Visual Detection of rpoB Mutations in Rifampin-Resistant *Mycobacterium tuberculosis* Strains by Use of an Asymmetrically Split Peroxidase DNAzyme

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Multidrug-resistant *Mycobacterium tuberculosis* is resistant to two first-line antituberculosis drugs, isoniazid and rifampin, resulting in the relapse of tuberculosis. *M. tuberculosis* grows very slowly, and thus traditional examination methods take time to test its drug resistance and cannot meet clinical needs. The use of a DNA probe makes it possible to test rifampin resistance. We developed an asymmetrical split-assembly DNA peroxidase assay to detect drug-resistant mutation of rifampin-resistant *M. tuberculosis* in the *rpoB* gene rapidly and visibly. A new strategy was also designed to eliminate the adverse effects caused by the complicated secondary structure of the target DNA and to improve the efficiency of the probes. This detection system consists of five group detections, covers rifampin-resistant determination region of the *rpoB* gene, and tests 40 kinds of mutations, including the most common mutations at codons 531 and 526. Every group detection or individual mutant allele detection can distinguish corresponding mutant DNA sequences from the wild-type DNA sequences.

Tuberculosis (TB) is caused by the tubercle bacillus. Approximately 2 billion people are currently infected with TB, and 2 million people die annually from this disease (6). As a single infectious agent, TB is the third leading cause of death by an infectious disease in low-income countries (31), after human immunodeficiency virus (HIV) and malaria. Drug-resistant TB is caused by a resistant strain of *Mycobacterium tuberculosis*, and its production and transmission is increasing the global TB burden. An investigation performed by the World Health Organization on drug-resistant TB (10) indicated that among the 6 to 8 million new cases of TB every year, ca. 10% of patients can tolerate one TB drug, and only 2% of patients can tolerate at least two TB drugs, mainly, isoniazid and rifampin. The mechanism of rifampin action involves the inhibition of transcription by binding to its target protein, which is the β subunit of the RNA polymerase. Resistance to rifampin is mainly caused by mutations in an 81-bp region of the *rpoB* gene, which encodes the β subunit of the RNA polymerase (5, 8, 16, 23). It has already been studied that single nucleotide polymorphisms (SNPs) in *M. tuberculosis* contribute to the resistance of rifampin and isoniazid (4). There is a correlation between mutations in the rifampin-resistance-determining region and phenotypic resistance, i.e., the sensitivity pattern does not have missense mutations, and 90% of the drug-resistant strains do have a mutation. This fact indicates that it may be possible to rapidly detect rifampin resistance in *M. tuberculosis* by using DNA probes.

Several assays based on DNA probes have been developed that can be used to detect resistance mutations. For instance, Piatak and coworkers designed five different molecular beacons (19, 29) that are complementary to different regions of the wild-type *rpoB* core. Detection of five colors in the assay indicates that there are no mutations in the sequence. However, if a color is not detected, this indicates that rifampin resistance may be present. In addition, Invogenetics developed the Inno-LiPA Rif. This TB detection method uses PCR-RDB to detect rifampin resistance (18). Furthermore, Troesch and coworkers used a DNA probe array to detect *M. tuberculosis* that was resistant to rifampin (22, 28). However, both of these methods are time-consuming or expensive.

DNAzymes can be used as DNA probes because of their specific catalytic activity. Sen and coworkers (25–27) discovered a DNAzyme that integrates hemin into the DNA G-quadruplex and has peroxidase activity that allows it to oxidize 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) diion (ABTS) using H$_2$O$_2$ to produce the colored radical anion ABTS$^-$. The reaction then undergoes a detectable color change. Willner and coworkers developed a platform that uses DNAzymes to detect different targets in the protein and DNA (14, 32). Kolpashchikov (12) and our lab (3) have independently developed symmetrical and asymmetrical split DNAzyme strategies that produce a visible reaction if a single mutant allele is present in the target DNA. Uttamchandani and coworkers (1) used Kolpashchikov’s strategy and asymmetrical PCR to establish a technique that can be utilized to detect DNA from *Salmonella* and *Mycobacterium*. The method described here is based on the technique developed in our group (3) and further clinical application testing. We describe here an asymmetrical, split-assembly DNA peroxidase assay (3) to rapidly and visibly detect rifampin-resistant *M. tuberculosis* containing multiple mutations. The mutations that are covered by this assay were chosen.

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from a Chinese publication (9) in which all of the reported mutations were included.

MATERIALS AND METHODS

Materials and reagents. Hemin (Acrose), HEPES (Amresco), dimethyl sulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd.), ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma-Aldrich], H₂O₂ (Sinopharm), oligonucleotides (Invitrogen Technology, Shanghai, China), high-fidelity PCR enzyme mix (catalog no. K0191; Fermentas), and a Gene-JET PCR purification kit (Fermentas) were used in these experiments.

Equipment. A Shimadzu UV-2550 UV-Vis spectrophotometer, a Thermo Multiskan MK3, and a Bio-Rad Thermocycler were used.

Bacterial strains. Wild-type M. tuberculosis H37Rv (strain ATCC 93009) was purchased from the Beijing Biological Product Institute, and the drug-resistant M. tuberculosis mutant strains 531-2G (Ser531Trp, TGG→TGG), 533-2C (Leu533Pro, CTG→CCG), 531-2T (Ser531Leu, TGG→TGG), 526-1T (His526Tyr, CAC→TAC), and 531-1A (Gln531Lys, CAA→AAA) were isolated from TB patient sputum samples at the Wuhan Medical Treatment Center. Wild-type and mutant strains were identified by DNA sequencing.

Preparation of bacterial genomic DNA. The bacteria were inactivated and killed at 100°C for 1 h, and the bacterial genomic DNA was prepared according to the protocol of the TIANamp Bacteria DNA kit (catalog no. DP302; Tiangen, People’s Republic of China). Briefly, the bacteria were incubated in enzyme solution (20 mg of lysosome/ml, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1.2% Triton X-100) at 37°C for 2 h. Then, 4 μl of RNase A (100 mg/ml) was added, followed by incubation at room temperature for 10 min. We then added 20 μl of proteinase K, followed by incubation at 55°C for 40 min. After centrifugation at 12,000 rpm for 1 min, the supernatant was collected, and the bacterial DNA was first pelleted by adding 220 μl of ethanol (96 to 100%) and then dissolved in 100 μl of double-distilled water.

Oligonucleotides. Probes A, B, L, and R were in 10 μM stock solutions prepared at 4°C for several months at −20°C. Probe SIB for mutant allele detection is prepared at 4°C as 30 μM stock solutions and then held for several months at −20°C. The probe mixtures for group detection were prepared as 5 μM stock solutions at 4°C and held for several months at −20°C.

Hemin and ABTS solutions. For the hemin solution, hemin was dissolved in DMSO to obtain a final concentration of 10 μM. For the ABTS solution, ABTS was dissolved in DMSO to obtain a final concentration of 250 mM.

Detection of the mutations of synthetic oligonucleotides. The reaction system was set within 180 s based on the absorbance of the oxygenation product ABTS⁺ at 414 nm in buffer (25 mM HEPES-NH₄OH [pH 8.0], 20 mM KCl, 200 mM NaCl, 1% DMSO), 100 nM probe A, 100 nM probe B, and 300 nM probe SIB for mutant allele detection (400 or 600 nM for group detection), in addition to 50 nM hemin, 2 mM H₂O₂, 2 mM ABTS⁺, and 100 nM target DNA.

Symmetrical PCR amplification. The 50-μl PCR mixture contained 1 μM concentrations of the primers rpoP1 and rpoP2, 10× PCR buffer with 15 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate (dNTP), 0.5 U of high-fidelity PCR enzyme mix, and 1 μl of genomic DNA. We used a Bio-Rad Thermo-Cycler to amplify the genomic DNA. The cycling steps were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension cycle at 72°C for 5 min.

Asymmetrical PCR amplification. A total of 50 ng (~0.7 μl of PCR amplification product after the first round) of 388-bp double-stranded DNA was added to the 50-μl PCR mixture along with 1 μM primer rpoTR9, 0.2 μM primer rpoTR8, 10× PCR buffer, 15 mM MgCl₂, 2 mM dNTP, and 0.5 U of high-fidelity PCR enzyme mix. The second PCR round consisted of 5 min at 95°C, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and then a final treatment at 72°C for 5 min.

RESULTS

Design and construction of assay. (i) Theory of the split peroxidase DNAzyme system. Split peroxidase DNAzyme is a type of DNA aptamer that is assembled by an unequal ratio (3:1) of probe A and probe B but also the competition probe (SIB) (3). To detect a single mutant allele, the probe system includes not only probe A and probe B but also the competition probe (SIB) (3) (Fig. 1). The 5′ terminus of probe SIB and probe A’s 3′ terminus that forms the G-quadruplex are partly complementary. Probe SIB’s 3′ terminus is complementary to the sequence of the wild-type DNA, while probe B’s sequence is complementary to the mutant. If the target DNA was rifampin resistant, then probes A and B would play a significant role in bonding with the target DNA and would form an activated DNAzyme, which would produce a visible color change. If the target DNA is wild type, then probes A and SIB would bind to the target DNA. Because the G-rich structures in the 3′ terminus of probe A are partly complementary to the 5′ terminus of probe SIB, the complex cannot combine with hemin to produce the necessary peroxidase activity. Therefore, the reaction remains colorless.

(ii) Design of a split DNAzyme probe to detect mutant alleles that confer rifampin resistance. The prominent characteristic of rifampin resistance is the centralization of mutant alleles. Ninety percent of the mutations occur in the 81-bp rpoB gene of the rifampin resistance region, and other mutations also occur (8, 13, 21). Huang et al. (9) sequenced 588 bp of the rpoB gene from 193 rifampin-resistant M. tuberculosis clinical strains and 3 artificially created rifampin-resistant strains. These researchers found that there are 40 different mutant alleles in the 81-bp rpoB gene of the rifampin-resistant region. The mutations include the known mutant alleles of rifampin resistance that are found in clinical strains from different countries (2, 11, 15, 20, 30). The mutant alleles we
studied are based on a report from Huang et al. (9). To reduce the number of probes, we designed the group detection strategy. We divided the target sequence into five regions (Fig. 2), each of which contains several mutation sites. The five regions cover the entire rpoB core sequence and all reported mutation sites. Five different split probe systems of each group are designed that correspond to these five regions. The probe system of group detection contained a common probe A, a series of probe Bs that were designed based on the mutant alleles, and the common competition probe SIB that hybridized to the wild type. The probe system of the specific mutant allele detection contained probe A, a specific probe B, and the competition probe SIB. The group detection determined which region the specific mutations belonged to, and the mutant allele detection determined the specificity to a mutant allele. The aim was to distinguish mutant DNA from wild-type DNA by using group detection. Furthermore, the mutant allele detection could differentiate the specific mutant from other mutants in the detected region.

(iii) Optimization design of the probe according to synthetic oligonucleotides. To detect mutations in the rpoB core region of M. tuberculosis, it was necessary to optimize the split peroxidase DNAzyme system, which included optimization of the probe A and probe B sequences and the concentration of probe SIB.

(iv) Optimization design of probe A. To increase the positive signal of the mutant detection and reduce the signal of the wild-type sequence, we optimized the design of probe A by altering its 3′-terminus sequence (A1, TGGGTAGGGGTGGG; A2, TGGGTAGGGGTTGGG; A3, TGGGTAGGGGTGGG; A4, TGGGTAGGGCGGG; and A5, TGGGTAGGGCGGG). The sequences of the 5′ terminus in probe SIB were also designed (SIB1, CCACT; SIB2, CCAACT; and SIB3, CCGCT). Therefore, five appropriate combinations were then made (A1/SIB1, A2/SIB1, A3/SIB2, A4/SIB2, and A5/SIB3). These sequences were applied to the detection system of group I (the “I” refers to the group number in the detection system). The activity of the complex was detectable from the absorbance of the reaction system at 414 nm, and the absorption value shown was the average of three independent experiments (see Table S1 in the supplemental material. Comparing the ratio of activity of complex A-B-C to its relative, A-SIB-C, we were able to determine the best combination. This indicated that the five combinations all produced good results, and the combination of A3/SIB2 was the best and thus was used in subsequent probe selection.

When there is a GGG segment in the matching region of probe A, it would establish an intramolecular G-quadruplex structure with the three GGG repeat in probe A’s 3′ terminus. Therefore, a strong background signal appears in the reaction. For instance, in group V, the intramolecular G-quadruplex structure in probe A could produce a significant background signal after bonding with hemin, as shown in Fig. S1, curve V-A1, in the supplemental material. We then added a sequence (CCAACC) to probe A’s 5′-terminus, which is partially complementary to its 3′ terminus (TGGGTAGGGCGGG), to prevent the formation of intramolecular G-quadruplex structures and significantly reduce the background signal (see Fig. S1, curve V-A, in the supplemental material).

(v) Optimization design of probe B. Probe B contained a segment at its 3′ terminus that was complementary to the target DNA, so that the mutant allele was located in the middle of the binding region, and also a GGG sequence in the 5′ terminus, which is involved in the formation of the G-quadruplex structure. Table S17 in the supplemental material shows the probe B of each
group and its corresponding mutation of the codon and amino acid. Most probes designed (3) by this method achieve the desired results; however, there are still several probes that did not produce an ideal effect. For example, some produced a low signal for the mutant allele or a high signal for the wild-type allele. The new design was aimed at distinguishing the mutant from the wild type effectively and completely. Therefore, we designed different solutions for different target DNA sequences and finally achieved satisfactory results.

In group I detection, for probe I-B532-2A1, the “I” refers to the group number in the detection system, the “532” refers to its corresponding codon in the rpoB gene, the “2” in 2A1 refers to the position of the mutant allele, and the “1” in 2A1 refers to the serial number of the different sequence attempts. The probe and its corresponding sequence (see Table S1 in the supplemental material) produced a low signal when it detected a mutant allele. After using RNAstructure 4.6 (http://rna.urmc.rochester.edu/RNAstructure.html) to predict the structure of I-B532-2A1, we discovered that it formed a stable secondary structure rather than bonding with the target DNA (see Fig. S2A in the supplemental material). In this detection system, the binding capacity of probe B and target DNA decreased, and the signal declined. In order to achieve a higher signal, we altered the connecting base of probe B from T to TT (I-B532-2A2) or A (I-B532-2A3), which disrupted the probe’s secondary structure. These data are presented in Table S2 and Fig. S2B in the supplemental material. From the results of calculation (energy), the energy of probe I-B532-2A3 (an “A” in place of the “T” in Fig. S2A in the supplemental material) is 1.9 kcal mol⁻¹, which is lower than for probes I-B532-2A2 and I-B532-2A3. Therefore, probe I-B532-2A3 formed an unstable hairpin structure, which was prone to bond with target DNA, and produced the best result (see Fig. S2B, line 3a, in the supplemental material).

In group IV detection, probe IV-B516-2G produced a high signal when exposed to wild-type DNA because it was able to strongly bind the wild-type sequence. To reduce the signal and the stability between probe IV-B516-2G and the wild-type allele, we attempted to remove one, two, or three bases from the 3’ terminus of probe B. Table S3 and Fig. S3 in the supplemental material show the results of the optimization. Reducing the probe by one, two, or three bases resulted in a significant decrease in the signal of the wild-type allele. At the same time, no significant negative effects were observed during the detection of the mutant allele. Finally, we chose a version of probe IV-B516-2G3 that was shortened by three bases at the 3’ terminus.

(vi) Optimization design of the concentration of probe SIB in group detections. A competition probe SIB was designed to block probe B from binding with the wild-type sequence. An excess of probe SIB was used to identify the mutant. Initially, the ratio of probe B to probe SIB was 1:3 in the mutant allele detection. However, during the detection of groups, various forms of probe B were introduced, which affected the concentration of probe SIB. Therefore, the appropriate concentration of probe SIB in the group detection should be determined in further studies. The concentration of the target DNA was 100 nM. The concentrations of probe A and each form of probe B were also 100 nM. The following concentrations for probe SIB were used: 0, 100, 200, 300, 400, 500, 600, 700, 800, and 900 nM (see Table S4 in the supplemental material). Figure S4a and b in the supplemental material show the results for groups I and II, respectively. The competition strategy was very effective. There was a strong signal without probe SIB. However, after the addition of 100 nM probe SIB, the signal declined sharply. As the concentration of probe SIB increased, the signal from the detection system decreased. Finally, we chose to use 600 nM probe SIB for group I and 400 nM probe SIB for group II. For group III, 400 nM probe SIB was used because it has fewer mutant alleles, and 600 nM probe SIB was used for groups IV and V.

Detection of synthetic oligonucleotides of mutations and wild-type DNA in each group. In this project, all of the mutant alleles were determined in the group detection and mutant allele detection. The compositions of probes are shown in Table S5 in the supplemental material, and the results from group I are shown in Fig. 3 and Fig. S5 in the supplemental material. The results for the other groups are shown in the supplemental material as follows: group II, Table S6 and Fig. S6; group III, Table S7 and Fig. S7; group IV, Table S8 and Fig. S8; and group V, Table S9 and Fig. S9. The results from the detection reaction indicated that we could easily distinguish the wild-type sequence and mutant sequences using five group detections and their mutant allele detections. The difference in the absorbance of each mutation and its related wild-type sequence for the mutant allele detections was >15-fold, and the highest was >60-fold. The discrimination within the group detections is >10-fold and is usually ~15-fold. The clear distinction between the mutant sequence and the wild-type sequence is observable by the naked eye.

Detection of rifampin resistance using genomic DNA. (i) Production of single-stranded DNA. The detection system required the production of single-stranded DNA, which was used as the target DNA and was produced by asymmetric PCR. To increase the sensitivity and to obtain enough quantity of target DNA, nested PCR was used. The first round of PCR was symmetrical PCR, in which a 588-bp double-stranded DNA product, including the rifampin resistance determination region, was amplified (9). Asymmetrical PCR was adopted as the second round, and

FIG 3 Mutant allele detection of wild-type and mutant alleles in cluster I. Samples 1a to 8a represent detection of the wild-type sequence, and samples 1b to 8b refer to the detection of mutant alleles.
a 157-nucleotide single-stranded DNA containing the rifampin resistance determination region was amplified (24) (see Table S10 in the supplemental material). By optimizing the ratio of the two primers, we were able to produce sufficient product and reduce the number of cycles from 70 to 40 (1). After the asymmetrical PCR, 50 μl of PCR product was added to the detection system directly without purification.

(ii) Detection of folded single-stranded DNA with complex secondary structure. After the optimization of five group detections and their mutant allele detections to the synthetic oligonucleotides, the technique was further developed so that it could be applied to clinical samples. The rpoB gene of the rifampin-resistant region contained several complementary sequences; these sequences could form complicated secondary structures, which would interfere with detection (7, 17). Therefore, two DNA probes—L and R—were designed to bond with the two sides of the target DNA and unwind its folded structure. The two probes were independently located on both sides of probes A and B and served as insulated probes. Probe L was complementary to the 5′ terminus of the target DNA, and its 5′ terminus was next to the 3′ terminus of probe B. Probe R was complementary to the 3′ terminus of the target DNA, and its 3′ terminus was located next to the 5′ terminus of probe A. Probes L and R eliminated the adverse effects caused by the complicated secondary structure of the target DNA.
DNA and extended the scope of DNA detection without the limit of length and secondary structure. Even without probes L and R, the reaction was still visible to the naked eye. After probes L and R were added, the signal increased to ~20 times that of the control, and the color change was more pronounced (Fig. 4). Table S11 in the supplemental material shows the composition of the probes. In Fig. 5, a bar chart displays the absorbance of the reaction system at 414 nm as detected in the 96-well plate. The probe sample contained all of the probes for detection but no target DNA. The control sample went through the PCR procedure without the addition of the template DNA. The result indicates that probes L and R increase the visibility of the signal.

(iii) Selection of the wild-type allele and its mutants by probes from clinical strains. We received five mutant samples of genomic DNA from clinical strains with different levels of rifampin resistance. These samples contained mutant strains 531-2G (Ser531Trp, TCG→TGG), 531-2T (Ser531Leu, TCG→TTG), 533-2C (Leu533Pro, CTG→CCG), 526-1T (His526Tyr, CAC→TAC), and 513-1A (Gln513Lys, CAA→AAA) and the wild-type strain H37Rv. These samples were used to detect the mutant alleles using their corresponding probe systems from the group. The detection results for mutant strain 531-2G are shown in Table S12 in the supplemental material and in Fig. 6. The results for mutant strains 531-2T, 533-2C, 526-1T, and 513-1A are shown in Table S13, Table S14, Fig. S10, and Fig. S11 in the supplemental material.

The results demonstrated that each of the mutant alleles was detected by the corresponding mutant allele detections. The sample containing the wild-type for sequence remained colorless, and the sample containing the mutant allele had a discernible green color change, which was at least nine times stronger than the signal from the wild-type allele according to the readout. The group detection was able to discern all five mutant alleles from the wild-type sequence, as indicated by a green color that was clearly observable by the naked eye. The optical density reading from the mutant allele detection was approximately nine times stronger than that from the wild-type sequence (Fig. 6A and see Fig. S10 in the supplemental material). The signal from the group detection containing the mutants was at least six times stronger than that from the reactions containing the wild type (Fig. 6B and see Fig. S11 in the supplemental material). The signal from mutant strain 531-2T, which is the most common mutant allele, was 14 times stronger than the wild-type signal (see Fig. S10A in the supplemental material). The results indicated that the method can be used to distinguish a mutant sequence from the wild-type sequence clearly and efficiently.

(iv) Detection of a mixture containing a single mutant and a wild-type allele. To expand the application and increase the usefulness of the method, we detected a resistant sequence in the presence of the wild-type allele. The SNP at codon 531 of rpoB (TCG to TGG) was mixed in various fractions with the wild-type sequence. The mixture contained the first-round PCR amplification product from the mutant and wild-type DNAs. The fractions of the mutants were as follows: 0, 1, 2, 5, 10, 20, 50, and 100%. Furthermore, 50 ng of the DNA mixture was added to the second round of asymmetric PCR amplification. Finally, the reaction was detected by the mutant allele detection for group I, which was used to determine mutant strain 531-2G. The results are shown in Table S15 and Fig. S12 in the supplemental material. It was clear that the resistant mutant was detectable when the fraction was 5%. When the fraction was >10%, the color change was easily observed.
DISCUSSION

We developed a method using an asymmetrically split peroxidase DNAzyme to determine the rifampin-resistant mutant allele in the genomic DNA. Several approaches merely focus on the major rifampin-resistant mutations (18, 22, 28), but our strategy is available for all mutants reported (9), not only the major alleles but also the minor ones. Another important advantage of this method lies in the adoption of the DNAzyme system, which allows instant and visible color change without the need of signal readout from an apparatus. The use of 0.03% Triton X-100 permits a quick result within 30 min of the PCR procedure (1). Furthermore, the sensitivity of the DNAzyme system eliminates the complicated procedure of post-PCR modification, such as the concentration and purification of the asymmetric PCR product.

Two systems—the PCR system and the detection system—are involved in the entire assay. Nested PCR was established to verify that there is enough quantity of target DNA in the detection system and to enhance the specificity and accuracy of the detection. Every PCR parameter, such as the ratio of the primers, the temperature, and the cycle number of each step, has been optimized carefully to increase production and efficiency. In the detection system, the initial tool for SNP analysis is the molecular-beacon probe (19); however, the requirement for the instrument and the individually fluorescence-labeled probe is definitely costly. The peroxidase DNAzyme was selected due to its robustness, its sensitivity, and its relatively low cost (3, 12, 14, 25, 26, 27, 32). Here, we apply the 3:1 split DNAzyme to detect target DNA. By adopting the competition strategy, i.e., introducing the probe SIB, the DNAzyme could identify the SNPs (3).

As has been reported, the secondary structure of analyte indeed interferes with the detection of single nucleotide. Some researchers have attempted to solve this problem. For example, Kolpashchikov and coworkers have developed a tricomponent sensor to open stem-loop structured DNA and make it available for hybridization with a molecular-beacon probe (7). In our approach, probes L and R are designed to hybridize to the fragment adjacent to the mutant allele site of the long target DNA and expose the detected region, which is accessible for hybridization with probes A and B (Fig. 4). Subsequent DNAzyme formation is ensured by unwinding the secondary structure of the analyte. Therefore, the design of probe L and R efficiently increases the hybridization of the probe system with target DNA (Fig. 5).

The probe design (3) of the detection system is simple and robust; most mutant detections using this approach have achieved a satisfactory result. However, there still exist a few obstacles. For example, probe V-A could form an intramolecular G-quadruplex structure due to the GGG repeat in its matching region, and this represents a significant background signal. This problem was solved by adding a sequence of CCAACC to its 5’ terminus that partially binds with its 3’ terminus (see Fig. S1 in the supplemental material). Another obstacle is that probe I-B532-2A1 could form a stable secondary structure and cause a low signal readout when the mutant is analyzed. In the present study, we modified the bases of the sequence and improved the signal of the mutant detection (see Fig. S2 in the supplemental material). In addition, the affinity of probe IV-B516-2G and the wild type is too strong to contribute to a high signal in the detection. After three bases were removed from the 3’ terminus, we were finally able to obtain the best result (see Fig. S3 in the supplemental material). The probe system ensures that the detection of wild-type sequence gives a low and imperceptible signal to avoid a false-positive result, and the mutant sample gives a high and observable signal. The fact that the signal for almost every mutant was at least 10-fold greater than that of the wild type in the detection of synthetic oligonucleotides indicates that the design of the probe system—i.e., probes A, B, L, R, and SIB—indeed enhances the application and sensitivity of this method.

We analyzed five M. tuberculosis clinical strains. All five mutant alleles could be distinguished from the wild-type sequence, as indicated by a green color that is clearly observable by the naked eye. The readout from the mutant sample in whatever detection was at least 6 times stronger than that from the wild-type sample (Fig. 6). As validated by experiments with five clinical samples, the assay proved to be effective, accurate, and practicable.

In order to determine the sensitivity of the assay, a mixture of wild-type and mutant strains was analyzed for resistance. This demonstrated that our method can also specifically detect individual alleles when the fraction of mutant is as low as 5% through apparatus or higher than 10% by detection with the naked eye (see Fig. S12 in the supplemental material).

In summary, the method described here is useful for distinguishing mutant rifampin resistance alleles from wild-type sequences. The use of PCR amplification and DNAzyme detection system could achieve a reliable and accurate result, without the need for expensive instrumentation. The design of probes L and R also offers a prototype technology for analyzing secondary-structure-folded analytes. Further work is still needed to simplify the PCR procedures described here and to apply the assay for other types of mutant detection.

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