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Multiplex strand displacement amplification (SDA) and detection of DNA sequences from *Mycobacterium tuberculosis* and other mycobacteria

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ABSTRACT

Strand Displacement Amplification (SDA) is an isothermal, *in vitro* method of amplifying a DNA target sequence prior to detection [Walker *et al* (1992) *Nucleic Acids Res.*, 20, 1691–1693]. Here we describe a multiplex form of SDA that allows two target sequences and an internal amplification control to be co-amplified by a single pair of primers after common priming sequences are spontaneously appended to the ends of target fragments. Multiplex SDA operates at a single temperature, under the same simple protocol previously developed for single-target SDA. We applied multiplex SDA to co-amplification of a target sequence (IS6110) that is specific to members of the *Mycobacterium tuberculosis*-complex and a target (16S ribosomal gene) that is common to most clinically relevant species of mycobacteria. Both targets are amplified 10^8-fold during a 2 hour, single temperature incubation. The relative sensitivity of the system was evaluated across a number of clinically relevant mycobacteria and checked for crossreactivity against organisms that are closely related to mycobacteria.

INTRODUCTION

One fifth of the world's population is infected with *M.tuberculosis*, the causative agent of tuberculosis, resulting in 3 million deaths annually. Although *M.tuberculosis* is the most significant of the mycobacterial pathogens, many other species such as *M.avium*, *M.intracellulare* and *M.kansasii* pose serious threats to immunocompromised individuals such as AIDS patients. Clinical diagnosis primarily involves growth by culture which typically requires 2–6 weeks due to the organisms' slow generation times.

Nucleic acid tests promise to provide faster and more sensitive diagnosis of mycobacterial infections. In some assays, genus-specific amplification by the polymerase chain reaction (PCR) is followed by species-specific detection through hybridization with labelled oligonucleotide probes (1–4). One advantage of this approach is that it provides comparable amplification efficiency across mycobacterial species because the two genus PCR primers hybridize to common mycobacterial sequences shared among many species. However, it is essential for the success of this approach that the PCR does not amplify DNA from closely related non-mycobacteria because these competing side reactions will lower Mycobacterium sensitivity (1). This approach also requires high stringency hybridization conditions during detection because mycobacterial species often must be differentiated through subtle sequence variations in the detector sequence region (4). Precise species identification following the PCR with genus primers can also be achieved by DNA sequencing (5) and restriction fragment length polymorphism (6), but these techniques are cumbersome. Wilton and Cousins (7) reported an alternative approach whereby genus and species specific mycobacterial sequences are co-amplified in a single PCR mixture containing 6 primer pairs. Products are then separated by electrophoresis and detected in a non-sequence specific manner by staining with ethidium dye, so target identification is based solely on molecular weight. More rigorous detection, based on hybridization of sequence specific probes, would be difficult because most of the products formed in this system arise from overlapping 16S ribosomal gene sequences.

We report here an isothermal, multiplex DNA amplification system, based on Strand Displacement Amplification (SDA), that allows one to detect the presence of DNA from organisms of the genus *Mycobacterium* and to simultaneously identify samples containing DNA from members of the *M.tuberculosis* complex (*M.tuberculosis*, *M.bovis*, *M.bovis*-BCG, *M.africanum* and *M.microti*).

Strand Displacement Amplification (SDA) is an isothermal method that provides 10^8-fold amplification of a target sequence during a 2 hour incubation at ~40°C (8, 12, 15). The technique is based upon the ability of a restriction enzyme to nick a hemimodified recognition site and the ability of a polymerase to displace a downstream DNA strand during replication. The method, depicted in Figure 1 for amplification of a single (double-stranded) target sequence, consists of two parts: (i) a target

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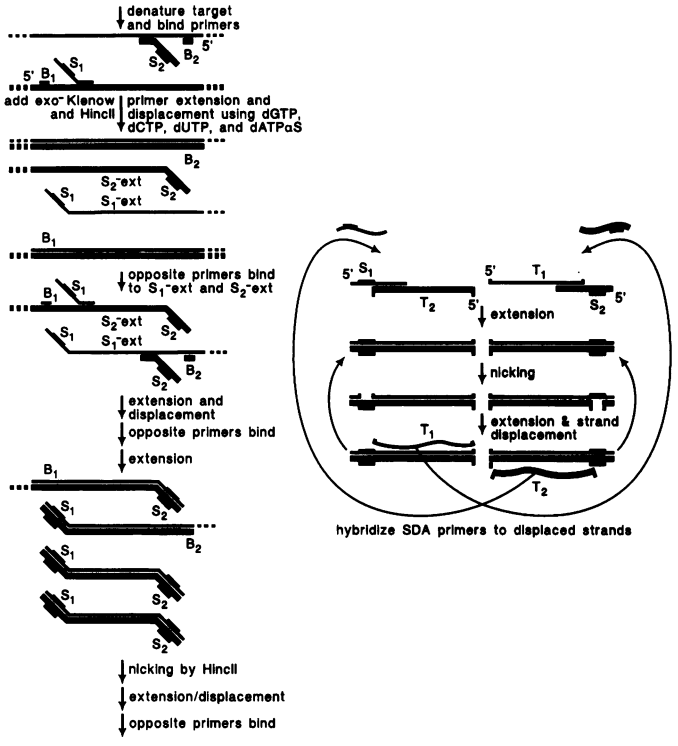

Figure 1. Schematic representation of single-target SDA. The target DNA sample is heat denatured in the presence of an excess of four primers (B_1, B_2, S_1 and S_2). S_1 and S_2 contain target binding regions at their 3'-ends and a recognition site (5'GTTGAC) for the restriction enzyme HincII located immediately 5' to the target binding regions (HincII recognition sites are designated by the raised boxes). S_1 and S_2 bind to opposite strands of the target sequence, flanking the region to be amplified. B_1 and B_2 are simply target binding sequences (containing no HincII recognition sites) and bind at positions 5' to S_1 and S_2. After the primers anneal to target at ~40°C, HincII is added along with an exonuclease deficient form of the Klenow fragment of *E.coli* DNA polymerase I (exo⁻ Klenow). At this point the remaining steps on the leftside of the figure proceed as a single cascade: Exo⁻ Klenow, which is present in large molar excess over the number of target sequences, simultaneously extends all four primers using dGTP, dCTP, dUTP and dATPαS. S_1 and S_2 are extended and then displaced during extension of B_1 and B_2. The displaced extension products of S_1 and S_2 (S_1-ext and S_2-ext) serve as targets for binding of 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 (bottom leftside of figure). HincII nicks the unmodified primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. Exo⁻ Klenow then extends the 3'-end at the nick and displaces the downstream strand. New S_1 and S_2 primers bind to the displaced strands and are extended. This is followed by additional nicking and strand displacement steps until the four duplexes at the bottom leftside of the figure converge into the 'steady-state' amplification cycle on the rightside of the figure. During each SDA cycle, the 3'-end of S_1 binds to the 3'-end of the displaced target strand T_2, forming a duplex with 5'-overhangs. Likewise, S_2 binds to T_1. Exo⁻ Klenow extends the recessed 3'-ends of the duplexes producing hemiphosphorothioate recognition sites that are nicked by HincII. These nicking and extension/displacement steps cycle continuously (short curved arrows on rightside of figure) because extension at a nick regenerates a nickable HincII recognition site. The strand displaced from the $S_1 \cdot T_2$ duplex is identical to T_1. Likewise, the displaced strand from the $S_2 \cdot T_1$ duplex is identical to T_2. Consequently, target amplification is exponential because each displaced T_2 bind a new S_1 primer while each displaced T_1 binds a new S_2 (long curved arrows on rightside of figure). 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 —■. Additional details are reported elsewhere (12, 15).

generation process (shown on the left) that makes copies of the target sequence flanked by nickable restriction sites, and (ii) the exponential amplification of these modified target sequences (shown on the right) by repeated nicking, strand displacement, and priming of displaced strands. Despite the complicated appearence of events depicted in Figure 1, SDA operates under a very simple protocol: Target DNA is heat denatured in the presence of all reagents except the restriction enzyme and polymerase. Amplification then proceeds at ~40°C after cooling and addition of the enzymes. Amplified products may then be detected by a variety of methods (12, 13).

In previous applications, SDA was used to amplify only a single target species in each reaction mixture. We have now extended the capability of SDA to allow highly efficient, simultaneous amplification two target sequences and an internal control molecule, designed to serve as a standard for judging the efficacy of individual amplification mixtures. In this multiplex SDA system, all three targets are amplified by a single pair of amplification primers, after an adapter-mediated target generation step is used to append common priming sequences to the ends of the different targets. The use of a single pair of amplification primers helps insure that the different targets are amplified at comparable rates. Furthermore, background amplification reactions, which compete against target-specific amplification and arise through errant priming events, are generally minimized by the use of a single pair of amplification primers.

We applied this adapter-mediated multiplex SDA system to the co-amplification of two mycobacterial target sequences. One target is a 16S ribosomal gene sequence that is common to virtually all clinically relevant mycobacterial species and differs considerably from the corresponding sequences in clinically relevant non-mycobacterial species, providing mycobacterial-genus specificity at the level of amplification. The second is the IS6110 element (14) which is specific to species belonging to the *M.tuberculosis* complex. The IS6110 sequence has no counter part in other organisms and thus provides amplification-level specificity for the *M.tuberculosis* complex. Because target specificity is achieved at the level of amplification, relatively non-stringent detection methods may be employed.

The multiplex SDA method follows the same simple experimental protocol employed for single-target SDA, and is generally applicable to any pair of target sequences.

MATERIALS AND METHODS

Bacterial strains

The sources of the bacterial strains were as follows: *M.tuberculosis* H37Rv (ATCC 27294), *M.bovis* (CDC 81), *M.bovis*-BCG (CDC 34), *M.avium* (ATCC 25291), *M.intracellulare* (ATCC 13950), *M.kansasii* (TMC 1201, LCDC 711), *M.gastri* (LCDC 1301), *M.fortuitum* (LCDC 2801), *M.paratuberculosis* (Linda), *M.chelonae* (TMC1543), *M.malmoense* (CDC 93), *M.szulgai* (TMC 1328), *M.flavescens* (LCDC 2601), *M.xenopi* (ATCC 19250, LCDC 1901), *M.terrae* (TMC 1450), *M.marinum* (TMC 1218, LCDC 801), *M.gordonae* (TMC 1318), *Corynebacterium diphtheriae* (ATCC 11913), *Corynebacterium xerosis* (ATCC 373), *Corynebacterium pseudodiphtheriticum* (ATCC 10700), *Nocardia asteroides* (ATCC 3308), *Nocardia brasiliensis* (ATCC 19296), *Nocardia orientalis* (ATCC 19795), *Streptomyces somaliensis* (ATCC

33201), *Streptomyces griseus* (ATCC 10137), *Streptomyces albus* (ATCC 3004), *Streptomyces gedanensis* (ATCC 4880), *Actinomyces israelii* (ATCC 10049), *Eubacterium lentum* (ATCC 43055), *Rhodococcus equi* (ATCC 6939), *Rhodococcus rhodochrous* (ATCC 13808), *Propionibacterium acnes* (ATCC 6919), *Actinoplanes auranticolor* (ATCC 15330), *Streptosporangium viridialbum* (ATCC 33328), and *Streptoverticillium alboverticillatum* (ATCC 29818).

DNA isolation

Stationary-phase cultures of mycobacteria in 60 mL of Middlebrook 7H9 broth were heat-killed 4 hours at 65°C and genomic DNA was isolated by enzymatic means (9). DNA from non-mycobacteria was isolated using proteinase K and hexadecyltrimethyl ammonium bromide (CTAB) (10). Concentations of genomic DNA were based upon published molecular weights (11) and 20 A_{260} units per mg of double-stranded DNA.

Strand displacement amplification

SDA was generally performed as previously described (8,12). [The IS6110 target (*M.tuberculosis* complex) and the 16S target (mycobacterial genus) are represented by the subscripts tb and g in the following discussion.] Previously we reported a single-target system for amplification of the IS6110 target using primers S_{tb1}, S_{tb2}, B_{tb1} and B_{tb2} (Table I) (12). For the present study, we first developed a single-target system to amplify the 16S target using primers S_{g1}, S_{g2}, B_{g1} and B_{g2} (Table I). We then developed a multiplex system to coamplify the IS6110 and 16S targets using S_{tb}, S_g, B_{tb1}, B_{tb2}, B_{g1}, B_{g2}, A_{tb} and A_g (Table I). The S_i, B_i and A_i primers were used as indicated at respective concentrations of 500, 25 and 50 nM.

In addition to primers, the multiplex SDA reactions (50 μL) represented in Figure 4 also contained 45 mM K_iPO_4 (pH 7.5), 0.1 mg/mL acetylated bovine serum albumin, 0.5 mM dUTP, 0.2 mM each dGTP, dCTP and dATPαS [2′-deoxyadenosine 5′-O-(1-thiotriphosphate], 6 mM $MgCl_2$, 12% (v/v) dimethyl sulfoxide (DMSO), 3% glycerol (contributed by the stock solutions of exo$^-$ Klenow and HincII), 50 ng of human placental DNA, 150 units (2 μL of 75 units/μL) of HincII (New England Biolabs), 2.5 units of exo$^-$ Klenow (United States Biochemical) and the indicated levels of bacterial target DNA. dUTP is used in place of dTTP in anticipation of an amplicon decontamination system based on uracil-DNA glycosylase (16). Samples were heat denatured for 2 minutes in a boiling water and then returned to 40°C before addition of HincII and exo$^-$ Klenow. SDA occurred over 2 hours at 40.0 ± 0.5°C in a temperature controlled water bath. The level of amplification observed is very sensitive to the exact temperature of incubation and K_iPO_4, pH, $MgCl_2$ and DMSO levels (8). A number of alternative reaction conditions produce equivalent results [e.g., 47.5 mM K_iPO_4, pH 7.6, 6.5 mM $MgOAc_2$ and 7.5% DMSO at 41°C or 45 mM K_iPO_4, pH 7.6, 5 mM $MgOAc_2$ and 10% DMSO at 41°C (data not shown)]. However, optimal K_iPO_4, pH, $MgOAc_2$ and DMSO levels are interactive, so they must be used in specific combinations as in the three examples listed above.

For experiments presented in Figure 5, conditions differed slightly from those described above (for Figure 4), in that the SDA reactions (50 μL) contained 350 ng human placental DNA, 7.5% (v/v) DMSO, and 47.5 mM K_iHPO_4 (pH 7.6). These samples also contained 500 copies of an 'SDA control' oligonucleotide (Table I), which is designed to be co-amplified with the other targets. All other components of the reaction were as described for experiments in Figure 4. After addition of enzymes, the reaction mixtures were incubated at 41°C for 2 hours.

Detection of amplified products

Following SDA, detection of amplified target molecules was performed (essentially as described (8,12)) by DNA polymerase-catalyzed extension of ^{32}P-labeled probes hybridized specifically to the central regions of the various targets. In the current work, samples were either probed simultaneously for the presence of both IS6110 and 16S targets (Figure 4), or these targets were detected seperately (Figure 5). In the first case, three detecter probes (D_{tb}, D_g and D_{ft}) were 5′ end-labeled together at a concentration of 1 μM each in 40 μL of 50 mM Tris−HCl (pH 8), 10 mM $MgCl_2$, 40 units of T4 polynucleotide kinase (New England Biolabs), and 300 μCi of gamma-^{32}P-ATP (3000 Ci/mmol, New England Nuclear); samples were incubated at 37°C for 30 min, and the kinase was then heat-killed by incubation at 95°C for two minutes; the labeled probes were used without further purification.

For simultaneous detection (Figure 4), a 5 μL aliquot of each SDA reaction was mixed with (i) 5 μL of 45 mM K_iPO_4 (pH 7.5), 0.1 mg/mL acetylated bovine serum albumin, 0.5 mM dUTP, 0.2 mM each dGTP, dCTP and dATPαS, 6 mM $MgCl_2$ and (ii) 1 μL of 1 μM each ^{32}P-detector probe (D_{tb}, D_g and D_{ft}). The samples were heat-denatured 2 minutes in a boiling water bath and then incubated 2 minutes in a 40°C water bath before addition of 2 μL of 1 unit/μL exo$^-$ Klenow. Samples were incubated 15 minutes at 40 °C and then 13 μL of 50% (w/v) urea, 20 mM Na_2EDTA, and 0.05% bromphenol blue/xylene cylanol were added. During the ^{32}P-primer extension reaction, D_{tb} binds to the IS6110 amplification product and is extended by the polymerase to a length of either 41 or 63 nucleotides, depending on whether the probe is hybridized to a nicked or unnicked target molecule. Similarly, a positive 16S signal results from hybridization and extension of D_g and D_{ft}, yielding products 30 and 51 nucleotides long. The various extension products were separated by electrophoresis on denaturing polyacrylamide gels, which were then analyzed by autoradiography (3 hour exposure at −70°C using Fuji RX film).

Essentially the same protocol was used for seperate detection of the amplified target molecules (Figure 5): all conditions were as described above for simultaneous detection except that only a single ^{32}P-labeled detector probe (either D_{tb}, D_g, or $D_{control}$, depending on target to be detected) was used in each primer extension reaction; individual extension reactions were then analyzed by denaturing polyacrylamide gel electrophoresis/autoradiography (16 h exposure at −70°C). The probes D_{tb} and D_g yield products as described above, while $D_{control}$ hybridizes only to the SDA control target and yields, upon extension, products containing 46 and 67 nucleotides.

RESULTS

Previously, we reported an SDA system for detection of DNA from members of the *M.tuberculosis* complex (*M.tuberculosis*, *M.bovis*, *M.bovis*-BCG, *M.africanum* and *M.microti*) (12,13).

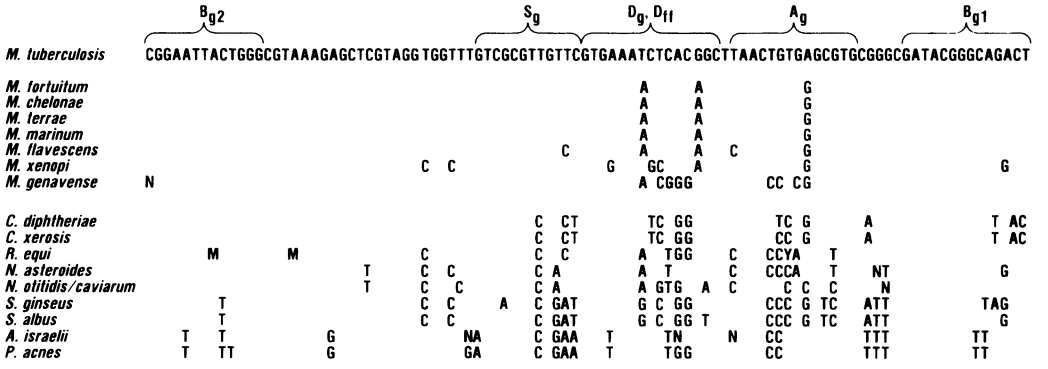

Figure 2. Sequence alignments for the mycobacterial genus target. A subsequence of the 16S ribosomal gene (*E.coli* nucleotide positions 556–652) (20) is shown for the indicated organisms (GenBank accession numbers are indicated). The *M.tuberculosis* sequence (x52917, nucleotide positions 507–603 is identical to that from *M.bovis* (x55589), *M.bovis*-BCG , *M.avium* (x52918), *M.intracellulare*(x52927), *M.kansasii* (x15916), *M.gastri* (x52919), *M.paratuberculosis* (x52934), *M.malmoense* (x52930), *M.szulgai* (x52926), *M.gordonae* (x52923), *M.leprae* (x53999), *M.ulcerans* (z13990), *M.asiaticum* (x55604), *M.scrofulaceum* (x52924) and *M.hemophilum*. Variations from the *M.tuberculosis* sequence are indicated for *M.fortuitum* (x52933), *M.chelonae* (x52921), *M.terrae* (m29568), *M.marinum*, *M.flavescens* (m29561), *M.xenopi* (m61664), *M.genavense* (x60070), *Corynebacterium diphtheriae*, *Corynebacterium xerosis* (m59058), *Rhodococcus equi* (m29574), *Nocardia asteroides* (x57949), *Nocardia otitidis/caviarum* (m59056), *Streptomyces griseus* (m76388, x55435), *Streptomyces albus* (x53163), *Actinomyces israelii* (x53228) and *Propionibacterium acnes* (m61903). [The sequence of our strain of *M.marinum* differs from the GenBank listing of *M.marinum* (x52920), which indicates a sequence identical to *M.tuberculosis*.] The target binding regions of the 16S primers and detector probes used in multiplex SDA are indicated (complete primer sequences are listed in Table I). 'N' represents unidentified nucleotides. 'Y' represents C or T. 'M' represents A or C.

As few as 5 genome copies of target DNA were detected using a target sequence from the IS6110 insertion element (14). In the present work, we describe a multiplex system that is able to co-amplify the IS6110 target and a subsequence of the 16S ribosomal gene that is conserved over most clinically relevant mycobacterial species.

The 16S rDNA target sequence is present in *M.tuberculosis*, *M.bovis*, M.bovis-BCG, *M.avium*, *M.intracellulare*, *M.kansasii*, *M.gastri*, *M.paratuberculosis*, *M. malmoense*, *M.szulgai*, *M.gordonae*, *M.leprae*, *M ulcerans*, *M.asiaticum* and *M.scrofulaceum* (Figure 2). A nearly identical sequence, differing at only 3 nucleotide positions, is also found in *M.terrae*, *M.chelonae*, *M.marinum* and *M.fortuitum*, while *M.flavescens*, *M.xenopi* and *M.genavense* exhibit greater divergence. This mycobacterial 'genus' target differs from corresponding sequences in organisms that are generally similar to mycobacteria (Figure 2).

Table I. List of primer and SDA control sequences

S_{tb1}	5'dTTGAAGTAACCGACTATTG*TTG*ACACTGAGATCCCCT
$S_{tb2} = S_{tb}$	5'dTTGAATAGTCGGTTACTTG*TTG*ACGGCGTACTCGACC
A_{tb}	5'dGTCGCGTTGTTCACTGAGATCCCCT
B_{tb1}	5'dCGCTGAACCGGAT
B_{tb2}	5'dTGGACCCGCCAAC
D_{tb}	5'dTCCGTATGGTGGATA
S_{g1}	5'dTTCCATAGTCCCAATCTTG*TTG*ACGCTCACAGTTA
$S_{g2} = S_g$	5'dTTCTATAGTCGGTTACTTG*TTG*ACGTCGCGTTGTTC
A_g	5'dGGCGTACTCGACCACGCTCACAGTTA
B_{g1}	5'dAGTCTGCCCGTATC
B_{g2}	5'dCGGAATTACTGGG
D_g	5'dGCCGTGAGATTTCAC
D_{ft}	5'dGCTGTGAGTTTTCAC
SDA Control	5'dGACGGCGUACUCGACCAGCGACGAUGUCUGAGGC-AACUAGCAAAGCTGAACAACGCGAC
$D_{control}$	5'dGCTTTGCTAGTTGCC

HincII recognition sequences are indicated in S_{tb1}, $S_{tb2} = S_{tb}$, S_{g1} and $S_{g2} = S_g$ by italics.

Conventional multiplex SDA

As described in Figure 1, amplification of a single target sequence by SDA requires a set of four primers (designated in Figure 1 as B_1, B_2, S_1 and S_2). Obviously, co-amplification of multiple target sequences by this method will generally require one set of four primers for every target to be amplified. In earlier work (12), we described a set of primers (S_{tb1}, S_{tb2}, B_{tb1}, B_{tb2}; Table I) for amplifying a 52 base-pair fragment of the IS6110 element of *M.tuberculosis*. We have since designed a set of primers (S_{g1}, S_{g2}, B_{g1} and B_{g2}; Table I) able to amplify a segment of the 16S rRNA gene that is unique to genus *Mycobacterium*. Although both the IS6110 and 16S rDNA fragments can be amplified $>10^8$ fold in seperate SDA reactions, the efficiency of 16S rDNA amplification is sharply reduced when one attempts to co-amplify both target sequences by combining all eight primers in a single reaction mixture (data not shown). In contrast, the IS6110 sequence is efficiently amplified in this mixture. It thus appears that the more efficient amplification of the IS6110 target outstrips the amplification of the 16S target in a competition for some limiting reaction component (probably the restriction enzyme HincII). Frequently we find that yields of all targets are diminished in convential multiplex reactions, apparently due to an increased level of background (non-target) amplification triggered by errant priming events, which generally become more prevalent as primer numbers increase. Clearly, efficient co-amplification of both targets will require a close balancing of amplification rates, and efficiencies will generally be improved by a minimizing the number of amplification primers.

Adapter-mediated multiplex SDA

In an effort to overcome the problems associated with convential multiplex SDA, we devised an alternative approach that makes use of adapter oligonucleotides to introduce common priming sequences onto the ends of the different target sequences (Figure 3). The various modified target molecules are then co-amplified,

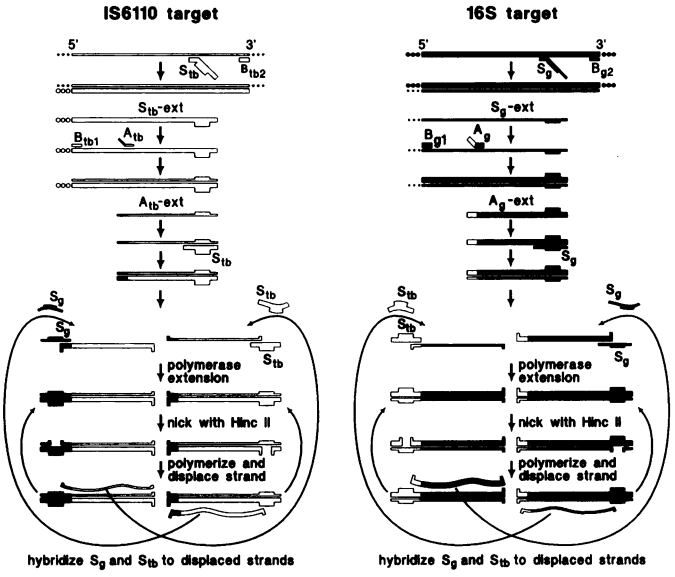

Figure 3. Multiplex SDA scheme for the IS6110 and 16S targets. [Complementary strands of the IS6110 target sequence are depicted by thick and thin open-box lines while the 16S target sequence is correspondingly represented by filled-box lines.] The genomic DNA sample is heat denatured in the presence of S_{tb}, S_g, B_{tb1}, B_{tb2}, B_{g1}, B_{g2}, A_{tb} and A_g. [The HincII recognition sites on S_{tb} and S_g are represented by the raised boxes.] As the sample is cooled to 40°C, S_{tb} and B_{tb1} bind to the IS6110 target segment, and likewise S_g and B_{g1} bind to the 16S target. The four primers are then extended by exo$^-$ Klenow using dGTP, dCTP, dUTP, and dATPαS. Extension of the upstream primers (B_{tb1} and B_{g1}) causes displacement of the downstream extension products from S_{tb} and S_g (S_{tb}-ext and S_g-ext), which are then free to bind primers B_{tb2} and B_{g2} and adapter primers A_{tb} and A_g. These primers are then extended by exo$^-$ Klenow, resulting in displacement of the extension products A_{tb}-ext and A_g-ext. The 5′-tail (in black) of the A_{tb} adapter primer is identical to the target binding region (3′-end) of SDA primer S_g, whereas the 5′-tail (in white) of A_g is identical to the target binding region of SDA primer S_{tb}. S_{tb} and S_g bind respectively to A_{tb}-ext and A_g-ext and are extended. These complexes represent intermediates in the SDA cycles (shown at the bottom of the figure), in which the IS6110 and 16S targets are exponentially amplified in the presence of an excess of S_{tb} and S_g.

with approximately equal efficiency, by a single pair of amplification primers.

Like conventional SDA (Figure 1), adapter-mediated SDA begins with a target generation process, shown in the top half of Figure 3, which converts a copy of the original target sequence into a form that can be amplified exponentially by the nicking/stand displacment cycle shown in the lower half of Figure 3. Unlike the conventional SDA (Figure 1), however, the target generation cascade in Figure 3 uses an adapter oligonucleotide to alter the primer specificity of the target sequence. Thus, on the lefthand side of Figure 3, a 16S priming sequence (in black) is appended to one end of the IS6110 target (in white), while, on the righthand side of Figure 3, an IS6110 priming sequence (in white) is appended to one end of the 16S target sequence (in black). These modifications permit both targets to be amplified exponentially by a single pair of amplification primers, namely S_{tb} and S_g which bind to sequences derived from the IS6110 and 16S targets, respectively.

The sequences of the various primers (S_{tb}, S_g, B_{tb1}, B_{tb2}, B_{g1}, B_{g2}, A_{tb} and A_g) involved in the target generation cascade are listed in Table I. A_{tb} is the adapter primer that introduces a 16S SDA primer sequence (i.e., the 3′-end of S_g = S_{g2}) onto the end of an IS6110 target fragment. Thus, its 3′ end (white in Figure 3, left side) consists of a 13 nucleotide sequence that binds to a segment of the IS6110 target, and its 5′ end (black in Figure 3, left side) consists of a 12 nucleotide sequence identical to the 3′ terminal sequence of amplification primer S_g, which is complementary to a segment of the 16S target. Similarly, adapter A_g, which introduces an IS6110 SDA primer sequence (3′-end of S_{tb} = S_{tb2}) onto the end of a 16S fragment, contains (i) a sequence of 14 nucleotides, at its 3′ end (black in Figure 3, right side), that bind directly to the 16S target and (ii) a 13 nucleotide sequence, on its 5′ end (white in Figure 3, right side), identical to the IS6110 target binding region of S_{tb}.

In the presence of appropriate primers and DNA polymerase, target generation originates spontaneously from both complementary strands of a given target, although Figure 3 depicts the process for only one strand. The top lefthand side of Figure 3 shows the generation of modified target molecules arising from the IS6110 sequence: After heat denaturation of the genomic target DNA, primers S_{tb} and B_{tb2} bind to one strand of the genomic IS6110 target sequence and are then extended by the exo$^-$ Klenow fragment. The S_{tb} extension product (S_{tb}-ext) is displaced as B_{tb2} is extended. B_{tb1} and A_{tb} primers then bind to the displaced S_{tb}-ext and are likewise extended, resulting in displacement of the A_{tb} extension product (A_{tb}-ext) which contains a 5′ terminal sequence identical to the target binding region of S_g and a 3′ terminal sequence complementary to S_{tb}. An S_{tb} primer then binds A_{tb}-ext, which forms a double-stranded, hemithiolated HincII recognition site resulting from polymerase incorporation of dGTP, dCTP, dUTP and dATPαS in all steps in Figure 3. S_{tb} is extended along A_{tb}-ext forming a double-stranded complex that is essentially identical to one of the intermediates in the exponential SDA cycle at the bottom of Figure 3. This complex automatically enters the SDA cycle, where the modified IS6110 target is amplified exponentially by primers S_{tb} and S_g, present in large molar excess. A completely analogous series of steps originates from the genomic 16S target sequence (rightside of Figure 3), leading to exponential amplification of this target.

This multiplex form of SDA is performed by the same simple protocol previously used for amplification of a single target sequence (12). Genomic target DNA is heat denatured in the presence of all reagents except HincII and exo− Klenow. After the sample returns to 40°C, the enzymes are added, and SDA proceeds at 40°C for 2 hours. Adapters (A_{tb} and A_g) are employed at a concentration (50 nM) 10-fold lower than amplification primers S_{tb} and S_g (500 nM). This helps ensure that adapters will not bind to displaced target strands before they are captured by S_{tb} or S_g.

Amplification of mycobacterial DNA

Adapter-mediated multiplex SDA was performed on an array of samples representing the most common mycobacterial pathogens (Figure 4). The mycobacterial species are arranged from left to right in Figure 4, generally according to decreasing clinical prevalence. Following SDA, each sample was simultaneously probed for the presence of both IS6110 and 16S targets: a cocktail containing three target specific ^{32}P-labeled probes (D_{tb}, D_g, and D_{ft}) was mixed with an aliquot (one-tenth total) of each SDA sample and this mixture was treated with DNA polymerase (exo− Klenow) to allow extension of probes hybridized to target. Probe D_{tb} is specific for the IS6110 sequence of the *M.tuberculosis*

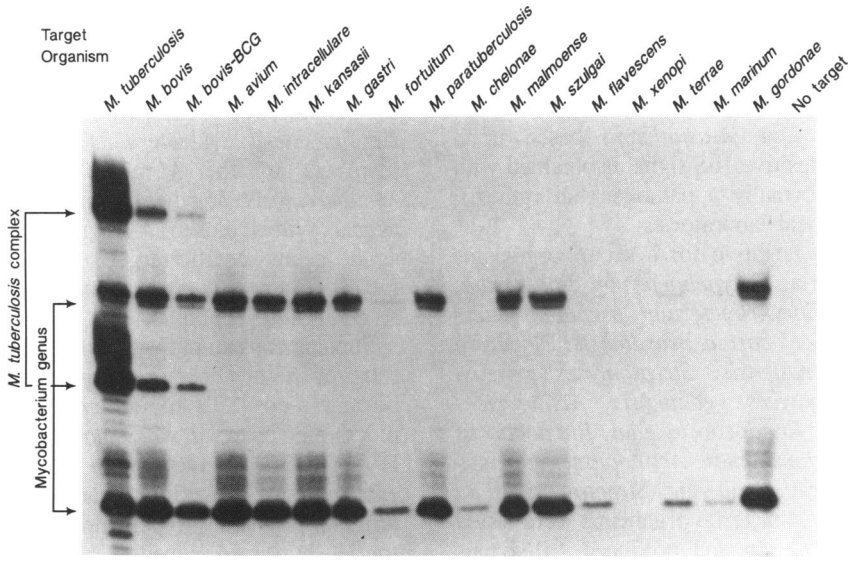

Figure 4. Specificity of multiplex SDA for mycobacterial species. Samples contained 1000 genome copies of DNA from the indicated mycobacterial species. 'No target' represents a sample without mycobacterial target DNA. One tenth of each SDA reaction was detected using ^{32}P-primer extension. ^{32}P-bands corresponding to amplification products for the IS6110 target (*M.tuberculosis* complex) and the 16S target (mycobacterial genus) are indicated.

complex, while D_g and D_{ft} hybridize to the two 16S target sequences represented by *M.tuberculosis*, *M.bovis*, *M.bovis*-BCG, *M.avium*, *M.intracellulare*, *M.kansasii*, *M.gastri*, *M.fortuitum*, *M.paratuberculosis*, *M.chelonae*, *M.malmoense*, *M.szulgai*, *M.flavescens*, *M.terrae*, *M.marinum* and *M.gordonae* (Figure 2). The primer extension mixtures were analyzed by denaturing polyacrylamide gel electrophoresis, which separates IS6110-specific extension products from those specific for the 16S targets (Figure 4).

Positive IS6110 and 16S signals were obtained for *M.tuberculosis*, *M.bovis* and *M.bovis*-BCG as expected since they are members of the *M.tuberculosis* complex. For *M.tuberculosis*, the IS6110 signal is stronger than the 16S signal because it contains ~10 copies of the IS6110 element (13) compared with a single copy of the 16S ribosomal gene. Roughly equivalent IS6110 and 16S signals are obtained with *M.bovis* and *M.bovis*-BCG because they contain one to two copies of the IS6110 element and a single copy of the 16S ribosomal gene. IS6110 signals are not obtained with any of the other mycobacterial species since they are not members of the *M.tuberculosis* complex. The SDA conditions and ^{32}P-detection protocol presented in Figure 4 provide detection of both targets starting with as few as ~5 genome copies of *M.tuberculosis* DNA in samples containing 50 ng of non-target (human) DNA (data not shown).

As expected positive 16S signals are detected for *M.avium*, *M.intracellulare*, *M.kansasii*, *M.gastri*, *M.paratuberculosis*, *M.malmoense*, *M.szulgai* and *M.gordonae*, since their 16S target sequences are identical to that of *M.tuberculosis*. Relatively weak signals are obtained with *M.fortuitum*, *M.chelonae*, *M.terrae* and *M.marinum*, which at first glance was not too surprising because adapter A_g forms a T·G mismatch with these species (Figure 2). However, we performed SDA experiments with a version of A_g that was a perfect match for these species and still obtained the same relatively weak signal intensities (data not shown). Perhaps the 16S sequences of these species possess a

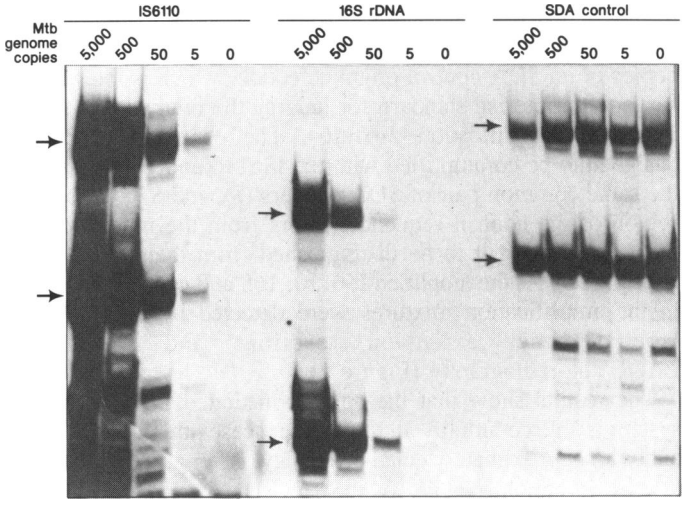

Figure 5. Autoradiogram showing the results of multiplex SDA of the IS6110 and 16S rDNA targets together with an internal SDA control oligonucleotide. Five amplification reactions were assembled containing 5000, 500, 50, 5, or 0 copies of genomic *M.tuberculosis* DNA and 500 copies of an internal control oligonucleotide. After amplification, 5 μL aliquots of each 50 μL reaction were seperately analyzed for the presence of one of three amplified sequences (IS6110, 16S rDNA, or SDA Control) by polymerase-catalyzed extension of a detector probe hybridized specifically to that sequence (see Materials & Methods); extension products and unreacted probes were seperated by electrophoresis on a denaturing polyacrylamide gel, which was then subjected to autoradiography. Arrows indicate expected (nicked and unnicked) product bands.

secondary structure that interferes with SDA. Surprisingly, the signal obtained with *M.flavescens* is as strong as that from *M.fortuitum* despite an additional A·C mismatch at the 3'-end of adapter Ag and a T·G mismatch at the penultimate 3'-position of SDA primer Sg. A 16S signal was not obtained with *M.xenopi*, which we expect is due to a poor match with the detector probes since its binding regions for S_g, A_g and B_{g1} are identical to those

of *M.fortuitum* for example. We did not test *M.genevense* and do not expect it to amplify based upon its published sequence (Figure 2). Positive 16S results are also expected for other mycobacterial species (e.g., *M.scrofulaceum*, *M.leprae*, *M.ulcerans*, *M.hemophilum* and *M.asiaticum*) based upon published 16S sequences. A positive 16S signal is obtained with *M.gordonae* which is not normally a pathogen but rather a common contaminant in clinical laboratories.

The multiplex system was negative for a set of organisms closely related to mycobacteria: *Corynebacterium diphtheriae*, *Corynebacterium xerosis*, *Corynebacterium pseudodiphtheriticum*, *Nocardia asteroides*, *Nocardia brasiliensis*, *Nocardia orientalis*, *Streptomyces somaliensis*, *Streptomyces griseus*, *Streptomyces albus*, *Streptomyces gedanensis*, *Actinomyces israelii*, *Eubacterium lentum*, *Rhodococcus equi*, *Rhodococcus rhodochrous*, *Propionibacterium acnes*, *Actinoplanes auranticolor*, *Streptosporangium viridialbum*, and *Streptoverticillium alboverticillatum*. All non-mycobacterial organisms were tested at 10^5 genomes per SDA reaction and produced ^{32}P-signals equivalent to background (data not shown).

Co-amplification of targets and internal control sequence

To test the system's sensitivity in the presence of a high level (350 ng/reaction) of non-target (human) DNA, which is known to depress target amplification (12), multiplex SDA was performed on samples containing various levels of genomic *M.tuberculosis* DNA, ranging from 0 to 5000 genome copies per reaction. All samples in these experiments also contained 500 copies of an SDA control oligonucleotide, which is intended to serve as an internal standard for judging the relative efficacy of individual amplification mixtures. The control sequence is designed to be co-amplified with the IS6110 and 16S targets by the same common pair of SDA primers (S_{tb} and S_g); the central region of the control sequence differs from the mycobacterial targets, allowing it to be distinguished from them. Following multiplex SDA, the amplified IS6110, 16S and control sequences in the amplification mixtures were detected by seperate ^{32}P-labeled-primer extension reactions and subsequent PAGE/autoradiography (Figure 5).

The results show that the adapter-mediated SDA multiplex system is able co-amplify all three sequences with high sensitivity and with approximately equal efficiency. After amplification, as few as 50 genome copies of the 16S rDNA target are readily detectable, while the IS6110 target can be detected in reactions containing as few as 5 genome copies. Because each *M.tuberculosis* genome contains (on average) ~10 copies of the IS6110 insertion element and one copy of the 16S rDNA gene, the observed ~10-fold difference in signal intensity between these two targets (e.g. compare signal intensities in the 50 genome copy lanes) implies that they really have nearly identical intrinsic amplification rates in this system. The internal control sequence (present at pre-amplification levels of 500 copies per reaction mixture) is also amplified at a comparable rate, judging from the fact that its signal intensity (all lanes) is comparable to the IS6110 and 16S lanes from reactions containing pre-amplification levels of 500 target copies.

DISCUSSION

We have developed a general and very sensitive SDA-based method for co-amplifying multiple DNA target sequences by means of a single pair of amplification primers. This multiplex process was applied to the amplification of various mycobacterial target sequences and forms the basis of a simple DNA-based test that enables one to perform a genus-level screening of samples for the presence of most clinically relavent mycobacteria and to simultaneously evaluate whether any such bacteria present are members of the *M.tuberculosis* complex (consisting of *M.tuberculosis*, *M.bovis*, *M.africanum*, *M.microti*, all causative agents of tuberculosis). A fragment of the 16S rRNA gene serves as the genus-specific target sequence, while a fragment of the IS6110 insertion element serves as the target specific for the *M.tuberculosis* complex.

Simultaneous amplification of multiple target sequences usually requires the presence of one pair of primers for every target to be amplified. This multiplicity of primers tends to promote mispriming events in which non-target DNA (profusely abundant in most clinical samples) can act as template for errant primer extension (7). Mispriming can also occur between the various primers, producing so-called primer dimers. Once formed, such spuriously primed sequences can undergo amplification as readily as bona-fide targets. In the late (post-exponential) stages of an amplification reaction, background and target-specific amplification begin to compete for the same pool of limiting reagents (which in the case of SDA is the restriction enzyme HincII), slowing further increases in target production. Formation of amplifiable background DNA generally increases as more amplification primers are added to a mixture. In solutions containing multiple primer pairs, the quantity of amplified background product formed can greatly exceed the level of amplified target, resulting in sharply diminished target yield. Thus, target signals are usually much lower in multiplex amplification reactions than in corresponding single-target reactions containing only single pairs of primers.

Multiplex amplification can also suffer from inequalities in the rates of amplification for the different targets. Extremely efficient amplification of one target can sharply curtail production of other target sequences. Hybridization efficiencies and other factors must be closely matched between the various targets to ensure that post-amplification target levels accurately reflect pre-amplification levels.

Figure 3 outlines the approach we developed for amplifying multiple target sequences using a single pair of amplification primers, which reduces competing background reactions due to mispriming and permits accurate balancing of amplification efficiencies for different targets. The process involves a series of spontaneous primer extension steps in which target sequences are converted by adapter primers into modified forms that contain arbitrary sequences affixed to their ends. The terminal sequences attached by this method are derived from the 5′ tail sequence of the adapter molecule. By choosing the sequence of these arbitary terminal segments, one can then control the identity of the primers required for target amplification. The method can thus be used to introduce common priming sequences onto the ends different target sequences, allowing them to be co-amplified by a common pair of amplification primers.

The adapter primers themselves cannot serve as amplification primers because they do not possess the HincII recognition sequences required for SDA. Consequently, adapter molecules do not promote background amplification in SDA (provided appropriate care is taken to avoid the possiblity of mutual priming between adapter and amplication primers.) This is in contrast to ligase-mediated PCR (17) and other PCR-based (18,19) methods for introducing common primers onto the ends of

different targets. In those cases, the adapter or linker oligonucleotides, which contain extendable 3′ ends, have the potential to act as errant amplification primers, leading to the possibility of increased levels of background amplification.

Despite its formidable appearance, the workflow for the adapter-mediated SDA multiplex system is very simple: A sample containing all the SDA reagents except enzymes is heat-denatured, cooled and then exo$^-$ Klenow and HincII are added. Target sequences are modified spontaneously and then automatically enter the amplification cycle with no outside intervention.

In the current work, the 5′ tail sequences of the adapters were chosen so that one end of the IS6110 target is modified to contain a sequence corresponding to an end of the 16S target. This modified IS6110 target can then be amplified by one SDA primer possessing the original IS6110 sequence specificity and a second SDA primer with specificity corresponding to one end of the 16S target. The 16S target is similarly modified to contain a terminal sequence corresponding to the unmodified end of the IS6110 target. With these terminal modifications, both target sequences can be amplified very efficiently by the same pair of amplification primers S_{tb} and S_g. Both targets were co-amplified from genomic *M.tuberculosis* DNA with high yield (108-fold amplification) and approximately equal efficiency (Figure 5), permitting detection of as few as a 5 genome copies of *M.tuberculosis* DNA (ten IS6110 copies per genome) or 50 copies of genomic DNA from other mycobacteria (one 16S rDNA copy per genome), even in the presence of high levels of non-target DNA.

The similarity of intrinsic amplification rates for the two targets seems to be a consequence of both targets sharing common priming sequences. In conventional multiplex reactions, where the two targets retained their original priming sequences and were co-amplified in the presence of four SDA primers (S_{tb1}, S_{tb2}, S_{g1} and S_{g2}), the IS6110 sequences was amplified in much higher yield than the 16S rDNA target (data not shown). Presumably hybridization rates or other rate-determining factors are more closely matched in the terminally modified targets. This close balancing of amplification rates may prove useful in situations where one seeks to deduce, from post-amplification target levels, the relative abundance of different targets present before amplfication.

The task of quantifying pre-amplification target levels may also be aided by including a control oligonucleotide in each reaction to serve as an internal calibration standard. For this application, it is important that the intrinsic amplification rate of the internal standard be comparable to that of the target sequences. We designed such an internal control for the current work, and found that the two target sequences (IS6110 and 16S fragments) and the control molecule can all be co-amplified with nearly equal efficiency (Figure 5), opening the possiblity of performing quantitative SDA on two independent targets in a single reaction vessel.

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