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Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system

(nucleic acid amplification/strand displacement/*Mycobacterium tuberculosis*)

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ABSTRACT **An isothermal *in vitro* DNA amplification method was developed based upon the following sequence of reaction events. Restriction enzyme cleavage and subsequent heat denaturation of a DNA sample generates two single-stranded target DNA fragments (T_1 and T_2). Present in excess are two DNA amplification primers (P_1 and P_2). The 3′ end of P_1 binds to the 3′ end of T_1, forming a duplex with 5′ overhangs. Likewise, P_2 binds to T_2. The 5′ overhangs of P_1 and P_2 contain a recognition sequence (5′-GTTGAC-3′) for the restriction enzyme *Hin*cII. An exonuclease-deficient form of the large fragment of *Escherichia coli* DNA polymerase I (exo$^-$ Klenow polymerase) [Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Friedman, J. M., Joyce, C. M. & Steitz, T. A. (1988) *Science* 240, 199–201] extends the 3′ ends of the duplexes using dGTP, dCTP, TTP, and deoxyadenosine 5′-[α-thio]triphosphate, which produces hemiphosphorothioate recognition sites on $P_1 \cdot T_1$ and $P_2 \cdot T_2$. *Hin*cII nicks the unprotected primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. The exo$^-$ Klenow polymerase extends the 3′ end at the nick on $P_1 \cdot T_1$ and displaces the downstream strand that is functionally equivalent to T_2. Likewise, extension at the nick on $P_2 \cdot T_2$ results in displacement of a downstream strand functionally equivalent to T_1. Nicking and polymerization/displacement steps cycle continuously on $P_1 \cdot T_1$ and $P_2 \cdot T_2$ because extension at a nick regenerates a nickable *Hin*cII recognition site. Target amplification is exponential because strands displaced from $P_1 \cdot T_1$ serve as targets for P_2 and strands displaced from $P_2 \cdot T_2$ serve as targets for P_1. A 10^6-fold amplification of a genomic sequence from *Mycobacterium tuberculosis* or *Mycobacterium bovis* was achieved in 4 h at 37°C.**

A variety of *in vitro* nucleic acid amplification techniques are being developed for clinical diagnosis of infectious and genetic diseases. Amplification techniques can be grouped into those requiring temperature cycling [PCR (1), ligase chain reaction (2–4), and transcription-based amplification (5)] and isothermal systems [self-sustained sequence replication (6) and a Qβ replicase system (7)] [for a comparative review, see Kwoh and Kwoh (8)]. Strand displacement amplification (SDA) is a DNA amplification technique that uses readily available enzymes and does not require temperature cycling. The technique is based upon the ability of a restriction enzyme to nick the unmodified strand of a hemimodified DNA recognition site, and the ability of a 5′–3′ exonuclease-deficient DNA polymerase to extend the 3′ end at the nick and displace the downstream strand. Exponential target DNA amplification is achieved by coupling sense and antisense reactions in which strands displaced from a sense reaction serve as target for an antisense reaction and vice versa.

Tuberculosis is one of the most common human infectious diseases, infecting an estimated one billion worldwide with ≈16 million active cases and 3 million deaths per year (9). Recently, it has received renewed attention due in part to its high incidence among AIDS patients. The disease is caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Culture-based diagnosis provides exquisite sensitivity and specificity but requires 3–6 weeks due to the slow growth rate of most pathogenic species of mycobacteria. Acid-fast organisms can be quickly identified in stained smears but with low sensitivity ($>10^4$ organisms per ml). Consequently, there is an acute need for a rapid and sensitive test.

The PCR meets these diagnostic requirements and has been applied to mycobacterial infections (10). An IS6110 insertion element, which is specific for *M. tuberculosis* and *M. bovis*, provides an effective target sequence for amplification and detection (11). SDA was applied to genomic DNA samples from *M. tuberculosis* and *M. bovis* using a portion of the IS6110 element as target sequence. We have achieved a 10^6-fold amplification, thereby establishing SDA as an isothermal alternative for amplifying specific DNA sequences.

MATERIALS AND METHODS

Materials. The large fragment of *Escherichia coli* DNA polymerase I (Klenow polymerase) was purchased from Boehringer Mannheim (sequencing grade). Exonuclease-deficient large fragment of *E. coli* DNA polymerase I (exo$^-$ Klenow polymerase) (12) was purchased from United States Biochemical. *Rsa* I was purchased from Bethesda Research Laboratories. *Hin*cII was purchased from New England Biolabs at a concentration of 64 units/μl and from Bethesda Research Laboratories at 50 units/μl. Ultra-pure human placental DNA was purchased from Sigma. 2′-Deoxynucleoside 5′-triphosphates (dGTP, dCTP, and TTP) and deoxyadenosine 5′-[α-thio]triphosphate (dATP[αS]) were purchased from Pharmacia. Oligodeoxynucleotides were synthesized on an Applied Biosystems model 380B instrument and purified by denaturing polyacrylamide gel electrophoresis. Aerosol-barrier pipet tips (PGC Scientific, Gaithersburg, MD) were used to minimize aerosol contamination.

Isolation of Genomic DNA from *M. tuberculosis* and *M. bovis*. Stationary-phase cultures of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) or *M. bovis* (kindly supplied by Salmon Siddiqi, Becton Dickinson Diagnostic Instrument Systems, Sparks, MD) in 60 ml of Middlebrook 7H9 broth were heat-killed at 65°C for 4 h. Genomic DNA was isolated by published procedures (13). Concentrations of genomic DNA from *M. tuberculosis* and *M. bovis* were calculated based upon respective genome sizes of 2.5×10^9

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Abbreviations: SDA, strand displacement amplification; dNTP[αS], dATP[αS], etc., deoxynucleoside 5′-[α-thio]triphosphate, deoxyadenosine 5′-[α-thio]triphosphate, etc.

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Da and 2.8×10^9 Da (14) and 20 A_{260} units per mg of double-stranded DNA.

Recombinant Plasmid Containing the IS6110 Sequence of *M. tuberculosis* and *M. bovis*. A 1208-base-pair (bp) fragment of the insertion sequence IS6110 (nucleotide positions 72–1279) (15), which is specific to *M. tuberculosis* and *M. bovis*, was isolated from genomic *M. tuberculosis* DNA by a PCR using primers containing recognition sequences for *Eco*RI and *Xba* I at their 5′ ends. A single copy of the IS6110 fragment was ligated into the *Eco*RI/*Xba* I site of pBluescript SK+ (Stratagene) and transformed into *E. coli* DH5αF cells (Bethesda Research Laboratories). Recombinant plasmid DNA was purified by standard alkaline lysis procedures and CsCl centrifugation (16).

SDA Reactions. Amplification reactions were performed on target DNA isolated from genomic *M. tuberculosis* DNA, genomic *M. bovis* DNA, or the recombinant plasmid containing a single copy of the insertion sequence IS6110 (15). *Rsa* I cleavage of these DNA samples liberated a 47-bp fragment (nucleotide positions 977–1023 of the insertion sequence IS6110), which served as target for SDA:

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5'-ACTCGACCTGAAAGACGTTATCCACCATACGGATAGGGGATCTCAGT
   TGAGCTGGACTTTCTGCAATAGGTGGTATGCCTATCCCCTAGAGTCA-5'
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Rsa I cleavage was performed using 10 units per μg of DNA in 100 μl of 50 mM Tris·HCl, pH 8/10 mM $MgCl_2$ for 1 h at 37°C followed by 2 min at 95°C. *Rsa* I-cleaved target samples were serially diluted into solutions containing *Rsa* I- cleaved human placental DNA (10 μg/ml) in 50 mM Tris·HCl, pH 7.4/6 mM $MgCl_2$/50 mM NaCl/50 mM KCl, thereby producing an array of stock target DNA solutions of various concentrations in the presence of a constant amount of human carrier DNA. SDA reaction mixtures (100 μl) contained various amounts of target DNA and 0.1 μg of human DNA in a solution of 100 units of *Hinc*II, 2.5 units of exo$^-$ Klenow polymerase, 1 mM dGTP, 1 mM dCTP, 1 mM TTP, and 1 mM dATP[αS], 50 mM Tris·HCl (pH 7.4), 6 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl, ≈1% glycerol (from enzyme stock solutions), and 1 μM SDA primers of the following sequences: 5′-d(TTGAATAGTCGGTTACTT*GTTGAC*ACTCGACCTGAAA) and 5′-d(TTGAAGTAACCGACTATT*GTTGAC*ACTGAGATCCCCT) (the *Hinc*II recognition sequence is italicized). Prior to addition of *Hinc*II and exo$^-$ Klenow polymerase, reaction samples were incubated 4 min at 95°C to denature the target fragment followed by 4 min at 37°C to anneal primers. Upon addition of *Hinc*II and exo$^-$ Klenow polymerase, amplification reaction mixtures were incubated 1–5 h at 37°C.

Detection of SDA Products. Amplified target DNA fragments and unamplified target standards were detected using a 5′-^{32}P-labeled probe: 5′-d(CGTTATCCACCATAC). The detector probe was ^{32}P-labeled using T4 polynucleotide kinase in a 50-μl reaction mixture containing 1 μM oligodeoxynucleotide probe, 6.9 mM Tricine (pH 7.6), 50 mM Tris·HCl (pH 8), 10 mM $MgCl_2$, 5 mM dithiothreitol, 2.3 μM [γ-^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml; 1 Ci = 37 GBq), 50 units of kinase (New England Biolabs). ^{32}P-labeling was carried out for 1 h at 37°C and terminated by heating at 95°C for 2 min.

The SDA reactions shown in Fig. 3 were terminated after 4 h at 37°C by heating 10 min at 65°C and products were detected as follows. The ^{32}P-labeled probe/kinase mixture (1 μl) was added to 100 μl of SDA reaction sample. The sample was denatured 4 min at 95°C and annealed 2 min at 37°C. The ^{32}P-labeled detector probe was extended to a diagnostic length upon addition of 2 μl of the Klenow polymerase (1 unit/μl) and incubation for 15 min at 37°C. The extension reaction was terminated by heating 10 min at 65°C followed by addition of 2.6 μg of native calf thymus DNA (Boehringer Mannheim) and 0.1 vol of 3 M sodium acetate. The DNA was precipitated with 3 vol of ethanol for 10 min at room temperature and recovered by centrifugation for 15 min in a bench-top microcentrifuge. DNA pellets were dissolved in 50% (wt/vol) urea/20 mM Na_2EDTA/0.5× TBE (16)/0.05% bromophenol blue/xylene cylanol and heated 2 min at 95°C.

The SDA reaction products shown in Fig. 4 were detected as follows. Samples (10 μl) from a 100-μl SDA reaction mixture were removed at indicated times and the reaction was terminated by heating 2 min at 95°C. The ^{32}P-labeled probe/kinase mixture (2 μl) was added to each 10-μl sample, and the mixture was heated 2 min at 95°C and then 2 min at 37°C. The ^{32}P-labeled detector probe was extended to a diagnostic length upon addition of 2 μl of the Klenow polymerase (1 unit/μl) and incubation for 15 min at 37°C. The extension reaction was terminated upon addition of 14 μl of 50% urea/20 mM Na_2EDTA/0.5× TBE/0.05% bromophenol blue/xylene cylanol.

Samples were analyzed by denaturing electrophoresis in a 10% (wt/vol) polyacrylamide gel [acrylamide:*N*,*N*′-methyleneacrylamide (18:1)] containing 50% urea and 0.5× TBE. ^{32}P-labeled gel electrophoresis bands were excised and radioactivity was quantified by liquid scintillation counting.

RESULTS AND DISCUSSION

SDA Design. The fundamental reaction scheme of SDA is depicted in Fig. 1. A single-stranded target DNA fragment and a DNA amplification primer bind at their complementary

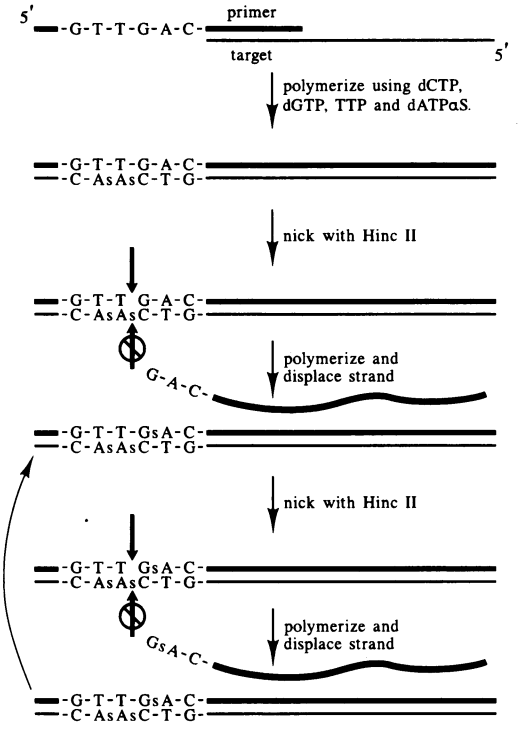


FIG. 1. Fundamental reaction scheme of SDA. A single-stranded target DNA fragment binds to an SDA primer containing a recognition sequence for *Hinc*II. DNA replication using dCTP, dGTP, TTP, and dATP[αS] produces a double-stranded hemiphosphorothioate recognition site. *Hinc*II nicks the unprotected primer strand at its recognition site, leaving intact the modified complementary strand (thin strand). DNA polymerase, lacking 5′–3′ exonuclease activity, extends the 3′ end at the nick and displaces the downstream fragment. The polymerization/displacement step regenerates a nickable recognition site; the 5′-GsA-3′ phosphorothioate linkage does not inhibit nicking of the thick strand. Nicking and polymerization/displacement steps cycle continuously producing single-stranded complementary copies of the target fragment. Oligodeoxynucleotide lengths are not drawn to scale.

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3′ ends, forming a duplex with a 5′ overhang at each end. The 5′ overhang of the primer strand contains a recognition sequence (5′-GTTGAC-3′) for the restriction enzyme *Hinc*II. An exonuclease-deficient form of the large fragment of *E. coli* DNA polymerase I (exo$^-$ Klenow polymerase) (12) extends the 3′ ends of the duplex using dGTP, dCTP, TTP, and dATP[αS], which produces a hemiphosphorothioate recognition site. *Hinc*II nicks the unprotected primer strand of the hemiphosphorothioate recognition site, leaving intact the modified complementary strand (thin strand in Fig. 1). The exo$^-$ Klenow polymerase extends the 3′ end at the nick and displaces the downstream complement of the target strand. The polymerization/displacement step regenerates a nickable *Hinc*II recognition site; the 5′-GsA-3′ phosphorothioate linkage does not inhibit nicking of the thick strand in Fig. 1. Nicking and polymerization/displacement steps cycle continuously producing single-stranded complements of the target strand.

Fig. 2 depicts SDA using two primers (P_1 and P_2) with a double-stranded target DNA fragment (T_1·T_2), which is generated by restriction enzyme cleavage of a DNA sample. After heat denaturation, single-stranded target fragments T_1 and T_2 bind to respective primers P_1 and P_2, which are present in excess. Both primers contain restriction enzyme recognition sequences located 5′ to the target binding sequences. Each primer·target complex (P_1·T_1 and P_2·T_2) cycles through nicking and polymerization/displacement steps in the presence of a restriction enzyme, DNA polymerase, three dNTPs, and one dNTP[αS] as illustrated in Fig. 1. SDA with a single primer produces copies of a target fragment at a linear amplification rate (Fig. 1). In contrast, amplification using two primers proceeds exponentially because strands displaced from the P_1·T_1 complex serve as target for primer P_2 and strands displaced from the P_2·T_2 complex serve as target for primer P_1 (Fig. 2).

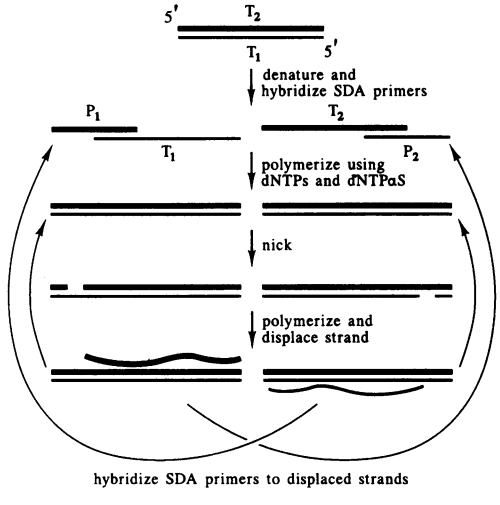


FIG. 2. Exponential SDA using two primers with a double-stranded target DNA fragment. After denaturation of a double-stranded target fragment, single-strand target fragments T_1 and T_2 bind to respective primers P_1 and P_2, which are present in excess. The primers contain recognition sites for the nicking restriction enzyme. DNA replication using three dNTPs and one dNTP[αS] produces double-stranded primer·target complexes with hemiphosphorothioate recognition sites, which are subsequently nicked by the appropriate restriction enzyme. DNA polymerase, lacking 5′–3′ exonuclease activity, extends the 3′ end at the nick and displaces the downstream strand. Nicking and polymerization/displacement steps cycle continuously due to regeneration of a nickable recognition site. Exponential amplification results because strands displaced from the P_1·T_1 complex serve as target for primer P_2 and strands displaced from the P_2·T_2 complex serve as target for primer P_1.

Restriction Enzymes and DNA Polymerases Suitable for SDA. Eckstein and coworkers (17) have identified many restriction enzymes that nick hemiphosphorothioate recognition sites. Only a small subset of these restriction enzymes function in SDA because of the special requirement that the polymerization/displacement step must regenerate a nickable recognition site. Phosphorothioate linkages incorporated into the primer strand must not inhibit subsequent nicking. Suitable restriction enzymes identified to date satisfy this requirement through nonpalindromic recognition sites. In addition to *Hinc*II used in the present study, *Hind*II, *Ava* I, *Nci* I, and *Fnu*4HI also function in SDA (G.T.W., M.C.L., and J.G.N., unpublished results).

To function adequately in SDA, a restriction enzyme must efficiently nick one strand of a hemiphosphorothioate recognition site and quickly dissociate from the nicked site to allow the DNA polymerase access. Many restriction enzymes efficiently nick a nonpalindromic hemiphosphorothioate recognition site but apparently dissociate too slowly from the nicked site for efficient operation in SDA (M.C.L., unpublished results).

Clearly, it is essential that the target DNA sequence does not contain a recognition sequence for the restriction enzyme used in SDA (*Hinc*II in the present investigation).

To function in SDA, a DNA polymerase must (*i*) initiate polymerization at a nick, (*ii*) utilize dNTP[αS]s, and (*iii*) displace a downstream strand (and lack 5′–3′ exonuclease activity). In addition to the exo$^-$ Klenow polymerase used in the present study, the Klenow polymerase (sequencing grade, Boehringer Mannheim) and the large fragment of *Bacillus stearothermophilus* (Bio-Rad) also function in SDA (G.T.W. and M.C.L., unpublished results).

SDA Primer Design. Each SDA primer contains a target binding region at its 3′ end. A recognition site for the nicking restriction enzyme is located 5′ to the target binding region. The recognition sequence is flanked on the 5′ side by a stretch of DNA typically ≈20 nucleotides long, the sequence of which is not critical. The 5′ end of a primer provides flanking DNA to facilitate binding of the nicking restriction enzyme to the recognition site. The 5′ end also must be long enough to provide a stable polymerase priming site after nicking by the restriction enzyme. The SDA primers in the current investigation are partially complementary at their 5′ ends. This was designed to reduce nonspecific binding. However, 5′ complementarity between primers is not essential for SDA.

SDA primers undoubtedly bind nonspecifically at 37°C to nontarget DNA present in the sample (human placental DNA in the current investigation). However, SDA not only requires that primers bind to DNA fragments with adequate homology but also requires an appropriately positioned 3′ end on the target strand to form a hemiphosphorothioate recognition site for the nicking restriction enzyme. Use of a 3′–5′ exonuclease-deficient DNA polymerase enhances primer specificity in SDA because such a DNA polymerase is less likely to extend nonspecific complexes containing mismatched 3′ ends.

In the PCR, primers can bind nonspecifically to one another, resulting in amplification of what are commonly referred to as primer dimers. These background amplification reactions can effectively attenuate target-specific amplification by competing for PCR reagents. Nonspecific binding between SDA primers can also result in amplification of primer dimers. Consequently, the terminal 3′ nucleotides of each SDA primer are chosen to minimize formation of primer dimers that can be extended by DNA polymerase. Again, use of a 3′–5′ exonuclease-deficient DNA polymerase reduces extension of primer dimers with mismatched 3′ ends. Extension of nonspecific complexes can be further reduced by using SDA primers whose 3′-hydroxyl groups are modified to prevent extension by DNA polymerase. It is not essential

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that an SDA primer be extended upon binding to a target fragment. The hemiphosphorothioate recognition site is still formed through extension of the 3′ end of the target fragment. Primers with modified 3′-hydroxyl groups also allow use of DNA polymerases possessing 3′–5′ exonuclease activity (e.g., the Klenow polymerase) by ensuring excess single-stranded primers are not degraded during SDA.

SDA of Genomic DNA from *M. tuberculosis* and *M. bovis*. *Rsa* I cleavage of genomic DNA from either *M. tuberculosis* or *M. bovis* produced a 47-bp target fragment for SDA. A set of samples containing various amounts of the target DNA fragment and 0.1 μg of *Rsa* I-cleaved human placental DNA was subjected to SDA using the exo$^-$ Klenow polymerase and *Hin*cII. Amplified target fragments were detected by DNA polymerase extension of a ^{32}P-labeled probe that is complementary to an internal segment of the target sequence. The ^{32}P-labeled detector probe {[5′-^{32}P]d(CGTTATCCAC-CATAC)} is extended to a length of either 35 or 56 nucleotides when hybridized to one of the following two strands produced during SDA: 5′-d(GACACTGAGATCCCCTATCCG-TATGGTGGATAACGTCTTTCAGGTCGAGTGTC) and 5′-d(TTGAAGTAACCGACTATTGTTGACACTGAGATC-CCCTATCCGTATGGTGGATAACGTCTTTCAGGTC-GAGTGTC). These two strands are respectively analogous to the displaced strand and the unnicked strand (containing the sequence 5′-GTTGsAC-3′) in Fig. 1.

Results from amplification of genomic DNA from *M. tuberculosis* and *M. bovis* are shown in Fig. 3. The number of genome copies (copies of the single *Mycobacterium* chromosome) initially present in each SDA sample is indicated above each lane in Fig. 3. There are 10–12 copies of the 47-bp target DNA fragment in the genome of *M. tuberculosis*. In comparison, there are 1–3 copies in the genome of *M. bovis* (ref. 18; D.D.S., unpublished observations). Consequently, band intensities from each of the *M. tuberculosis* samples are ≈10-fold higher than the corresponding *M. bovis* samples due to this difference in the number of target fragments per genome. Intensities of the SDA product bands decrease as a function of decreasing initial target over the array of *M. tuberculosis* or *M. bovis* samples. For the experiments shown in Fig. 3, a maximum signal is exhibited for amplification of $>10^3$ genome copies of *M. tuberculosis* and $>10^4$ genome copies of *M. bovis* due to the limiting amount of the ^{32}P-labeled detector probe. Reactions performed without addition of target DNA exhibit faint SDA-specific ^{32}P-labeled bands in Fig. 3 due to accidental contamination of reagents with ≈10 target molecules (≈1 *M. tuberculosis* or ≈10 *M. bovis* genome copies). It is extremely difficult to maintain reagents that are free of target DNA contaminants on the scale of 10 molecules. Contamination control is a common problem with the PCR as well (19).

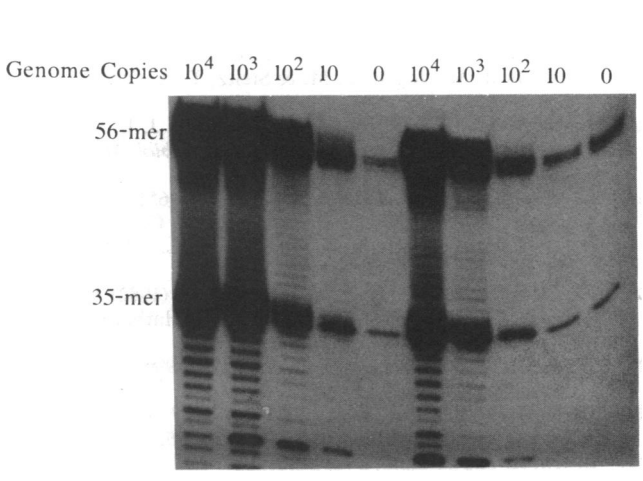


FIG. 3. Denaturing gel electrophoresis analysis of SDA products. SDA reaction mixtures contained 0.1 μg of *Rsa* I-cleaved human placental DNA and various amounts of target genomic DNA from either *M. tuberculosis* or *M. bovis*. SDA reactions proceeded for 4 h. Amplified target fragments were detected by extension of a ^{32}P-labeled probe to the indicated lengths. The number of copies of the single *Mycobacterium* chromosome initially present in each SDA reaction is shown above each lane.

A recombinant plasmid containing a single copy of the IS6110 sequence served as a standard for estimating the degree of amplification in SDA reactions. (The plasmid was used as a standard because it was available in sufficient quantity.) Comparison of amplified samples with standards allows us to estimate an amplification factor of 10^6-fold. A constant amplification factor is observed for each sample, regardless of the initial amount of target.

The current method of detection using a ^{32}P-labeled probe allows detection of as few as $\approx 10^8$ DNA molecules. This sensitivity in conjunction with 10^6-fold amplification allows clear detection of as few as 10 genome copies of *M. tuberculosis* and 100 genome copies of *M. bovis* using the 47-bp *Rsa* I fragment from the insertion element IS6110 as target.

We have not yet examined the replication fidelity of the exo$^-$ Klenow polymerase during SDA. Detection using a target-sequence-specific ^{32}P-labeled probe indicates fidelity is sufficient for diagnostic applications. However, fidelity may be a concern in other applications. Only a few pertinent investigations are published. The exo$^-$ Klenow polymerase is 4- to 7-fold less accurate than the Klenow polymerase (20). *E. coli* DNA polymerase I exhibits a 20-fold decrease in fidelity when dCTP is replaced with dCTP[αS] (21). This decrease in fidelity was attributed to inhibition of the 3′–5′ exonuclease proofreading activity of DNA polymerase I by dCTP[αS]. The fidelity of avian myeloblastosis virus DNA polymerase and DNA polymerase β, which lack 3′–5′ proofreading activity, was not altered by dCTP[αS] substitution. Finally, *Taq* DNA polymerase accurately incorporates dNTP[αS] using a template containing phosphorothioate nucleotides as judged by sequencing PCR products (22).

SDA Reaction Kinetics. Reaction kinetics were studied using the recombinant plasmid that contains a single copy of the IS6110 target fragment. A time-course profile for the initial stage of an SDA reaction is shown in Fig. 4*A Inset*.

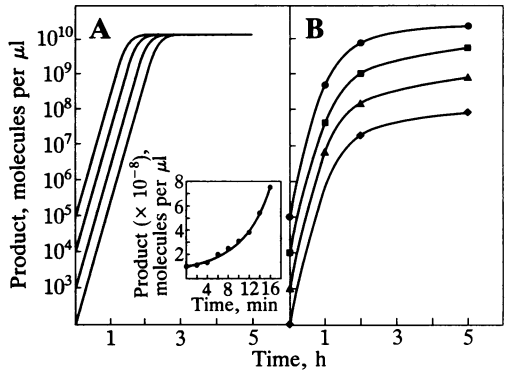


FIG. 4. Time-course profiles of SDA reactions. The SDA target was a recombinant plasmid containing a single copy of the IS6110 fragment. (*A Inset*) Observed exponential amplification for an SDA reaction with 1×10^8 initial target molecules per μl at time 0. The observed doubling time is 5.3 min. (*A*) Semilogarithmic plot of theoretical time courses for SDA reactions with various amounts of initial target DNA. Calculated curves (Eq. 1) are based upon a doubling time of 5.3 min and an SDA reagent at a limiting concentration of 1×10^{10} molecules per μl. (*B*) Semilogarithmic plot of observed time courses for experimental SDA reactions with various amounts of initial target DNA.

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Exponential amplification is observed with a doubling time (cycle time) τ of 5.3 min ($P_t = P_0 2^{t/\tau}$, where P_t is the number of product molecules at time t and P_0 is the number of target molecules at time 0). The following sequential steps occur within each 5.3-min cycle as illustrated in Figs. 1 and 2: (*i*) nicking of the hemiphosphorothioate recognition site by *Hinc*II, (*ii*) dissociation of *Hinc*II from the nicked site, (*iii*) polymerization and strand displacement by the exo$^-$ Klenow polymerase, (*iv*) hybridization of the displaced strand to the other primer, and (*v*) extension of the displaced strand and primer forming another hemiphosphorothioate recognition site.

The net result of each cycle is a doubling of product number (P) and consumption of reagents (R):

$$P + R \rightarrow 2P.$$

If reagents are not replenished during the course of the reaction, the level of product at time t is given by:

$$P_t = (R_0 Q e^{kt})/(1 + Q e^{kt}), \quad [1]$$

where $Q = P_0/(R_0 - P_0)$, P_0 is the initial product (target) concentration, and R_0 is initial concentration of limiting reagent. The apparent rate constant k is related to the cycling time τ by $k = (\ln 2)/\tau$.

An SDA reaction should proceed exponentially until the concentration of some reagent R becomes limiting. Fig. 4*A* is a theoretical time course profile for SDA reactions with various amounts of initial target DNA. The curves were calculated from Eq. 1 based upon a cycle time τ of 5.3 min and a limiting reagent concentration R_0 of 10^{10} molecules per μl. Product concentration is expected to increase exponentially until it reaches a level ≈5-fold less than R_0. The exponential phase is then followed by a much less efficient amplification phase as the product concentration approaches R_0. The final product concentration should be independent of the initial target concentration.†

Time courses of experimental SDA reactions are shown in Fig. 4*B*. Each experimental SDA reaction exhibits exponential amplification over the first 60 min. By the 2-h mark, each reaction has switched from exponential amplification to a much less efficient amplification phase, during which product concentration asymptotically approaches a level that depends on the initial amount of target. In each reaction, the number of target molecules is amplified by a constant amount (≈10^6-fold after 5 h), regardless of the number of initial targets. This results in a dose–response relationship between initial and final target levels, in sharp contrast to the results predicted by the theoretical curves in Fig. 4*A*. A similar dose–response relationship was reported for another amplification technique referred to as self-sustained sequence replication (6).

In SDA reactions, addition of fresh enzymes at the 1-h mark does not prolong the exponential phase, indicating enzyme degradation is not responsible for the reduction in amplification efficiency after that point. The observed dose–response relationship probably results from amplification of nontarget DNA (human placental DNA) that is present in large excess over specific target sequences. Although the intrinsic efficiency of this background amplification is expected to be very low, the high level of nontarget DNA may lead to significant accumulation of undesired products. These background reactions should not interfere with target amplification early in the reaction when reagents are present in excess. However, the exponential amplification phase (during which the number of target sequences doubles every 5.3 min) can only be sustained while the concentration of limiting reagent R remains at least 5- to 10-fold higher than amplified product levels (see Eq. 1 and Fig. 4*A*). Competing background reactions will prematurely deplete reagent levels and as a consequence will shorten the period during which exponential amplification of target sequences can occur. In addition, competing reactions will limit the final quantity of amplified target sequences that can be produced because reagents converted to background products will be unavailable for the production of target sequences. Consequently, a dose–response relationship will result because the degree to which target amplification can compete against background reactions will depend on the ratio of target to nontarget sequences present at the beginning of the reaction.

†Fig. 4*A* is intended to be a qualitative illustration of the reaction kinetics expected for SDA. Eq. 1 is quantitatively correct only if reagent R is consumed in the rate-determining reaction step. (We have yet to establish the contribution of individual steps to the overall reaction rate.) If R does not participate in the rate-determining step, we still expect the same limiting behavior defined by Eq. 1, namely, (*i*) exponential growth during the early phase of the reaction with a doubling time of 5.3 min and (*ii*) a final product concentration that is independent of initial target levels and approaches a value of R_0. Furthermore, the growth of P_t should begin to deviate from an exponential increase at the same level of P_t, regardless of initial target concentration.

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