klenow and 150 units of *HincII* (the average concentrations used in this study) correspond to $\sim 10^{12}$ and 10^{13} molecules. An extremely simple mechanistic model predicts that a 1 : 10 ratio of exo^- Klenow to *HincII* would not ensure that exo^- Klenow binds first to the nicked site following *HincII* dissociation. Because exo^- Klenow is not expected to have a particular affinity for an unnicked *HincII* recognition sequence, one might expect that high exo^- Klenow/*HincII* ratios would be optimal were it not for increased background amplification at high exo^- Klenow levels. The exo^- Klenow concentration must also not be so high that it nonspecifically blocks *HincII* access to its recognition site. Obviously, complicated factors must be considered to explain optimal polymerase and restriction enzyme concentrations.

Exo^- Klenow tends to add extra nucleotides to the end of duplex DNA (terminal transferase activity).⁽⁸⁾ Addition of extra nucleotides is a particular concern in SDA because displaced strands must be subsequently extended on the primer (S_1 or S_2) that captures it. If a displaced strand contains an extra 3' nucleotide, it will base-pair with the underlined T (T) in the *HincII* site 5'-GTTGAC on S_1 or S_2 . In the presence of dGTP, dCTP, TTP, and dATP α S, exo^- Klenow adds dA α S and dG with approximately equal tendency to the ends of duplex DNA.⁽³⁾ An extra 3'-dA α S on the displaced strand will naturally base-pair with the T in the *HincII* site 5'-GTTGAC. In contrast, however, an extra 3'-dG on the displaced strand will form a dG·T base pair in the *HincII* site. Such a dG·T mismatch at the 3' end of the displaced strand may impede extension on the SDA primer. Furthermore,

the resultant dG·T-containing *HincII* site may not be efficiently nicked (it may undergo double-stranded cleavage⁹). Therefore, addition of an extra dG to the 3' end of the duplex may produce a displaced strand that does not serve as target, thereby nullifying that cycle of SDA (Fig. 1, bottom).

However, buffer conditions can be adjusted such that extra nucleotides are not efficiently added to the 3' ends of duplexes. Therefore, this extra nucleotide phenomenon is not necessarily detrimental to SDA, although it should be considered when designing SDA systems that use a restriction enzyme other than *HincII*. For example, *NciI* can be used in conjunction with the recognition site 5'-CCGGG using dATP, TTP, dGTP, and dCTP α S.⁽¹⁾ Addition of an extra 3'-dA to the ends of displaced strands, which is highly favored over an extra 3'-dG, would produce a dC·dA mismatch at the underlined C (C) in the *NciI* site 5'-CCCGGG.

Klenow, which possesses 3' to 5' exonuclease activity, does not add extra nucleotides to the ends of duplexes.⁽⁸⁾ However, Klenow does not work as well as exo^- Klenow in SDA because its 3' to 5' exonuclease activity degrades free primers and promotes mispriming on nontarget DNA.

Buffer/Salt Conditions

SDA reactions usually contain 50 mM K_2PO_4 (pH 7.4) which serves as a buffer and K^+ source for the enzymes. In preparing this buffer, one must be careful not to raise the K^+ concentration any higher than necessary while adjusting the pH because K^+ concentrations >100 mM are detrimental to SDA. We mix 13 ml of 0.5 M KH_2PO_4 with 87 ml of 0.5 M K_2HPO_4 to form 0.5 M K_2PO_4 (pH 7.6) (10 $\times$ stock solution), which corresponds to a final 1 $\times$ concentration of 94 mM K^+ in the SDA reaction. The pH of this 10 $\times$ stock solution decreases to pH $\sim$ 7.4 upon dilution to the final 1 $\times$ concentration of 50 mM K_2PO_4 . Changes in pH from 7.4 to 7.6 have minimal effect.

An earlier version of SDA used 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 50 mM KCl instead of 50 mM K_2PO_4 (pH 7.4).⁽¹⁾ Use of K_2PO_4 produces higher amplification factors. However, a drawback in using K_2PO_4 is that magnesium precipitates with phosphate at concentrations higher than the final SDA

concentrations. Consequently, when assembling an SDA reaction sample, MgCl_2 is not added until all other reagents except enzymes have been added to ensure that K_2PO_4 and MgCl_2 do not encounter one another at elevated concentrations.

SDA reactions usually contain 6 mM MgCl_2 and 4 mM total dNTP. The free Mg^{2+} concentration is $\sim$ 2 mM because dNTPs quantitatively bind Mg^{2+} . Other concentration combinations of MgCl_2 and dNTPs may produce favorable results. However, because the dNTP and free Mg^{2+} concentrations are coupled, the two concentrations cannot be changed independently. The reader should also be aware that precipitation of magnesium phosphate may be more prevalent at other concentrations of MgCl_2 , dNTPs, and K_2PO_4 .

Bovine serum albumin (BSA) is usually included at a final concentration of 0.1 mg/ml to stabilize enzymes and prevent their adsorption to polypropylene microcentrifuge tubes, although indication of its inclusion was inadvertently omitted from previous publications.^(1,2) Amplification of the 52-mer pBR322 target decreases 23% for a 2-hr reaction when addition of BSA is omitted. However, the final BSA concentration for this reaction was still 0.6 μ g/50 μ l as a result of a contribution from the stock vendor solution of *HincII*.

Temperature

Reactions are usually performed at 37–42°C. Higher temperatures reduce the stability of *HincII* and exo^- Klenow. Lower temperatures enhance background amplification due to mispriming. The SDA sample must be at its incubation temperature (37–42°C) before addition of *HincII* and exo^- Klenow. Assembly of reactions at lower temperatures promotes mispriming and, thus, background amplification, which attenuates target-specific amplification.

The optimal organic solvent concentration usually decreases with higher temperature, as both destabilize nucleic acid hybridization. Often, a few combinations of temperature and organic solvent produce equivalent results. Temperatures higher than 37°C were not tried with these pBR322 targets.

The target denaturation step at $\sim$ 95°C before addition of *HincII* and exo^- Klenow should be limited to $\sim$ 2 min to

guard against magnesium phosphate precipitation, which is promoted at high temperature. However, it is essential that the sample actually achieves $\sim 95^{\circ}\text{C}$ during that 2-min incubation to denature the target fully.

Time

SDA typically converts from an exponential to a linear mode after ~ 2 hr because at that point amplified product (both target-specific and background) reaches a level that renders *HincII* no longer in excess.^(1,3) The reactions in Figure 2 were performed for 3 hr to reach the highest level of amplified product with the longer targets that do not amplify as well. However, the extra hour produced only about twofold more product (Table 2).

Each SDA cycle (Fig. 1, bottom) consists of (1) primer binding to a displaced target fragment, (2) extension of the primer/target complex by exo^{-} Klenow, (3) nicking of the resultant hemiphosphorothioate *HincII* site, (4) dissociation of *HincII* from the nicked site, and (5) extension of the nick and displacement of the downstream strand by exo^{-} Klenow. For short target fragments (~ 50 nucleotides), the cycle time is ~ 3.5 min, with the rate-limiting step being *HincII* dissociation from the nicked site (G.T. Walker, unpubl.). The extension/displacement step may become somewhat rate limiting for longer target fragments (>100 nucleotides). However, rate-limiting extension/displacement behavior would probably reflect inefficient displacement of longer fragments owing to poor processivity of exo^{-} Klenow rather than a slow intrinsic rate of nucleotide incorporation. Low processivity during extension/displacement requires multiple polymerase initiation events. Each initiation event must compete with reannealing of the displaced strand reannealing to the template strand.

SUMMARY

The most attractive feature of SDA is its operation at a single temperature, which removes the need for instrumented temperature cycling as with PCR and the ligase chain reaction.⁽¹⁰⁾ Highly reproducible temperature profiles, over a large array of samples, can burden the accuracy and expense of an amplification technique. However, the expense of a

temperature cycler is offset somewhat by the cost of additional enzymes used in isothermal techniques. In comparisons with isothermal, transcription-based techniques,⁽¹¹⁾ SDA requires fewer enzymes and has a simpler mechanism. SDA may also be more robust than transcription-based processes because it is not susceptible to contaminating ribonuclease activity. This is generally more of a concern when using clinical samples.

The most significant disadvantage of SDA is its inability to efficiently amplify long target sequences. Until this shortcoming is eliminated, SDA will be assigned to the diagnostic laboratory along with the ligase chain reaction. Currently, SDA cannot compete with PCR in research applications such as the isolation of gene sequences. The second disadvantage of SDA is that it operates at relatively low (nonstringent) temperatures, which produces considerable background reactions. Consequently, SDA reaction products cannot be analyzed routinely by ethidium-stained gel electrophoresis, as is used commonly with PCR, unless the target sample contains a large number of initial targets.

NOTE

Just before submission of this article, I was informed that the updated sequence of pBR322⁽¹²⁾ varies from the published sequence on which I designed the SDA primers.⁽¹³⁾ The updated sequence contains a C instead of a T at position 1894 and single-nucleotide deletions at positions 1899 and 1915 (nucleotide positions as in ref. 13). Unfortunately, primer S_{2b} binds at one of the discrepant regions. According to the updated sequence,⁽¹²⁾ primer S_{2b} should be 5'-dTACAAGTAACCGACTATTGTTFA_CGGGATTCTGTTCA. Previous experience suggests that a single mismatch toward the 5'-end of the target-binding region of S_{2b} should reduce SDA efficiency by 2- to 10-fold. Therefore, the amplification factor for the S_1/S_{2b} primer pair in Table 2 may improve with the above updated version of S_{2b} . The binding positions of primers S_{2c} , S_{2d} , and B_2 in Table 1 reflect the updated pBR322 sequence.⁽¹²⁾

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