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Improving isothermal DNA amplification speed for the rapid detection of *Mycobacterium tuberculosis*

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ABSTRACT

In this study, we report the development of a helicase-dependent amplification assay for rapid detection of *Mycobacterium tuberculosis* (MTB). By applying a step-by-step optimization method, the amplification time from an input of 2-copy MTB genomic DNA was reduced from about 60 min to less than 30 min.

Tuberculosis (TB) is a highly contagious bacterial infection induced by various strains of mycobacterium known as the *Mycobacterium tuberculosis* (MTB) complex. Over the past two decades, many nucleic acid amplification methods have been published for the direct detection of MTB. Among them, several isothermal nucleic acid amplification methods have been developed for their advantage of not requiring any expensive thermocycler. For example, BD Biosciences developed the BDProbeTec™ MTB assay, a method based on the isothermal strand displacement amplification (SDA) (Down et al., 1996). According to several evaluation studies, this method possesses a high degree of specificity and sensitivity (Ichiyama et al., 1997, McHugh et al., 2004), but it has a disadvantage of requiring a costly amplifier/analyzer for the detection of the amplification product. More recently, Aryan et al. published a Loop-Mediated isothermal AMplification (LAMP)-based assay with a sensitivity of about 1 genome copy per assay without any costly instruments (Aryan et al., 2010). However, the sensitivity of this LAMP-based assay is dependent on an initial heat denaturation of the template at 95 °C for several minutes before the isothermal amplification. The amplification requires a rather long incubation time of 60 to 90 min. The assay also lacks an internal control for monitoring inhibition in input samples, thus increasing the possibility of a false negative result.

Here we describe a highly sensitive and rapid amplification assay for the detection of *M. tuberculosis*, based on the Helicase-Dependent Amplification or HDA (Vincent et al., 2004). Several articles have been published on using HDA for the detection of various pathogenic organisms (Chow et al., 2008, Gill et al., 2008, Gill et al., 2007, Goldmeyer et al., 2008, Tong et al., 2008). However, all of these published HDA methods require at least one hour amplification time.

For the development of the HDA-based MTB assay, the target gene selected was the insertion sequence 6110 (IS6110), which is conserved in the TB causing bacteria belonging to the MTB complex and has been used previously (Aryan et al., 2010, Down et al., 1996). Primers targeting IS6110 in *M. tuberculosis* genome were screened using the IsoAmp® II Universal tHDA kit (BioHelix, Beverly, MA, USA) and MTB genomic DNA (gDNA) from strain H37Ra (ATCC #25177D-5) as input template. The pair of primers, IS6110-F and IS6110-R (Table 1), displayed the highest amplification efficiency during initial screening and was selected for further optimization.

The speed of the HDA-based MTB assay was optimized by successive modifications of the amount of primers, Enzymes and Ficoll® PM 400 (Sigma-Aldrich, St. Louis, MO, USA) in the amplification reaction. The amount of each component was first optimized using real-time HDA by selection of the reaction conditions resulting in the fastest amplification of 20 copies of TB gDNA. The speed and sensitivity of each condition were then further tested using 200, 20 and 2 copies of gDNA (see Fig. 1). The amplification reactions were monitored in real-time using an ABI 7300 real-time thermocycler (Applied Biosystems, Foster City, CA, USA), in the presence of 0.2× Evagreen (Biotium Inc., Haywood, CA, USA) reporter dye and 1× ROX (Invitrogen, Carlsbad, CA, USA) reference dye. The reactions were incubated for 60 to 90 min at 65 °C–66 °C followed by melting curve analysis. As shown in Fig. 1, the speed of the amplification was significantly increased by the increase of the primer concentration from 75 nM to 200 nM each (Fig. 1, Green line) and the increase of the IsoAmp® III Enzyme mix (BioHelix, Beverly, MA, USA) from 1× to 3×

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Table 1
Nucleotide sequences used in this study.

Name	Sequence (5′–3′)	Orientation	Label	Target
IS6110-F	CAACAAGAAGGCGTACTCGACCTGA	F	–	IS6110 and IC
IS6110-R	CTCGCTGAACCGGATCGATGTGTACT	R	BioTEG	IS6110 and IC
IS6110-FITC	CGTTATCCACCATACGGATA[6-Fam-Q]	F	FITC	IS6110
IC-DIG	TATCACGCTTTCGAGTGTC[AmC7-Q-Dig]	F	DIG	IC

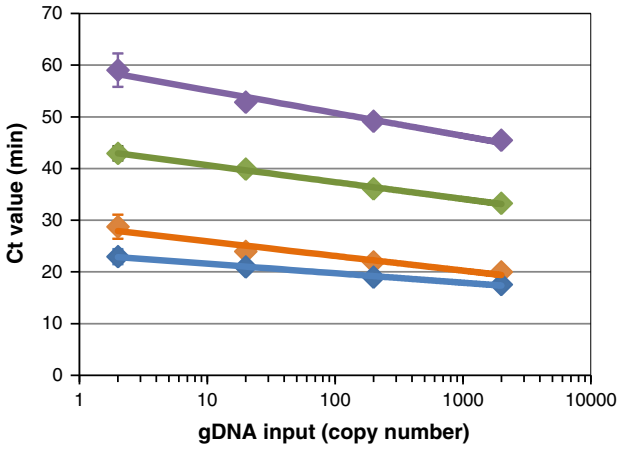

Fig. 1. Reaction speed optimization of the HDA-based MTB assay. The amplification of 2, 20, 200 and 2000 copies of MTB gDNA in various buffer compositions was monitored by real-time and the C_t values were determined as the time for the amplification signal to cross the threshold value of 0.1. Purple, 10 mM KCl, 20 mM Tris–HCl [pH 8.8 at 25 °C], 4.4 mM $MgSO_4$, 40 mM NaCl, 400 µM each dNTP, 4.65 mM dATP, 75 nM IS6110-F, 75 nM BioTEG-IS6110-R and 1× IsoAmp® III Enzyme Mix; Green, same as purple except that the amount of primers is increased to 200 nM each; Orange, same as green except that the amount of IsoAmp® III Enzyme Mix is increased to 3×; Blue, same as orange with the addition of 5% Ficoll 400. Each reaction was done in triplicates and two negative controls (NC) were included in each run, which did not yield any amplification signal.

(Fig. 1, Orange line). Acting as a molecular crowding reagent, Ficoll® PM 400 increases the effective concentrations of all the reagents and the addition of 5% Ficoll 400 increased the amplification speed further resulting in detection of 2 copies of gDNA in about 22 minutes in the real-time assay (Fig. 1, Blue line).

An internal control (IC) was incorporated into the assay to monitor amplification failures due to inhibitions. The IC sequence was constructed as a random sequence with a similar GC content and the same length as the IS6110 target, flanked by the same primer sequences of IS6110-F and IS6110-R. The IC sequence was cloned in the pCRII-blunt-TOPO vector (Invitrogen). The amount of the internal control plasmid in the final assay format was determined to be 10^5 copies per assay because at this amount, the amplification of IC was robust and did not interfere with the amplification of the target at the limit of detection (LoD, 2 genome copies, Fig. 2).

The sensitivity of the HDA-based MTB assay was determined by amplification of different amounts of gDNA for 30 min at 65 °C in a heat block followed by detection of the specific amplicons using the BESt™ cassette amplicon detection system (BioHelix) (Chow et al., 2008, Goldmeyer et al., 2008). The cassette detection requires 2 probes, each labeled with FITC or DIG, for the specific hybridization with the target or the IC amplicon respectively (Table 1). For amplicon detection using the BESt™ cassette, the 5′ end of IS6110-R primer was labeled with biotin (BioTEG-IS6110-R). The primer amounts were modified individually to increase the synthesis of biotin-labeled single-stranded DNA amplicon by using asymmetrical conditions and

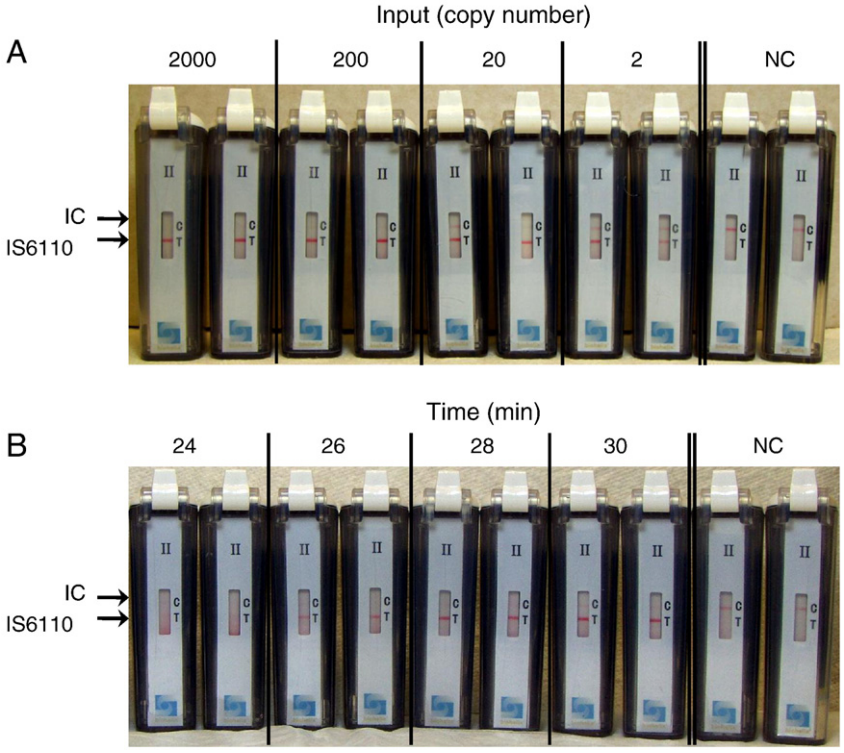

Fig. 2. Sensitivity of the HDA-based MTB assay. A. 2000, 200, 20 or 2 copies of gDNA were amplified using the HDA-based MTB assay for 30 min at 65 °C and analyzed by cassette detection. B. 20 copies of gDNA were amplified using the HDA-based MTB assay for 24, 26, 28 or 30 min at 65 °C and analyzed by cassette detection. Each reaction was done in duplicates. Two negative controls (NC) were included in each run and incubated for 30 min at 65 °C.

Table 2
Bacterial strains used for primer specificity test in this study.

Bacterial species (no. of isolates tested)	ATCC number	HDA results
Klebsiella aerogenes (4)	700603, BAA1706, 700721, BAA-1075	–
Proteus mirabilis (1)	12453	–
Burkholderia cepacia (1)	BAA-245	–
Stenotrophomonas maltophilia (1)	13637	–
Ralstonia solanacearum (1)	11696	–
Acinetobacter sp. (1)	49467	–
Serratia marcescens (1)	27137	–
Xanthobacter autotrophicus Py2 (1)	BAA-1158	–
Aeromonas hydrophila (1)	7966	–
Providencia stuartii (1)	33672	–
Salmonella enterica (1)	10708	–
Pseudomonas alcaligenes (1)	14909	–
Pseudomonas chlororaphis (1)	55670	–
Pseudomonas oryzihabitans (1)	43271	–
Pseudomonas pseudoalcaligenes (1)	700437	–
Pseudomonas stutzeri (1)	17587	–
Pseudomonas taetrolens (1)	4683	–

The reaction conditions were the same as those used in the experiments shown in Fig. 1 as Blue line. The reaction mix was incubated at 65 °C in an ABI 7300 real-time PCR thermocycler, and amplification of specific target was monitored by the EvaGreen fluorescent signal, followed by melting curve analysis. For these primer specificity tests, probes or internal control were not included in the reaction. Positive control reactions containing 5 or 50 copies of the TB genomic DNA were included in each real-time HDA run.

the optimal amplicon detection was achieved using 125 nM and 150 nM of IS6110-F and BioTEG-IS6110-R respectively. Reactions were assembled using the optimal amount of each reagent and were incubated at 65 °C in a heat block for 30 minutes. After the amplification the reaction tube was placed directly into a type II BESt™ cassette for amplicon detection (Goldmeyer et al., 2008). Closing the cassette-lid forcing the tube to release amplicon into a vertical-flow DNA strip in the closed device allowed colorimetric detection of amplicons without cross contamination. The readout was analyzed after 20 min. As shown in Fig. 2A, as low as 2 copies of gDNA were efficiently amplified and detected by the HDA-based MTB assay. A time course assay showed that 20 copies of gDNA could be efficiently amplified and detected after 26 min of amplification (Fig. 2B).

The specificity of the HDA-based MTB assay was tested using 5×10^5 copies per reaction of genomic DNA isolated from 20 non-MTB bacterial strains (Table 2). They were all negative in HDA amplification after 60-minute incubation and cross-reactivity was not observed.

In conclusion, the amplification speed of the HDA-based MTB assay has been significantly improved in this study by optimization of various reaction conditions, including primer concentration, molecular crowding reagents, and amplification enzymes. By combining these speed-enhancing methods, the amplification time was significantly reduced from original 60 min to less than 30 min from as low as 2 copies of genomic DNA input (Fig. 1). This is the first report of an effective method for systematic optimization of the HDA reaction speed, resulting in dramatically reduced amplification time. The assay is performed at a constant temperature of 65 °C and does not require heat denaturation of the template, making it truly isothermal. Detection is achieved using an instrument-free, self-contained disposable cassette, minimizing the possibility of cross contamination. Therefore, the entire test procedure only requires an inexpensive heat block and should be particularly suitable for resource limited settings.

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