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 Hincl 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. 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 Hincl. Amplification then proceeds at -37°C after cooling and addition of the enzymes.

Past publications on SDA 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. S1 was paired with either S2a, S2b, S2c, or S2d so that the four target sequences would have one end in common. Single versions of B1 and B2 were used with all four target fragments by designing them to bind at target locations 5' to those of S1 and S2d. 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. 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 Hincl is no longer in excess. 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.

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 Hincl 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. 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, 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 S1 and S2 at positions 3' to the Hincl site of each primer, a primer-dimer will be formed that is exponentially amplified.
because it contains a nickable HincII site at each end. These primer–dimers amplify very efficiently owing to their short length, thereby attenuating target-specific amplification, especially for longer target sequences. Some of the S₁ and S₂RNA 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>pBR322 hybridization (nucleotide positions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1801-1813</td>
</tr>
<tr>
<td>$S_{2a}$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1852-1839</td>
</tr>
<tr>
<td>$S_{2b}$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1896-1883</td>
</tr>
<tr>
<td>$S_{2c}$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1950-1938</td>
</tr>
<tr>
<td>$S_{2d}$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1998-1985</td>
</tr>
<tr>
<td>$S_1$ and $S_2b$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>1781-1795</td>
</tr>
<tr>
<td>$S_1$ and $S_2a$</td>
<td>5'-dGCTCGGGATGATCTGAGTC</td>
<td>2018-2006</td>
</tr>
</tbody>
</table>

*HincII recognition sites are underscored.

### TABLE 2  
SDA Conditions for Amplification of pBR322

<table>
<thead>
<tr>
<th>Primers</th>
<th>Percent DMSO (vol/vol)*</th>
<th>exo$^-$ Klenow (units)</th>
<th>Target length (base pairs)$^{c}$</th>
<th>Amplification factor</th>
<th>Duration of SDA (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$S_1$ and $S_{2a}$</td>
<td>6</td>
<td>3.5</td>
<td>52</td>
<td>4.5 x 10^8-fold</td>
<td>2</td>
</tr>
<tr>
<td>$S_1$ and $S_{2b}$</td>
<td>6</td>
<td>3.5</td>
<td>52</td>
<td>8.1 x 10^6-fold</td>
<td>3</td>
</tr>
<tr>
<td>$S_1$ and $S_{2b}$</td>
<td>10</td>
<td>6</td>
<td>96</td>
<td>1.4 x 10^7-fold</td>
<td>3</td>
</tr>
<tr>
<td>$S_1$ and $S_{2c}$</td>
<td>12</td>
<td>8</td>
<td>150</td>
<td>2.9 x 10^7-fold</td>
<td>3</td>
</tr>
<tr>
<td>$S_1$ and $S_{2d}$</td>
<td>15</td>
<td>10</td>
<td>198</td>
<td>3.0 x 10^5-fold</td>
<td>3</td>
</tr>
</tbody>
</table>

*All reactions also contained $B_1$ and $B_2$.

### GENERAL EMPERICAL ASPECTS OF SDA

#### Primer and Target Sequences

The sequences of $S_1$ and $S_2$ dramatically affect amplification efficiency. $S_1$ and $S_2$ usually have 11- to 14-nucleotide target-binding regions, allowing for primer extension within the relatively low stringency conditions of SDA (37°C). As mentioned previously, the 3'-ends of $S_1$ and $S_2$ must not bind, forming primer–dimer combinations, because they are amplified efficiently at the cost of target amplification.

The S' sequences of $S_1$ and $S_2$ 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 S' sequences do not fortuitously promote background amplification that competes with target-specific annealing. Historically, we either chose S' se-
sequences that did not form obvious inter- or intraprimmer secondary structures or we designed $S_1$ and $S_2$ 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 $S_1$ and $S_2$ from pernicious hairpin or duplex formation.

The sequences of $B_1$ and $B_2$ are not critical to amplification efficiency. However, they must not anneal to any of the other four primers ($B_1$, $B_2$, $S_1$, or $S_2$) in a manner that attenuates target-specific hybridization.

The target sequence must not contain recognition sites for the nicking restriction enzyme used in SDA. Fortunately, HindII 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 HindII sites.) Therefore, only exact HindII recognition sites in the target sequence are of concern. However, it is important to search for all four HindII sites ($5'$-GTPyPuAC).

**Primer Concentrations**

$S_1$ and $S_2$ 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. $B_1$ and $B_2$ are typically used at lower concentrations (50 nM) to ensure that they do not anneal and extend before $S_1$ and $S_2$. During the entire SDA incubation period, $B_1$ and $B_2$ 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 concent...
centrations of 5–10 U/µl. We purchase *HincII* at high concentration (50–75 U/µl) from New England Biolabs and Life Technologies, Inc. *HindIII*, which is identical to *HincII* except from a different strain of *Haemophilus influenzae*, is available from Boehringer Mannheim at ≥40 U/µl. 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 ~10^{12} and 10^{14} 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). Addition of extra nucleotides is a particular concern in SDA because displaced strands must be subsequently extended on the primer (S₁ or S₂) 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′-CCGGG on S₁ or S₂. In the presence of dGTP, dCTP, TTP, and MgCl₂, exo−*Klenow adds dA and dG with approximately equal tendency to the ends of duplex DNA. An extra 3′-dG on the displaced strand will naturally base-pair with the T in the *HincII* site 5′-CCGG. 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 cleavage). 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. 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′-CCGGG.

*Klenow*, which possesses 3′ to 5′ exonuclease activity, does not add extra nucleotides to the ends of duplexes. 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 KPO₄ (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 KH₂PO₄ with 87 ml of 0.5 M K₂HPO₄ to form 0.5 M KPO₄ (pH 7.6) (10× stock solution), which corresponds to a final 1× concentration of 94 mM K⁺ in the SDA reaction. The pH of this 10× stock solution decreases to pH ~7.4 upon dilution to the final 1× concentration of 50 mM KPO₄. 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 KPO₄ (pH 7.4). Use of KPO₄ produces higher amplification factors. However, a drawback in using KPO₄ is that magnesium precipitates with phosphate at concentrations higher than the final SDA concentrations. Consequently, when assembling an SDA reaction sample, MgCl₂ is not added until all other reagents except enzymes have been added to ensure that KPO₄ and MgCl₂ do not encounter one another at elevated concentrations.

SDA reactions usually contain 6 mM MgCl₂ and 4 mM total dNTP. The free Mg²⁺ concentration is ~2 mM because dNTPs quantitatively bind Mg²⁺. Other concentration combinations of MgCl₂ and dNTPs may produce favorable results. However, because the dNTP and free Mg²⁺ 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 MgCl₂, dNTPs, and KPO₄.

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. 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 µg/50 µl 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 ~95°C before addition of *HincII* and exo−*Klenow* should be limited to ~2 min to
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 \( \text{HincII} \) 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 hemi-phosphorothioate \( \text{HincII} \) site, (4) dissociation of \( \text{HincII} \) 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 \( \text{HincII} \) 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 renatting of the displaced strand renatting 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 given 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-\( \psi \)GGAGATTCTGTTCA. 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.\(^{12}\)

**REFERENCES**

6. The computer program Oligo by National Biosciences, Hamel, MN.
Empirical aspects of strand displacement amplification.

G T Walker

*Genome Res.* 1993 3: 1-6

References

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