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Rapid, High-Throughput Detection of Rifampin Resistance and Heteroresistance in *Mycobacterium tuberculosis* by Use of Sloppy Molecular Beacon Melting Temperature Coding

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Rifampin resistance in *Mycobacterium tuberculosis* is largely determined by mutations in an 80-bp rifampin resistance determining region (RRDR) of the *rpoB* gene. We developed a rapid single-well PCR assay to identify RRDR mutations. The assay uses sloppy molecular beacons to probe an asymmetric PCR of the *M. tuberculosis* RRDR by melting temperature (T_m) analysis. A three-point T_m code is generated which distinguishes wild-type from mutant RRDR DNA sequences in approximately 2 h. The assay was validated on synthetic oligonucleotide targets containing the 44 most common RRDR mutations. It was then tested on a panel of DNA extracted from 589 geographically diverse clinical *M. tuberculosis* cultures, including isolates with wild-type RRDR sequences and 25 different RRDR mutations. The assay detected 236/236 RRDR mutant sequences as mutant (sensitivity, 100%; 95% confidence interval [CI], 98 to 100%) and 353/353 RRDR wild-type sequences as wild type (specificity, 100%; 95% CI, 98.7 to 100%). The assay identified 222/225 rifampin-resistant isolates as rifampin resistant (sensitivity, 98.7%; 95% CI, 95.8 to 99.6%) and 335/336 rifampin-susceptible isolates as rifampin susceptible (specificity, 99.7%; 95% CI, 95.8 to 99.6%). All mutations were either individually identified or clustered into small mutation groups using the triple T_m code. The assay accurately identified mixed (heteroresistant) samples and was shown analytically to detect RRDR mutations when present in at least 40% of the total *M. tuberculosis* DNA. This was at least as accurate as Sanger DNA sequencing. The assay was easy to use and well suited for high-throughput applications. This new sloppy molecular beacon assay should greatly simplify rifampin resistance testing in clinical laboratories.

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* is increasing worldwide (9, 27, 37). Rapid methods to detect drug resistance are needed to quickly identify drug-resistant strains and to implement appropriate therapy (4, 16, 36). *M. tuberculosis* does not naturally contain plasmids, and almost all cases of clinical drug resistance are caused by single-nucleotide polymorphisms (SNPs) or small insertions/deletions in relevant genes (28). In the case of rifampin resistance, 95 to 98% of rifampin-resistant clinical strains have mutations in the 80-bp rifampin resistance determining region (RRDR) of the *M. tuberculosis* RNA polymerase beta (*rpoB*) gene (9, 11, 12, 15, 20, 28). PCR and probe-based molecular genotyping assays can be used to detect these resistance-inducing mutations. Such genotypic assays are potentially more rapid than labor-intensive culture-based drug susceptibility tests. Genotypic drug susceptibility testing has shown good overall concordance with the phenotypic antibiotic susceptibility tests of MDR and XDR clinical strains (6), and in a recent study, a genotypic test actually correlated better with clinical outcome than standard phenotypic susceptibility testing (36). When combined with automated sample processing systems, such as the Xpert MTB/RIF test (14), genotypic susceptibility tests can significantly reduce testing turnaround time, increasing patient notification rates and decreasing time to treatment (4, 32).

The Xpert MTB/RIF assay is one example of a genotypic test that is being increasingly used to screen for rifampin resistance (33). However, the single-use cartridge design of the Xpert assay limits its use for laboratory-based high-throughput testing. Several widely used reverse blot hybridization assays, such as the INNO-LIPA Rif.TB assay (Innogenetics, Belgium) and the MTBDRplus (Hain, Germany) assay (1, 5, 15, 19, 21, 23, 29, 34), are available for laboratory-based rifampin resistance screening; however, these assays are complicated by their open hybridization format. Open hybridization systems require a relatively cumbersome work process, including rigorous physical separation of different work areas (2, 23) due to the risk of handling open PCR amplicons in a molecular diagnostic laboratory. Open systems also require a relatively large number of probes to test for relevant resistance-associated mutations. This requirement complicates assay chemistry and hybridization parameters. In contrast to reverse blot hy-

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bridization assays, melting temperature (T_m)-based assays enable mutation detection in closed systems. These assays take advantage of the fact that fully complementary strands of DNA have higher melting temperatures than DNA heteroduplexes which contain one or more nucleotide mismatches. T_m assays are well suited for busy diagnostic laboratories, because they can be performed in homogeneous closed systems without the risk of carryover amplicon contamination, are amenable to multiplexing, and are easily adapted to a high-throughput format. Fluorescence resonance energy transfer (FRET) probes, dually labeled probes, TaqMan, and molecular beacon probes have all been used in T_m assays to detect drug resistance mutations in *M. tuberculosis*, as has the high-resolution T_m analysis (HRMA) of PCR products using DNA intercalating dyes (9, 10, 13, 18, 20, 24, 26, 27). However, *M. tuberculosis* drug resistance assays which use T_m analysis have been largely limited to tests of the most commonly encountered mutations (20, 24, 27). We have previously shown that a new generation of mismatch-tolerant probes, called sloppy molecular beacons (SMBs), can be efficiently used to identify mutations in the *M. tuberculosis* genome associated with fluoroquinolone resistance (8). The assay had high sensitivity and specificity, and the thermodynamic properties of SMBs made it possible to develop an assay with easily interpretable T_m curves. The assay was also able to detect fluoroquinolone resistance mutations in clinical samples that contained mixtures of drug-susceptible and drug-resistant DNA. We have now applied the same technology to detect rifampin resistance in *M. tuberculosis*. Here, we show that our approach enables the rapid, high-throughput, single-well detection of mutations in the *rpoB* RRDR of *M. tuberculosis.* We tested the ability of the assay to detect 44 different *rpoB* mutations that have been described previously to be associated with rifampin resistance, and we validated its performance on a panel of clinical samples representing a diverse collection in terms of geographical distribution and the mutation types. The ability of the assay to specifically identify rifampin-resistant clinical strains and to detect heteroresistance was also evaluated, and its performance was compared to conventional antibiotic susceptibility test results and verified with targeted sequencing.

MATERIALS AND METHODS

Clinical DNA samples. Two clinical sample sets were tested to include a wide variety of mutations and geographic origins. The first sample set consisted of 440 sequential *M. tuberculosis* isolates cultured from patients enrolled in a natural history study of MDR tuberculosis (NCT00341601 at clinicaltrials.gov) in the National Masan Tuberculosis Hospital in Changwon, Republic of Korea, for which reliable conventional drug susceptibility tests and/or DNA sequencing of the *M. tuberculosis* RRDR were available. The second sample set consisted of 149 selected *M. tuberculosis* cultures obtained from the WHO TDR Tuberculosis Specimen Bank, maintained by the United Nations Children's Fund/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, which includes cultures from Africa, Asia, Australia, Europe, and Latin America (8), for a total of 589 DNA samples tested. The TDR samples were chosen to represent a wide repertoire of RRDR mutants and a broad geographical distribution for both RRDR mutants and rifampin-susceptible controls. Conventional rifampin susceptibility was performed using the LJ proportions method with 40 μg/ml used as the critical rifampin concentration defining resistance (31). Rifampin resistance results were available for 561 out of the 589 samples. The RRDR sequence, and in most cases the entire *rpoB* gene sequence, was available for all 589 isolates. We resequenced a subset of RRDR mutants, representing all of the different mutations tested in our study, to confirm the original DNA sequencing results. The results of the DNA sequencing performed as part of this current study were used in the rare event of discordance with the prior sequencing result. Each DNA sample was quantified with a Nanodrop microvolume spectrophotometer (Thermo Scientific), and 2 to 5 ng of the DNA sample was used for PCR. All samples were independently coded and randomly redistributed to blind the sample sequence and origin before performing each test assay. The results of the assay used in this investigation were not reported to the treating physicians and were not used to guide treatment decisions.

DNA samples from human origin, NTM, and Gram-positive and Gram-negative bacteria. Pure human genomic DNA obtained from the ATCC repository (Manassas, VA); 121 clinical nontuberculous mycobacterial (NTM) cultures, representing 26 species, isolated from patients at the National Masan Tuberculosis Hospital; a laboratory strain of *M. smegmatis*; 18 NTM isolates obtained from the ATCC repository (Manassas, VA), consisting of *M. abscessus*, *M. scrofulaceum*, *M. celatum*, *M. haemophilum*, *M. asiaticum*, *M. kansasii*, *M. avium*, *M. flavescens*, *M. szulgai*, *M. terrae*, *M. fortuitum*, *M. intracellulare*, *M. marinum*, *M. xenopi*, *M. thermoresistibile*, *M. simiae*, *M. trivial*, and *M. malmoense*; and DNA extracted from 18 species of Gram-negative and Gram-positive bacteria representing the most common bloodstream infections and nosocomial pathogens obtained from various sources (7) were selected to test for analytical specificity. DNA was isolated from pure clinical strains by boiling a loopful of culture in Instagene matrix solution (Bio-Rad). The DNA was quantified as described above and tested using 100 to 400 ng for each PCR. To test for the capability of the assay to detect *M. tuberculosis* RRDR mutations in the presence of a 10-fold excess of background NTM DNA, 10^5 genome equivalents of *M. tuberculosis* DNA harboring common RRDR mutations found in our study (516GTC, 526TAC, 531TTG, and 533CCG) was mixed with 10^6 genome equivalents of NTM DNA. Human DNA was tested in 10^5-fold excess above the level of the target *M. tuberculosis* DNA.

SMBs and primers. A 172-bp fragment (nucleotides 1226 to 1397, with numbering based on the gene start site according to the *Escherichia coli* DNA nomenclature) containing the *rpoB* RRDR was amplified using the target primer *rpoB*-F (5′-agacgttgatcaacatccg-3′) and the antisense primer *rpoB*-R (5′-acctccagcccggcacgctcacgt-3′). These primers were designed to be specific to the *M. tuberculosis* complex and were verified by an alignment of *M. tuberculosis* and all of the NTM *rpoB* sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/). Three SMB probes, rpo1 (5′-tetramethylrhodamine-**cgacc**gCccatgaattggctcagctggctggtgAc**ggtcg**-BHQ2-3′), rpo2 (5′-cyanine 5-**ggcgcg**aaccAcgacagcgggttgttctggtccatgaa**cgcgcc**-BHQ2-3′), and rpo3 (5′ −6 carboxyfluorescein-**cgcgcg**caTcAccAacagtcggTgcttgtgggtcaacc**cgcgcg**-BHQ1-3′) (where the underlined boldface sequences represent the stem portion of the SMB, the lowercase sequences represent the probe portion of the SMB, uppercase letters represent the mutations introduced into the probe region to obtain a stable stem-loop structure, and BHQ represents Black Hole Quencher), were targeted against the *rpoB* RRDR. The SMBs were designed using the *in silico* DNA folding program at http://mfold.rna.albany.edu/?q=mfold/dna-folding-form, and the probe-target hybrid folding program at http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-melting was used to predict the possible probe-target hybrid structures and T_ms. The probes were designed to generate a maximum T_m difference between wild-type and mutant sequences in their respective target regions of the RRDR so as to enable their unambiguous identification. Primers were obtained from Sigma Genosys, and the SMB probes were from Biosearch, CA.

Using artificial oligonucleotides containing *rpoB* RRDR mutations to test assay probes. Not all of the RRDR mutations described in the literature were available to us in the form of clinical *M. tuberculosis* DNA. To enable us to test the assay against the broadest range of RRDR mutations, we created oligonucleotides containing either wild-type or mutant RRDR sequences, including 44 clinically relevant mutations identified as being present at a frequency of at least 0.1% in clinical settings (based on an extensive literature search), to test the ability of the assay probes to

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TABLE 1 Three-probe T_m (°C) code of the assay in tests of artificial oligonucleotide targets with wild-type and mutant RRDR sequences[a]

Sample			T_m (°C) of:			dT_m (°C) of:		
No.	Name	Mutation	rpo1	rpo2	rpo3	rpo1	rpo2	rpo3
1	WT		76.0	75.3	71.2			
2	507	GGC-GAC	71.3	75.2	71.2	4.7	0.0	0.0
3	510	CAG-CAC	69.7	74.4	71.9	6.3	0.8	−0.7
4	511 A	CTG-CCG	70.7	75.0	71.3	5.2	0.2	−0.1
5	511 B	CTG-CGG	71.3	75.0	71.4	4.7	0.3	−0.2
6	512	AGC-ACC	70.7	75.2	71.4	5.3	0.1	−0.3
7	513 A	CAA-AAA	70.9	75.0	71.2	5.1	0.3	0.0
8	513 B	CAA-CCA	71.1	74.9	71.6	4.9	0.4	−0.4
9	513 C	CAA-CTA	71.1	74.9	71.6	4.9	0.4	−0.4
10	514 A	TTC insertion	71.3	75.7	71.3	4.7	−0.5	−0.1
11	514 B	TTC-TTG	70.2	74.5	71.0	5.8	0.8	0.1
12	514 C	TTC-TTT (silent)	70.5	74.5	71.0	5.5	0.8	0.2
13	515	ATG-ATT	73.5	72.5	71.7	2.5	2.8	−0.5
14	516 A	GAC-GTC	75.6	71.3	71.4	0.4	7.0	−0.2
15	516 B	GAC-TAC	74.8	72.1	71.4	1.2	3.2	−0.2
16	516 C	GAC-GGC	77.0	73.0	71.3	−01.0	2.3	−0.1
17	516 D	GAC-GCC	75.4	71.8	71.5	0.6	3.5	−0.3
18	516 E	GAC-TTC	75.1	71.4	71.5	0.9	3.9	−0.4
19	516 F	GAC-AAC	74.9	72.6	71.2	1.1	2.7	−0.1
20	517	CAG-CTA	75.2	67.3	72.0	0.8	8.0	−0.8
21	518	AAC-TAC	74.9	70.0	71.7	1.1	5.3	−0.6
22	522 A	TCG-TTG	76.2	73.2	70.2	−0.2	2.1	1.0
23	522 B	TCG-CCG	75.1	71.9	72.5	0.9	3.4	−1.3
24	522 C	TCG-TGG	76.0	72.4	71.2	0.0	2.9	−0.1
25	526 A	CAC-TAC	76.0	75.2	65.8	0.0	0.1	5.4
26	526 B	CAC-GAC	75.7	75.0	64.1	0.3	0.3	7.1
27	526 C	CAC-CGC	75.8	75.0	67.9	0.2	0.3	3.3
28	526 D	CAC-CTC	76.4	75.4	65.0	−0.5	−0.1	6.2
29	526 E	CAC-CAG	76.3	75.3	65.1	−0.3	−0.1	6.1
30	526 F	CAC-AAC	75.9	75.1	65.7	0.0	0.2	5.5
31	526 G	CAC-TGC	75.1	75.6	63.0	0.9	−0.4	8.1
32	526 H	CAC-CCC	76.3	75.1	64.0	−0.3	0.2	7.2
33	526 I	CAC-ACC	76.0	75.2	62.9	−0.1	0.1	8.3
34	527	AAG-AGG	76.0	75.2	67.6	0.0	0.1	3.6
35	529	CGA-CAA	76.6	75.3	65.5	−0.6	0.0	5.7
36	531 A	TCG-TTG	75.2	75.4	74.7	0.8	−0.1	−3.5
37	531 B	TCG-TGG	75.5	75.4	73.8	0.4	−0.2	−2.6
38	531 C	TCG-GCG	76.2	75.5	71.9	−0.2	−0.2	−0.7
39	531 D	TCG-TAC	76.3	75.4	74.0	−0.3	−0.2	−2.8
40	531 E	TCG-CAG	75.5	75.7	74.9	0.5	−0.4	−3.8
41	531 F	TCG-TAG	75.4	75.3	72.7	0.6	−0.1	−1.5
42	533 A	CTG-CCG	75.6	75.6	72.1	0.4	−0.3	−1.0
43	533 B	CTG-ATG	74.7	75.6	73.3	1.3	−0.3	−2.1
44	514, 531	TTC-TTT, TCG-TTG	71.1	75.7	74.9	4.9	−0.4	−3.7
45	516, 522	GAC-GGC, TCG-TTG	77.5	69.8	70.7	−01.5	5.5	0.5

[a] Each T_m value represents an average of 10 separate reactions. WT, wild-type RRDR sequence. The shaded cells represent the dT_m values obtained for the probes detecting the corresponding mutations.

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identify different RRDR sequence types. The 44 different mutations spanning the RRDR from codons 507 to 533 included SNPs, insertions, and double mutants (Table 1). The T_m of each of the three SMB probes in the presence of each RRDR oligonucleotide target was measured. Approximately 500 ng of individual oligonucleotide targets was added to a reaction mixture containing 0.8 ng/μl of each SMB probe, 4 mM $MgCl_2$, 1× PCR buffer (10× Stoffel Buffer; Applied Biosystems), and 5% glycerol. T_m analysis was performed using a Light Cycler 480 II real-time PCR system (Roche Molecular Systems Inc.) using the following assay parameters; denaturation at 95°C for 5 min, followed by cooling down to 45°C and then gradual heating to 85°C, with continuous monitoring of fluorescence during the process at a rate of 10 data acquisitions per degree centigrade. T_m calls were performed at the end of the reaction using the automated T_m calling software (Light Cycler 480 software), and resulting T_m values for each probe were determined. The three T_m values (one for each SMB probe) that were generated in the presence of an RRDR target where then used to define a three-point T_m code for each RRDR mutant or wild-type sequence.

Sloppy molecular beacon T_m assay on clinical DNA. All PCR-T_m analyses of DNA from clinical samples were performed and decoded by two experimenters who were blinded to the nature and identity of the samples. Each DNA sample was quantified using a Nanodrop microvolume spectrophotometer (Thermo Scientific), and 2 to 5 ng of the DNA sample was used for each PCR except where indicated otherwise. PCR was

performed in 384-well plates using a Roche Light Cycler 480 II real-time PCR system (Roche Molecular Systems Inc.) in 20-μl reaction volumes containing 1 μM target primer and 50 nM antisense primer, 0.8 ng/μl of each SMB probe, 4 mM $MgCl_2$, 250 mM deoxynucleoside triphosphates (dNTPs), 1× PCR buffer, 5% glycerol, 0.06 U/μl of AmpliTaq Gold Stoffel DNA polymerase (Applied Biosystems), and 2 to 5 ng of sample DNA or an equivalent volume of water. PCR was carried out with the following steps: activation of the enzyme for 2 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 65°C for 30 s, and extension at 72°C for 10 s. Following PCR cycling, post-PCR-T_m analysis was performed by denaturation at 95°C for 5 min, followed by cooling down to 45°C and then gradual heating to 85°C, with continuous monitoring of fluorescence during the process at a rate of 10 data acquisitions per degree centigrade. T_m calls were performed at the end of the reaction using the automated T_m calling software (Light Cycler 480 software), and resulting T_m values for each probe were determined. A nontemplate control using sterile water instead of DNA as the template was used as the DNA-negative control, and a DNA-positive control using 1 ng of genomic DNA from *M. tuberculosis* H37Rv as the template was also included in each assay plate.

Assay LOD. The analytical sensitivity of the assay was determined using serial dilutions of three different genomic DNA samples. One sample had a wild-type RRDR sequence, and one sample each had the RRDR mutations 516GTC and 531TTG, which were chosen because these two mutations occurred frequently in our clinical DNA panel and in the literature. Each DNA sample was serially diluted to represent a concentration range from 10^6 to 10 genome equivalents per reaction. The *rpoB* SMB assay was performed 10 times at each dilution, and the minimum DNA concentration which produced correct T_m codes 100% of the time was determined as the limit of detection (LOD) of the assay for that RRDR type.

DNA mixtures containing wild-type and 531TTG mutant DNA. DNA mixtures containing wild-type and mutant DNA were prepared to evaluate the performance of the assay in mixed samples containing both wild-type and RRDR mutant DNA (heteroresistance). This situation might occur clinically in a dual *M. tuberculosis* infection or during the *in vivo* evolution of rifampin resistance caused by tuberculosis treatment (17). Various amounts of the 531TTG mutant genomic DNA (a representative highly prevalent RRDR mutation) was added to wild-type *M. tuberculosis* DNA to generate DNA mixtures containing 10 to 90% mutant DNA (in 10% increments) in a total DNA amount of 10 ng (10^6 genome equivalents). SMB T_m shift assays and Sanger sequencing of the RRDR were performed on each mixed DNA type to compare the ability of the SMB assay to detect the RRDR mutant in this mixture to that of Sanger DNA sequencing.

Sanger DNA sequencing. DNA samples were amplified using *rpoB* gene-specific primers as described above, except for the fact that 0.5 μM both forward and reverse primers, 2.5 mM $MgCl_2$, and 0.03 U/μl of AmpliTaq Gold DNA polymerase (Applied Biosystems) were used. The amplified products were checked by gel electrophoresis and then purified using a PCR purification kit (Qiagen) by following the manufacturer's instructions. The purified PCR products were subjected to bidirectional sequencing using the *rpoB* gene-specific forward and reverse primers in a 3130XL Genetic Analyzer (Applied Bio-systems) using a BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Human subject approval. This study was approved by the National Masan Tuberculosis Hospital, NIAID, and UMDNJ institutional review boards, and all subjects gave written informed consent (UMDNJ IRB protocol number 0120090104).

RESULTS

SMB design parameters produce a gradient of T_m values which differentiate mutant from wild-type RRDR sequences. We have previously demonstrated the utility of SMB T_m-based assays to detect fluoroquinolone resistance-associated mutations in *M. tuberculosis* (8). To identify rifampin resistance-associated mutations in the *M. tuberculosis* RRDR, we designed three SMB probes, rpo1, rpo2, and rpo3, which contained 28- to 32-bp probe sequences and 5- to 6-bp stem sequences. The probes were designed to generate stable stem-loop structures while avoiding the formation of secondary structures in the loop region. The rpo1 probe targeted RRDR codons 507 to 516, rpo2 targeted codons 514 to 523, and rpo3 targeted codons 524 to 533. Each probe was designed to specifically detect all clinically relevant resistance-associated RRDR mutations spanning the codons targeted by the respective probes. A stable stem-loop structure is necessary for optimal binding kinetics between the SMB probes and the *M. tuberculosis* DNA target and for the production of distinct T_m values corresponding to each RRDR mutation. We stabilized the stem-loop structures for all three probes by introducing mutations into the probe loop to disrupt secondary structures and palindromes (Fig. 1A) as described previously (7, 8). The mutations introduced were chosen either to be complementary to a known

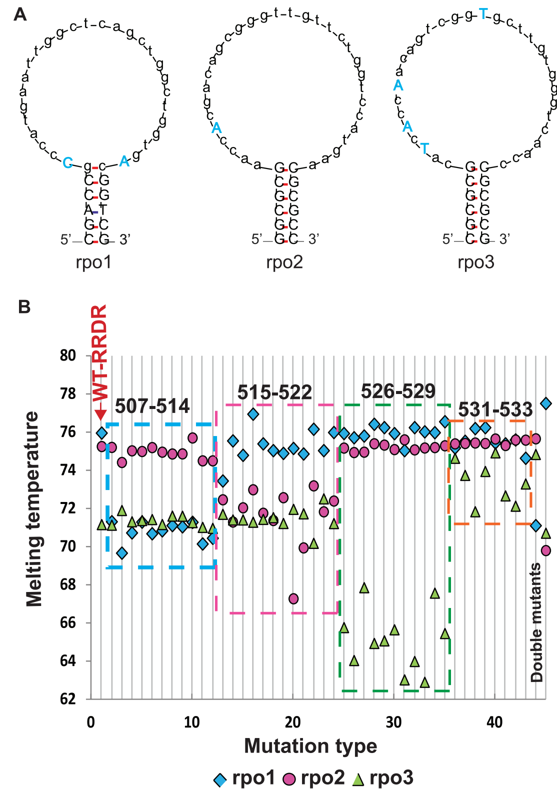

FIG. 1. Probe structures and T_m profiles against artificial targets. (A) The stable stem-loop structures of the three SMB probes used in the assay are shown. Related and unrelated mutations in the loop region introduced to obtain a stable stem-loop structure are shown in blue uppercase letters. Uppercase letters in black show the stem regions. (B) Three-probe T_m code of the assay tested against artificial targets with the wild-type RRDR or known RRDR mutations. Each horizontal line containing a square, circle, and triangle represents a unique 3-point T_m code corresponding to a single RRDR sequence. Line 1 shows results for a wild-type RRDR target, and lines 2 to 45 show results for mutant RRDR targets. The sequences of the mutations tested correspond to those shown in rows 1 to 45 of Table 1. Mutants are detectible by the presence of a substantial shift in the T_m value of either one or more of the three T_m points. Lines 2 to 12 show RRDR sequences harboring mutations in codons 507 to 514. Lines 13 to 24, RRDR sequences harboring mutations in codons 515 to 522; lines 25 to 35, RRDR sequences harboring mutations in codons 526 to 529; lines 36 to 43, RRDR sequences harboring mutations in codons 531 to 533; lines 44 and 45 show two RRDR double mutants.

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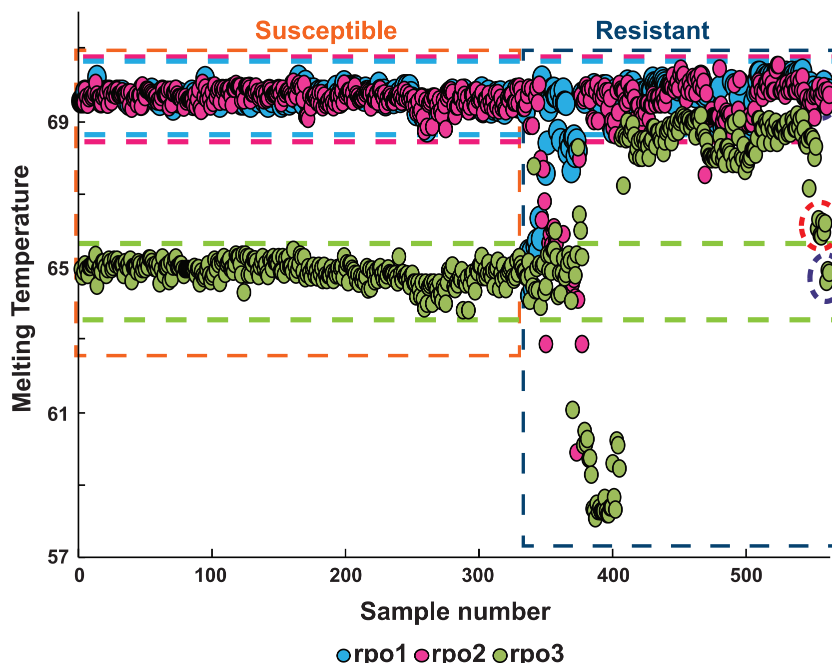

FIG. 2. Three-point T_m profile of 589 clinical DNA study samples. The results of the assay tested against the clinical *M. tuberculosis* study isolates are shown. The horizontal green, pink, and light-blue lines represent the T_m zones corresponding to the wild-type RRDR sequence for each of the three individual probes. Samples with one or more of the three T_m values that fall outside these T_m zones are easily identified as rifampin resistant. Rifampin-susceptible and rifampin-resistant isolates can be distinctly clustered based on their individual T_m profiles, as shown enclosed within the orange and blue rectangles. The red circle indicates the samples with the 533CCG mutation, which was distinctly and independently clustered from the wild-type samples in spite of having a relatively low dT_m value. The purple circle indicates the three rifampin-resistant samples with mutations in the *rpoB* gene outside the RRDR.

RRDR mutation or to be unrelated to any known nucleotide at that position, depending on assay requirements and on the resulting probe structure. Among the rifampin resistance-associated mutations introduced, probe rpo1 included a 516GGC mutation instead of wild-type sequence and rpo3 included 531TTG and 533ATG mutations instead of wild-type sequence. The inclusion of RRDR resistance-associated mutations within the SMB probes produced a gradient of probe-target relatedness, with certain common RRDR mutations producing T_m values higher than wild-type RRDR values and other (less commonly occurring) RRDR mutations producing T_m values lower than that of wild-type RRDR (Table 1 and Fig. 1B).

We measured the T_m of all three SMBs in the presence of wild-type RRDR and each of the RRDR mutant targets using artificial oligonucleotides. A three-point T_m code for each oligonucleotide target was generated by this procedure (Table 1). We found that the T_m code for the wild-type target could be distinguished from RRDR mutant targets in all cases. For 41 of the 44 RRDR mutants, at least one of the three SMBs had a T_m value difference (dT_m) of at least 2.1°C from the wild-type RRDR value (Table 1 and Fig. 1B). Many dT_ms were even higher. Targets containing common double mutants also had at least one probe with a dT_m of at least 2.1°C (Table 1 and Fig. 1B). Three less commonly occurring RRDR mutations, 531GCG, 531TAG, and 533CCG, were detected with dT_ms of −0.7, −1.5, and −1.0°C, respectively. However, these mutants produced larger dT_ms when chromosomal DNA from clinical *M. tuberculosis* isolates was tested (described below). The RRDR mutants could also be conveniently grouped into T_m code clusters corresponding to the codons tested by the individual probes (Fig. 1B and Fig. 2).

Assay T_m profiles of clinical samples compared to DNA sequencing. We evaluated the ability of the SMB assay to detect RRDR mutations against a panel of 589 clinical samples of diverse geographical origins (Table 2). The test panel included 236 RRDR mutant samples and 25 different RRDR mutation genotypes distributed over 11 codons, including the common clinical mutations, double mutants, codon insertions, and deletions (Table 2). The predominant mutation type was 531TTG (62%), followed by 516GTC (8.8%), 526TAC (7%), and 533CCG (4%), with the other genotypes being contained in the remaining 18% of the RRDR mutants. The assay identified 236/236 (sensitivity, 100%; 95% confidence intervals [CIs], 98.0 to 100.0%) RRDR mutants correctly, including 234/234 samples that contained only mutant DNA and 2/2 samples that were identified as heteroresistant mixed infections, by DNA sequencing. For 24 out of the 25 mutations, the average dT_m values were between 2.4 and 9.7°C, as either a positive or a negative shift from the T_m of the wild-type sequence, for at least one of the three probes (Table 2). The 10 samples with a 533CCG mutation had dT_ms ranging from −1.0 to −1.5°C. Thus, our tests of clinical samples identified all RRDR mutants, in contrast to our tests of oligonucleotides where 3/44 RRDR mutations showed smaller dT_ms, making them more difficult to detect. Assay specificity was also high, with 353/353 (100%; 95% CI, 98.7 to 100.0%) samples with wild-type RRDR sequences being correctly identified as having wild-type RRDR by the SMB assay. The 353 wild-type samples had mean dT_m values for rpo1, rpo2, and rpo3 of 69.7 ± 0.18, 69.7 ± 0.21, and 64.9 ± 0.29°C, respectively (Table 2).

All of the wild-type and mutant DNA samples generated an identifiable three-T_m code, which enabled the grouping of the

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TABLE 2 Three-probe T_m (°C) code of the assay in tests of clinical *M. tuberculosis* DNA[a]

Sample		T_m (°C) of:			SD for probe no.:			dT_m (°C) for probe no.:			No. of isolates
No.	Mutation type	rpo1	rpo2	rpo3	1	2	3	1	2	3	
#1	Wild type	**69.7**	**69.7**	**64.9**	0.18	0.21	0.29				353
2	511CTG-CCG	65.0	69.6	64.8	0.70	0.60	0.55	4.6	0.1	0.0	3
3	513CAA-CTA	65.9	69.8	64.7	0.64	0.35	0.51	3.8	−0.1	0.1	2
4	513CAA-AAA	65.7	69.8	64.7	0.11	0.16	0.43	4.0	−0.1	0.2	2
5	513-514CAA insertion	64.4	68.9	64.5	0.00	0.00	0.00	5.3	0.8	0.4	1
6	516GAC-AAC	69.8	67.7	65.1	0.00	0.00	0.00	−0.1	2.0	−0.2	1
7	516GAC-GTC	69.3	65.4	64.9	0.44	0.50	0.49	0.4	4.3	0.0	21
8	516GAC-CTC	67.6	62.9	64.5	0.00	0.00	0.00	2.1	6.8	0.4	1
9	516GAC-TAC	68.3	64.7	64.5	0.36	0.33	0.87	1.4	5.0	0.4	7
10	518AAC deletion	69.8	62.9	65.3	0.00	0.00	0.00	−0.1	6.8	−0.4	1
11	522TCG-TTG	69.7	66.8	64.3	0.00	0.00	0.00	0.0	2.8	0.5	1
12	526CAC-CGC	69.8	69.9	60.0	0.00	0.20	0.01	−0.2	−0.2	4.9	4
13	526CAC-TGC	69.8	70.1	59.5	0.00	0.00	0.00	−0.1	−0.4	5.4	1
14	526CAC-CTC	69.8	69.4	58.9	0.18	0.50	0.56	−0.2	0.3	5.9	3
15	526CAC-TAC	69.6	69.6	59.0	0.34	0.30	0.14	0.1	0.1	5.9	17
16	526CAC-GAC	69.8	69.7	58.3	0.08	0.44	0.17	−0.1	0.0	6.5	4
17	531TCG-CAG	69.9	69.9	68.6	0.01	0.02	0.06	−0.2	−0.2	−3.7	2
18	531TCG-TTG	69.8	69.6	68.6	0.55	0.53	0.42	−0.1	0.0	−3.7	147
19	531TCG-TGG	70.0	69.9	67.3	0.00	0.00	0.00	−0.3	−0.2	−2.4	1
20	533CTG-CCG	69.8	69.8	66.1	0.18	0.14	0.23	−0.1	−0.1	−1.2	10
21	513CAA-CTA, 523GGG-GAG	65.8	68.0	64.4	0.00	0.00	0.00	3.9	1.7	0.5	1
22	515ATG-ATT, 516GAC-TAC	66.7	64.8	64.4	0.00	0.00	0.00	3.0	4.9	0.5	1
23	515ATG-GTG, 516GAC-GGC	70.2	66.3	65.1	0.00	0.00	0.00	−0.5	3.4	−0.2	1
24	516GAC-GGC, 518AAC-CAC	68.5	59.9	65.4	0.00	0.00	0.00	1.2	9.7	−0.5	1
25	516GAC-TAC, 531TCG-TTG	68.2	64.1	68.3	0.00	0.00	0.00	1.5	5.5	−3.4	1
26	516GAC-GGC, 533CTG-CCG	72.2	67.9	66.2	0.23	0.19	0.31	−2.5	1.8	−1.4	2

[a] SD represents the standard deviations of the T_m values of each of the three probes for the different clinical samples harboring the same RRDR mutations. The shaded cells represent the dT_m values obtained for the probes detecting the corresponding mutations.

RRDR mutants into smaller clusters that contained one or more possible mutations as observed with the artificial targets (Fig. 2 and Table 2). Specifically, the assay clustered single mutants, insertions, and deletions into the following groups: 511/513 mutant, 513-514CAA insertion, 516AAC/522TTG, 516GTC, 516CTC, 516TAC, 516GGC, 518AAC deletion, 526CGC, 526TGC/CTC/TAC, 526GAC, 531CAG/TTG, 531TGG, and 533CCG (Table 2 and Fig. 1B). Double mutants were identified by the presence of characteristic dT_ms from more than one probe (Table 2).

Comparison to phenotypic drug susceptibility testing. The performance of the *rpoB* SMB assay was compared to phenotypic drug susceptibility testing in the 561 samples for which rifampin drug susceptibility testing results were available (Fig. 2). Sensitivity for resistance was 222/225 (98.7%; 95% CI, 95.8 to 99.6%), and specificity was 335/336 (99.7%; 95% CI, 98.1 to 99.9%). Among the rifampin-resistant false negatives, three contained *rpoB* mutations outside the RRDR (251TTC, 331CCC, and 572TTC). The single rifampin-resistant false positive occurred in a sample that was found to have an *rpoB* 511CCG mutation upon DNA sequencing. Clinical isolates with 511CCG mutations are prone to be misreported as rifampin susceptible by phenotypic rifampin susceptibility assays (31). Thus, this particular clinical strain may have been truly rifampin resistant. In fact, two other isolates in our study with the 511CCG mutation were reported to be phenotypically rifampin resistant. Unfortunately, cultures of this isolate were not available for repeat phenotypic susceptibility testing. The SMB assay and phenotypic drug susceptibility results were concordant for all of the other 557 samples tested.

Analytical specificity of the assay. The analytical specificity of the assay was further tested using DNA extracted from 121 clinical and 18 reference strains and 1 laboratory NTM strain, as well as 18 species of Gram-positive and Gram-negative bacteria. None of the NTMs, except for *M. malmoense*, generated a perceptible signal from any of the 3 probes. Very high concentrations of *M. malmoense* DNA (100 to 200 ng) caused the rpo2 probe to generate a T_m of 70°C, but no measurable T_m values were generated for the rpo1 and rpo3 probes. *M. smegmatis* also generated a profile similar to that of *M. malmoense* which is an rpo2 T_m of 70°C, even at lower DNA concentrations of up to 1 ng. This triple-T_m code (0, 70, and 0) was quite distinct from all of the other T_m codes that we had obtained on tests of both wild-type and mutant RRDR *M. tuberculosis* DNA. None of the Gram-positive or Gram-negative bacteria tested produced a measurable signal. To test for possible interference from NTM DNA, we mixed 10^6 genome equivalents of *M. malmoense*, *M. abscessus*, *M. avium*, *M. chelonae*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, and *M. smegmatis* DNA with 10^5 genome equivalents of *M. tuberculosis* DNA containing RRDR mutations at 516GTC, 526TAC, 531TTG, or 533CCG. Each RRDR mutant was correctly identified despite the presence of excess NTM DNA (see Table S1 in the supplemental material). Further increasing the *M. malmoense* DNA concentration to a 20- to 50-fold excess compared to the concentration of *M. tuberculosis* DNA caused the rpo2 probe to generate a double T_m peak in the presence of the *M. tuberculosis* 516GTC and 516AAC RRDR mutants (see Table S1). When a double peak was visualized, one of the double peaks corresponded to the T_m expected for the *M. tuber-*

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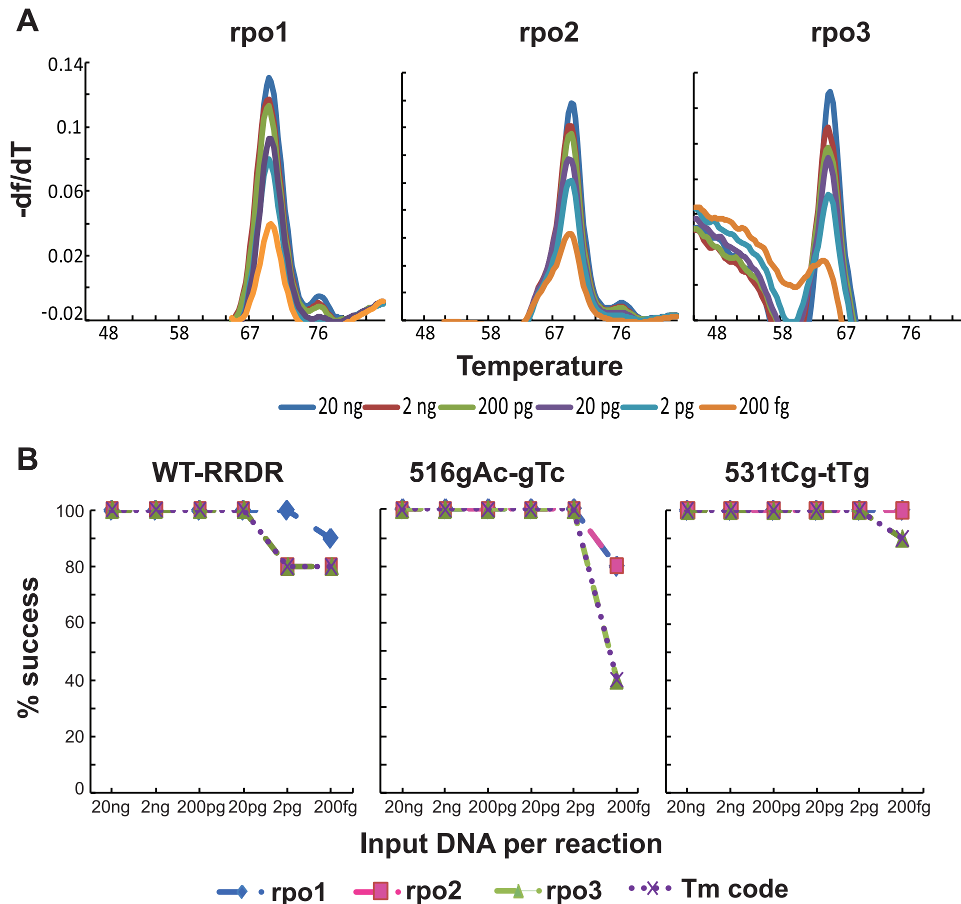

FIG. 3. Analytical sensitivity of the assay. (A) Assay curves with limiting amounts of wild-type DNA target. Melting temperature profiles of the three SMB probes on serial dilutions of *M. tuberculosis* H37Rv genomic DNA with wild-type *rpoB* RRDR. (B) Limit of detection (LOD) of the assay against wild-type and RRDR mutant DNA. T_m values for each probe are shown for assays performed against the wild-type RRDR and two different RRDR mutant DNA samples. Assays were tested with a range of DNA concentrations as indicated. The LOD of the assay for each RRDR sequence type is defined by the LOD of the probe with the lowest analytical sensitivity. As shown, the LOD for wild-type RRDR was 20 pg, and the LOD for the 516GTC and 531TTG mutant RRDR samples was 2 pg.

culosis RRDR mutant, and the other peak corresponded to the T_m expected for *M. malmoense* DNA (data not shown). *M. malmoense* DNA alone, when used at a 20- to 50-ng concentration, generated a single peak with a T_m of 70°C corresponding to the rpo2 probe, and this did not mimic the wild-type profile or any of the RRDR mutant three-probe T_m profiles (see Fig. S1A in the supplemental material). Results identical to those for *M. malmoense* were obtained when 10 ng of the laboratory strain of *M. smegmatis* DNA was spiked into *M. tuberculosis* DNA containing the 516GTC and 516AAC mutations (see Table S1). These results strongly suggest that NTM coinfections will not affect assay results regarding *M. tuberculosis* rifampin susceptibility. The assay was also found to be unaffected by the presence of as much as a 10^5-fold excess of human genomic DNA compared to the concentration of *M. tuberculosis* DNA. The assay's capacity for mutation detection also remained unaltered, as tested with a mixture containing a large excess of human DNA mixed with clinical sample DNA containing the RRDR mutation 516TAC, suggesting that human genomic DNA from sputum should not interfere with the assay's performance (see Fig. S1B).

Assay LOD. Genomic DNA from *M. tuberculosis* containing wild-type *rpoB* RRDR and the two most common RRDR mutations, 516GTC and 531TTG, were chosen to determine the limit of detection of the *rpoB* SMB assay. DNA samples were serially diluted from 10 ng to 100 fg per microliter in 10-fold decrements, and the SMB assay was performed on 2 μl of each individual dilution 10 times. We observed that the T_m values obtained at different concentrations were independent of the initial amount of target DNA put into the reaction for *M. tuberculosis* with wild-type (Fig. 3A) and mutant RRDR (data not shown), when T_m peaks could be measured. Each probe had a characteristic LOD below which no T_m value could be observed. We defined the assay LOD as the DNA concentration below which at least one probe could not consistently measure a T_m (probe rpo3 in this case). For the wild-type and mutant DNA samples, the assay could detect 20 and 2 pg of DNA correctly 100% of the time (Fig. 3B), respectively, which is approximately 2,000 and 200 *M. tuberculosis* genome equivalents, respectively. These results suggest that the assay is able to detect rifampin resistance in DNA extracted from all smear-positive and many smear-negative patient samples.

Detection of 531TTG mutation in DNA mixtures. The ability of the *rpoB* SMB assay to detect the RRDR mutant in this mixture was compared to that of Sanger DNA sequencing. The SMB assay detected the presence of the RRDR mutation down to a concen-

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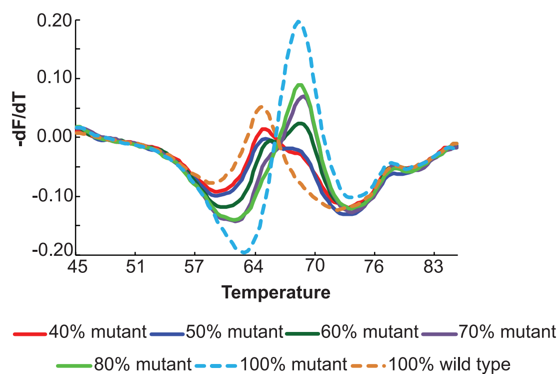

FIG. 4. Detection of mixed-sequence heteroresistance. The assay was performed with various mixtures of wild-type and mutant DNA, as indicated. Double T_m peaks, indicating mixtures of wild-type and mutant target, were seen when as little as 40% mutant sequence was present.

tration of 40% mutant DNA, as indicated by the double peak of T_m values (Fig. 4). The mutant T_m peak became increasingly prominent as more mutant DNA was present in the mixture. DNA mixtures with 10, 20, and 30% mutant DNA showed a single wild-type T_m peak and were indistinguishable from a sample containing 100% wild-type DNA (Fig. 4). Using the same DNA mixtures to perform the bidirectional sequencing of the *rpoB* amplicon, the unequivocal presence of the mutant DNA was apparent only on sequencing chromatograms when at least 50% mutant DNA was present. Thus, our SMB assay detected mixed infections at least as well as Sanger DNA sequencing as far as the most common 531TTG mutation was concerned.

DISCUSSION

The assay used in this study detected the reported RRDR mutations with high sensitivity and specificity. The assay was also analytically very specific for *M. tuberculosis* when tested against a large panel of NTM and other bacteria. Heteroresistance was detected at least as well as that detected by Sanger DNA sequencing. Finally, the assay LOD appeared to be sufficiently sensitive for use in all smear-positive and many smear-negative sputum samples from tuberculosis patients. To our knowledge, our study describes the first T_m shifting assay for rifampin-resistant *M. tuberculosis* with this high a level of performance. The mismatch-tolerant SMB probes used in this study offer several advantages over the current generation of probe-based drug susceptibility tests. Unlike open hybridization assays, our assay is performed in a closed system, which greatly simplifies sample testing and minimizes cross-contamination. Unlike HRMA detection methods, which detect mutations by recognizing subtle melting curve variants (9, 27), our assay produces clear and consistent T_m peaks, which enables highly reproducible T_m value identification.

Several assays have been described previously which utilize FRET probes or dually labeled probes and post-PCR T_m shifts to identify resistance-associated mutations (9, 10, 13, 18, 20, 24, 26, 27). A recent study by Luo et al. used dually labeled probes and a common fluorophore to label and identify mutations associated with isoniazid and rifampin resistance using T_m curve patterns, which is similar to our approach. However, the dually labeled probes used by those authors produced overlapping T_m peaks that are potentially difficult to analyze (24). This system is also unlikely to be able to identify most mixtures of wild-type and mutant DNA sequences. In contrast, the peaks generated by SMBs were sharp and consistent even when our assay was performed starting from very small numbers of genomic DNA copies. Assays which use FRET probes are constrained by the requirement to design probes in pairs (one anchor and one sensor probe). This requirement can cause some mutations to be masked by the anchor probes (18) and in some cases results in T_m differences that are less than 0.5°C between wild-type and mutant sequences (25). FRET probes also require the monitoring of fluorescence ratios, which generates complicated T_m patterns that can be easily confounded by DNA mixtures (20). A recent study used shared-stem molecular beacons and TaqMan probes to identify *rpoB* RRDR mutations using post-PCR T_m calculations (18). However, the assay required at least two independent reactions and four different probes to cover the entire RRDR. Furthermore, the T_m differences and the resolution provided by the shared-stem molecular beacons was much smaller than that observed with other dually labeled probes and by our assay, decreasing mutant discriminatory capacity (18).

Mismatch-tolerant SMB probes created according to our design principles allow versatility in sequence recognition while maintaining a robust capacity to discriminate mutations. The three-point T_m code produced by our assay either specifically identified RRDR mutations or grouped them into easily identifiable mutation clusters. This ability to subtype different RRDR mutations may prove useful in epidemiological investigations or where different rifamycins confer different levels of resistance (20, 28, 30, 35). The assay did not depend on high-resolution T_m capabilities. Unlike other assays which focus mainly on identifying the most common clinically prevalent mutations (20, 24, 27), we designed our assay to detect both the common and uncommon mutations over the entire RRDR sequence. The large probe regions permitted by SMB design made it possible for us to use just three probes to target the entire 80-bp *rpoB* RRDR sequence. The assay identified every type of mutation tested, including nucleotide transitions, transversions, deletions, and insertions, along with several double mutations.

There are some limitations to the current form of our assay. First, our assay cannot specifically differentiate an infrequently occurring synonymous mutation at RRDR codon 514 from nonsynonymous mutations at codon 514, which are associated with rifampin resistance (13). Should synonymous mutations appear more frequently than are currently reported in clinical settings, our assay could be redesigned to generate a unique three-point T_m code for that particular mutation. Second, the average dT_m of the rpo3 probe for the 533CCG mutant was 1.16°C. While sufficient to reliably detect 533CCG mutations, this dT_m was the smallest of all the dT_ms generated by mutations in our assay. It is possible that the dT_m for 533CCG mutations could be improved by additional rounds of probe redesign. Third, we have not yet tested our assay on DNA directly extracted from human sputum samples. It is possible that PCR inhibitors present in sputum degrade the assay LOD. However, many methods exist to extract *M. tuberculosis* DNA from sputum for sensitive real-time PCR assays (3, 22). Therefore, we do not expect this to be a major developmental hurdle.

In summary, we have developed a sensitive, specific, and rapid assay that is strongly amenable to a high-throughput format which can detect all of the clinically significant mutations in the *rpoB* RRDR of *M. tuberculosis.* This assay is specific for *M. tuberculosis*, simple, and robust and does not require any high-resolu-

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tion melting software, thus it is compatible with various real-time PCR platforms. The SMB T_m coding approach we describe is likely to be generally useful for rapid mutation detection in clinical microbiology.

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D. Alland is among a group of inventors who earn royalties for molecular beacon usage.

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