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Detection of Isoniazid-, Fluoroquinolone-, Amikacin-, and Kanamycin-Resistant Tuberculosis in an Automated, Multiplexed 10-Color Assay Suitable for Point-of-Care Use

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ABSTRACT Extensively drug-resistant (XDR) tuberculosis (TB) cannot be easily or quickly diagnosed. We developed a rapid, automated assay for the detection of XDR-TB plus resistance to the drug isoniazid (INH) for point-of-care use. Using a simple filter-based cartridge with an integrated sample processing function, the assay identified a wide selection of wild-type and mutant sequences associated with XDR-TB directly from sputum. Four new large-Stokes-shift fluorophores were developed. When these four Stokes-shift fluorophores were combined with six conventional fluorophores, 10-color probe detection in a single PCR tube was enabled. A new three-phase, double-nested PCR approach allowed robust melting temperature analysis with enhanced limits of detection (LODs). Finally, newly designed sloppy molecular beacons identified many different mutations using a small number of probes. The assay correctly distinguished wild-type sequences from 32 commonly occurring mutant sequences tested in *gyrA*, *gyrB*, *katG*, and *rrs* genes and the promoters of *inhA* and *eis* genes responsible for resistance to INH, the fluoroquinolone (FQ) drugs, amikacin (AMK), and kanamycin (KAN). The LOD was 300 CFU of *Mycobacterium tuberculosis* in 1 ml sputum. The rate of detection of heteroresistance by the assay was equivalent to that by Sanger sequencing. In a blind study of 24 clinical sputum samples, resistance mutations were detected in all targets with 100% sensitivity, with the specificity being 93.7 to 100%. Compared to the results of phenotypic susceptibility testing, the sensitivity of the assay was 75% for FQs and 100% each for INH, AMK, and KAN and the specificity was 100% for INH and FQ and 94% for AMK and KAN. Our approach could enable testing for XDR-TB in point-of-care settings, potentially identifying highly drug-resistant TB more quickly and simply than currently available methods.

KEYWORDS 10-color assay, three-phase PCR, XDR-TB, point-of-care test

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Drug-resistant tuberculosis (TB) is an increasing threat worldwide (1–5). The most worrisome cases of TB are caused by multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains, which are already resistant to rifampin (RIF) and isoniazid (INH) and which acquire additional resistance to the fluoroquinolones (FQs) and any one of the newer injectable drugs, amikacin (AMK), kanamycin (KAN), or capreomycin (CAP). These highly resistant strains are termed extensively drug resistant (XDR) *M. tuberculosis* (6). XDR-TB is very difficult to treat and can lead to high rates of mortality (7–11), especially when an XDR-TB diagnosis is missed and appropriate treatment is delayed (10). Rapid diagnostic assays for the detection of XDR-TB are urgently needed to address these problems.

Culture and drug susceptibility testing of *M. tuberculosis* are expensive, time-consuming, and labor-intensive and present a serious biohazard to laboratory workers (12, 13), resulting in fewer accredited facilities in countries where *M. tuberculosis* is endemic. Even when available, culture-based susceptibility testing can take from weeks to months to complete. *M. tuberculosis* may also be tested for drug resistance using the alternative rapid, sensitive, and safer genotypic assays, which detect resistance by identifying mutations known to confer resistance to the first- and second-line drugs in a majority of clinical strains (14–23) and minimizes the biohazard. Genotypic testing approaches that can be reduced to a few manual steps are more amenable to use at the point of care, which can dramatically expand their availability to medically underserved populations.

The GeneXpert MTB/RIF (Xpert MTB/RIF) assay is an example of a genotypic assay that identifies *M. tuberculosis* while it simultaneously detects RIF resistance, if present (24). The Xpert MTB/RIF assay can be performed directly from clinical samples. It is entirely self-contained in a disposable test cartridge, and it can be operated by personnel with minimal training to provide on-demand results at the point of care in less than 2 h. Although the current assay is performed using a desktop GeneXpert MTB/RIF instrument that requires uninterrupted external power, an inexpensive, handheld, and battery-operated alternative platform for this assay recently announced by Cepheid should make it possible to perform Xpert MTB/RIF assays at the point of care in a clinical setting. The Xpert MTB/RIF assay has a clinical sensitivity of 96.8 to 99% for smear-positive sputum samples and 65.4 to 78.7% for smear-negative sputum samples (25, 26), and it identifies RIF resistance with approximately 94% sensitivity and 98% specificity (27). However, the drug resistance portion of the Xpert MTB/RIF assay provides information only on RIF resistance, and a rapid easy-to-use assay for the detection of other types of drug resistance in *M. tuberculosis* is still urgently needed.

An evolving body of work has indicated that most cases of resistance to the major TB drugs can be associated with approximately 25 mutations in six genes and promoter regions. Approximately 90% of cases of INH resistance can be detected by the presence of mutations in *katG* codon 315 and nucleotides −8 to −15 of the *inhA* promoter (28); approximately 90% of cases of FQ resistance can be detected by the presence of mutations in codons 88 to 94 of the *gyrA* gene, with mutations in codons 500 and 538 to 540 of the *gyrB* gene identifying resistance in an additional few percentage of cases (29–33). Mutations in positions 1401 and 1402 of *rrs* identify approximately 80% of cases of AMK resistance, and the same *rrs* mutations plus mutations at positions −8 to −37 of the *eis* promoter identify resistance in approximately 80% of cases of KAN resistance (34–36). The presence of a wild-type sequence (or of a few rare, previously identified polymorphisms that do not encode resistance) indicates susceptibility to the same drugs with close to 100% specificity when discordant results are carefully investigated.

Several molecular tests that detect most of the mutations responsible for XDR-TB have recently been developed (4, 14, 16–20, 23, 37–39). However, all of these tests require sophisticated laboratory facilities, making them unsuitable for near-patient TB diagnosis so that treatment may begin immediately (40, 41). Designing an Xpert MTB/RIF-like test to identify XDR mutations at the point of care has been challenging due to the difficulty in identifying all of the clinically relevant mutations associated with

drug resistance in six separate genes or promoter regions plus an internal control in a single reaction tube. The need to detect resistance mutations directly from smear-negative clinical sputum samples that may contain small numbers of *M. tuberculosis* bacilli further challenges the assay design. Here, we describe the development of such a technology for use at the point of care which addresses and resolves all these challenges. This new assay is designed to complement the existing Xpert MTB/RIF assay, supplying additional information on the INH, FQ, KAN, and AMK resistance of isolates in clinical samples that are already positive for TB (with or without RIF resistance) using the Xpert MTB/RIF test. New large-Stokes-shift dyes, innovative three-segment nested thermal cycling, and melting temperature (T_m)-based mutation detection are used in combination to produce a 10-color, eightplex PCR which simultaneously amplifies seven genes and detects over 25 different mutations indicative of XDR-TB directly from sputum. The performance of this highly multiplexed assay in an analytical laboratory setting and its preliminary validation in a field setting are also described.

RESULTS

Large Stokes-shift fluorescent chemistries increase usable detection channels. Our assay for the detection of XDR-TB (referred to here as the XDR assay) required nine different probes to identify mutations in seven *M. tuberculosis*-specific amplicons. An additional 10th probe was needed to confirm amplification of the assay's internal control (IC). The number of probes that can be distinguished in a single multiplexed homologous amplification assay is limited by the number of fluorophores that can be reliably resolved. Most homologous detection systems differentiate among fluorophores using an on-axis detection strategy, whereby each fluorophore has a distinct excitation and emission spectrum (Fig. 1A, CF1 to CF6). On-axis detection is usually limited to four fluorophores, given the narrow range of wavelengths available over the visible spectrum (Fig. 1A, 6-carboxyfluorescein [FAM]-CF5). However, we were able to resolve up to six different fluorophores, using an on-axis strategy, by adding one fluorophore with excitation in the near ultraviolet (UV) (Fig. 1A, CF1) and another fluorophore that emitted in the near infrared (IR) (Fig. 1A, CF6) (24). We sought to further increase multiplexing by developing what we have termed off-axis fluorophores that produce a larger Stokes shift (farther toward the red) than conventional fluorophores. These proprietary off-axis fluorophores have excitation spectra similar to those of on-axis fluorophores, but their unique combination of excitation and emission spectra enabled us to assign each one to a distinct channel that distinguished them from on-axis fluorophores. Large Stokes-shift fluorophores CF7 (which excites blue and emits yellow), CF8 (which excites green and emits orange), CF9 (which excites blue and emits orange), and CF10 (which excites green and emits IR) (Fig. 1A) were created to utilize four off-axis channels, thereby increasing the detection capacity of the GeneXpert MTB/RIF system to 10. Each of these large-Stokes-shift fluorophores produced strong signals when linked to the oligonucleotide probes in aqueous buffer as well as narrow excitation and emission spectra (Fig. 1B to E). We also developed a proprietary quenching molecule with sufficient spectral overlap to efficiently quench all of the new fluorophores, allowing us to incorporate the new off-axis, large-Stokes-shift fluorophores into molecular beacon probes.

Achieving highly multiplexed melting temperature analysis and PCR in an integrated sample processing cartridge. We have previously described a series of real-time PCR assays that together detect most cases of resistance to FQs, AMK, and KAN in *M. tuberculosis* (34, 42). Resistance was detected by hybridizing sloppy molecular beacons (SMBs) to amplicons of drug resistance targets and then measuring the melting temperature (T_m) of the SMB-target hybrid. Drug-susceptible wild-type targets were identified by the presence of characteristic T_m values. Drug resistance mutations in these targets were identified by a shift in T_m values away from the wild-type T_m. However, the drug resistance assays were performed in multiple tubes, and our desire to add INH resistance testing as well as an internal control assay would have increased

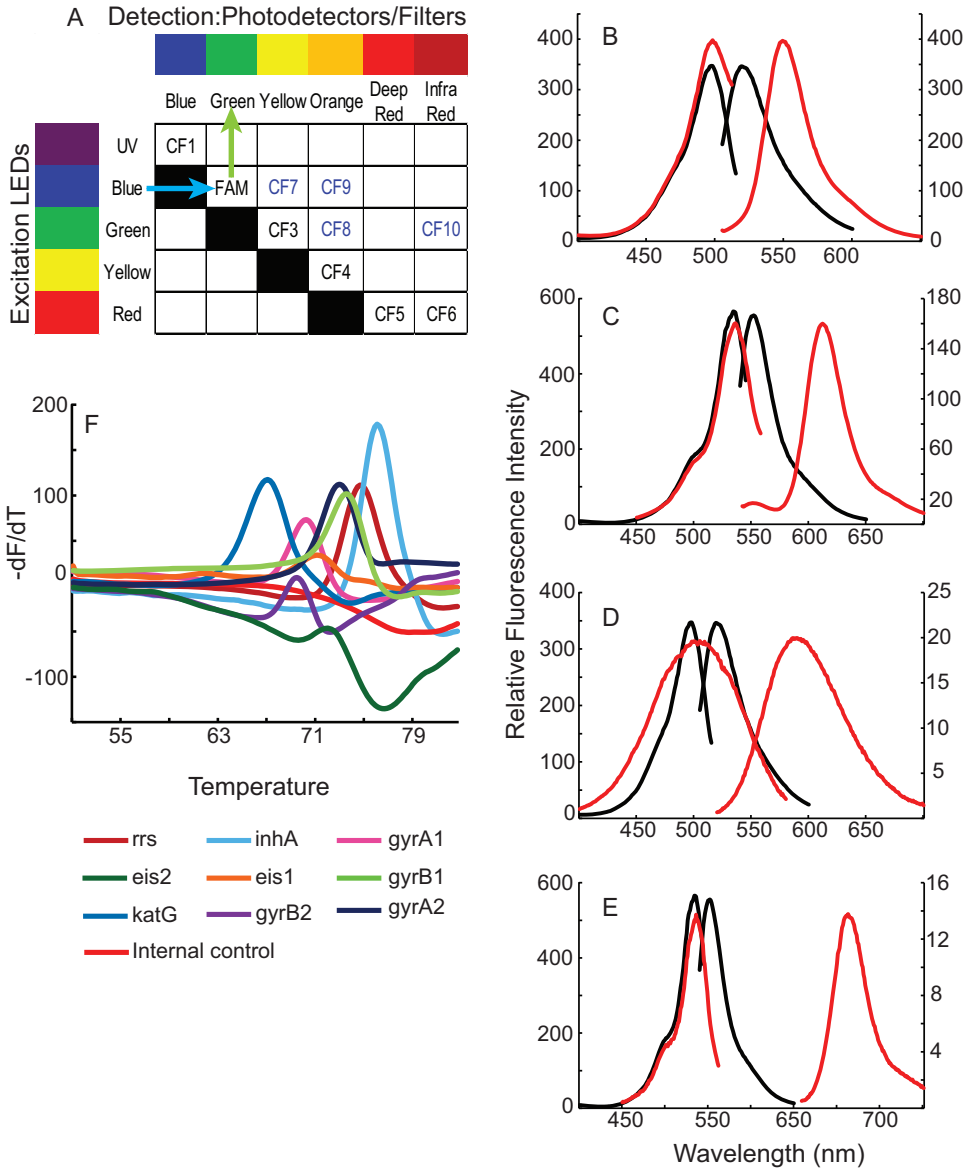

FIG 1 Highly multiplex mutation detection using a combination of on-axis and off-axis fluorophores. (A) Schematic showing the method for resolving up to 20 different fluorophores using only five excitation light-emitting diodes (LEDs) and six detection channels. In the current assay, 4 large off-axis Stokes-shift fluorophores (CF7 to CF10) are combined with 6 on-axis fluorophores (CF1 to CF6) to resolve 10 different fluorophores. UV, ultraviolet. (B to E) The excitation and emission spectra of the large-Stokes-shift fluorophores are shown (red curves) in comparison to the excitation and emission spectra of on-axis fluorophores (black curves) with similar excitation maxima, as shown by the overlapping of the excitation maxima of the different fluorophore types in each panel. The scale for the on-axis fluorophores is shown on the left-hand *y* axis; the scale for the off-axis fluorophores is shown on the right-hand *y* axis. (B) FAM and CF7; (C) CF3 and CF8; (D) FAM and CF9; (E) CF3 and CF10. (F) Melt curves of 10 different fluorophore-labeled sloppy molecular beacons specific to drug resistance targets in *M. tuberculosis* and the assay internal control target, simultaneously detected in a real-time PCR instrument normally designed to detect only six fluorophores. Note the absence of cross talk between channels. -dF/dT indicates the negative first derivative of the normalized fluorescent melt intensity of the probes with respect to temperature.

the number of required tubes even further. We therefore investigated whether we could make use of our 10 resolvable fluorophore-quencher pairs to combine all the different resistance detection assays into a single assay tube in the GeneXpert MTB/RIF system with an enhanced spectral resolution. We labeled 10 SMBs with the 10 fluorophores (and the universal quencher) designed to be detected in 1 of the 10 channels shown in Fig. 1A. Nine of the SMBs had a probe region that was strongly complementary (with no more than 3 mismatches) to short regions in the *inhA* promoter (one SMB)

or the *katG* gene (one SMB) (for INH resistance detection), the *gyrA* (two SMBs) or *gyrB* (two SMBs) gene (for FQ resistance detection), and the *rrs* gene (one SMB) or *eis* promoter (two SMBs) (for KAN and AMK resistance detection). A 10th SMB was designed to be complementary to a region of the *Bacillus globigii* genome and was used as part of an IC for sample processing and PCR. All 10 SMBs as well as the primers (see Tables S1 and S2 in the supplemental material) and all necessary reagents needed to amplify the eight target amplicons in a two-step heminested PCR were integrated into a filter-based sample processing/real-time PCR detection cartridge identical to that used in the Xpert MTB/RIF assay, except for the differences in PCR reagents (24). The full assay was then tested on clinical sputum samples spiked with known numbers of CFU of drug-susceptible *M. tuberculosis*, using a GeneXpert MTB/RIF instrument designed to perform all the sample processing and PCR steps automatically. The first phase of the PCR was a conventional symmetrical PCR, while the second, nested phase was performed as an asymmetrical PCR (43) to allow the efficient generation of single-stranded amplicons, which are the best targets for T_m analysis. Our results confirmed that the 10-fluorophore set allowed us to differentiate among these 10 SMBs using the same excitation light-emitting diodes, filters, and detectors contained in a GeneXpert MTB/RIF instrument originally designed to differentiate only six fluorescent probes. The 10 individual first-derivative melt curve profiles were clearly visible without any interfering cross talk from one channel to the other (Fig. 1F).

Three-phase nested single-primer amplification dramatically enhances the LOD. Multiplex PCR can adversely affect assay sensitivity, and we were unable to achieve a limit of detection (LOD) better than 1,639 CFU/ml sputum using a two-phase heminested and nested PCR (Fig. 2A), yet for the XDR assay to be used as a reflex test for positive Xpert MTB/RIF assays, its ideal LOD should be roughly equivalent to the Xpert MTB/RIF assay LOD of 132 CFU/ml (24). To this end, we devised a novel three-phase amplification scheme to further improve the assay LOD. The first amplification phase consisted of a symmetric PCR. This was followed by a second round of nested or heminested symmetric PCR. Finally, a third linear amplification was performed, but this time only one (nested) target primer was used to amplify each amplicon, generating single-stranded products to which the probes could specifically hybridize (Fig. 3). This three-phase amplification approach enabled us to fully optimize the first two PCR phases for sensitive symmetric amplification and to optimize the third amplification phase for the production of single-stranded targets that are ideal for probe-based T_m analysis. The total on-instrument assay time was approximately 110 minutes, permitting a time to the result that was approximately equal to that of the Xpert MTB/RIF assay. We found that three-phase amplification improved the LOD by almost 1 log unit to 300 CFU/ml (Fig. 2B). Even with counts below the established LOD, the assay showed 83%, 70%, and 20% success rates at 250 CFU/ml, 125 CFU/ml, and 62.5 CFU/ml, respectively. All T_m peaks remained strong and easily interpretable even in assays with samples that had counts below the LOD but that were positive (Fig. 4). Importantly, none of the 60 assays that were performed with samples containing *M. tuberculosis* at the sub-LOD target concentrations and that did not detect *M. tuberculosis* showed any T_m artifacts that could be erroneously interpreted as peaks corresponding to mutant sequences. Similarly, the $>$200 negative-control assays without added bacilli, performed over a period of 10 months, were negative for all the *M. tuberculosis*-specific probes. The IC assay remained positive for all the negative samples (controls or replicates with counts below the LOD), indicating a successful PCR (Fig. 4I).

Accurate mutation detection. The nine different *M. tuberculosis*-specific SMB probes in the Xpert MTB/RIF cartridge used for the XDR assay were designed to detect at least 32 different wild-type and mutant sequences in our test panel known to be associated with clinical INH, FQ, and aminoglycoside resistance (Table 1). We tested the performance of the assay with DNA from an extensive panel of MDR and XDR clinical isolates with a variety of common mutations in the target region, including double and triple mutations in the *gyrA* quinolone resistance-determining region (QRDR) (Table 1),

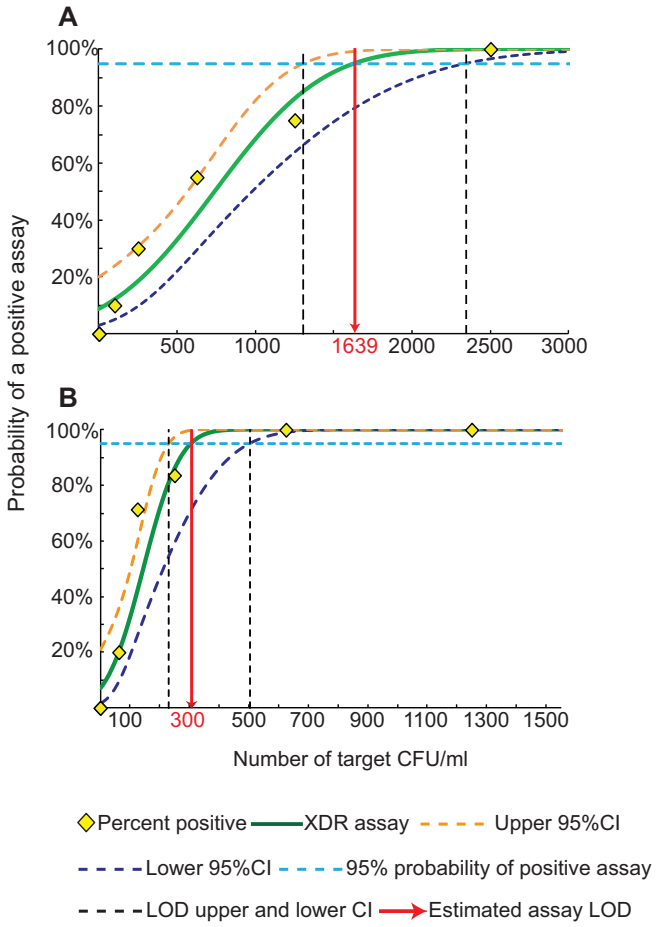

FIG 2 Assay limit of detection. One-milliliter sputum samples were spiked with the indicated numbers of *M. tuberculosis* CFU, and the success rate of the assay with each number of CFU was tested. The LOD was defined as the lowest number of CFU associated with a 95% probability of a successful assay. (A) Two-phase assay; (B) three-phase assay. CI, confidence interval.

as well as 50 different pan-susceptible clinical strains with wild-type sequences in all the target genes. Experiments were performed in eight different GeneXpert MTB/RIF system bays over a period of 3 months to confirm the reproducibility of each T_m value and the mutation detection capacity of the assay. The assay generated clear T_m peaks in the presence of each wild-type and mutant sequence (Fig. 5), with standard deviations (SDs) for each T_m being no more than ±0.3°C (Table 1). Mutant targets caused the T_m of the corresponding SMB to shift at least 2°C (equivalent to at least 6 standard deviations) above or below the T_m of the wild-type target. These substantial differences in T_m values between wild-type and mutant sequences (dT_ms) enabled us to generate probe-specific T_m windows (Table S6) unique to either wild-type or mutant sequences and to easily differentiate between drug-susceptible and drug-resistant isolates.

There is increasing evidence that different resistance mutations may be associated with different MICs and may confer variable susceptibilities to different drugs within a given drug category (34, 35, 44–46). We investigated whether the T_m generated by each SMB could be used to specifically identify individual mutations (Table 1). Indeed, the two *gyrA* SMBs could distinguish between the S91P/A90V, D94A, D94G, and D94N/Y mutations and the two *gyrB* SMBs could distinguish between the D500V, D500N, and N538T/E540V/D mutations. Similarly, the *rrs* SMB could distinguish between nucleotide 1401 and 1402 mutations, the two different phylogenetic *gyrA* polymorphisms at position 95 could be specifically identified, and double and triple mutations in *gyrA*

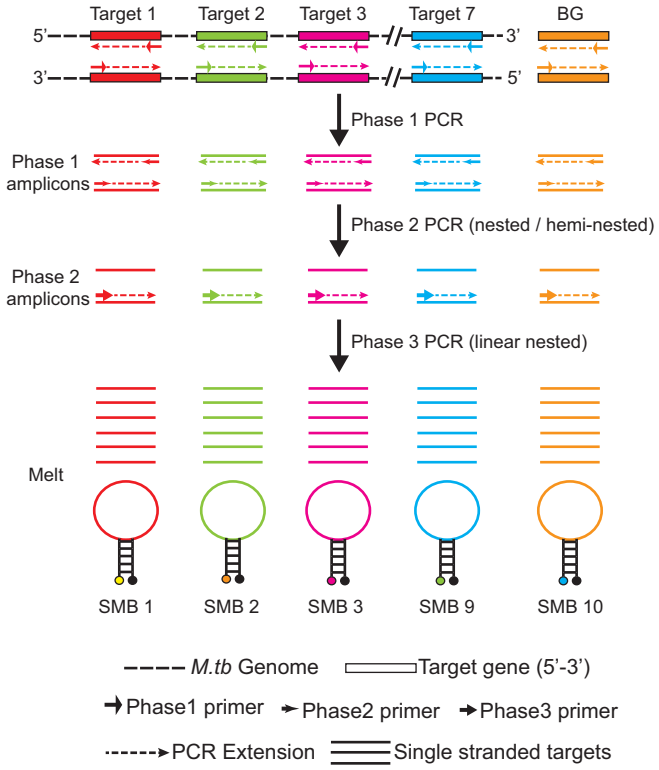

FIG 3 The three-phase, eightplex, 10-color PCR assay scheme. A sevenplex PCR amplifies the genes from *M. tuberculosis* (*M.tb*), and the eighth assay amplifies the *Bacillus globigii* (BG) internal control. Ten differently colored SMB probes target the eight amplicons generated.

could also be identified as such. We were also able to distinguish among individual *inhA* and *eis* promoter mutations by virtue of their different T_m patterns (Table 1).

Detection of heteroresistance. Patients infected with a mixture of susceptible and resistant clones (heteroresistant clones) have been increasingly described in both MDR-TB and XDR-TB cases (47–50). Detection of heteroresistance can be challenging for molecular assays; however, these mixtures of sequences may be detected as double peaks in T_m-based detection systems (42). We tested the ability of our XDR assay to detect heteroresistance in a series of DNA mixtures containing 0 to 100% mutant DNA against a background of wild-type DNA with a total of 10,000 genome equivalents (mutant plus wild type) per reaction. The DNA mixtures were distributed randomly and coded, and the assay was performed in a blind manner. The mutations tested were D94G and D94A in the *gyrA* QRDR, C(−15)T in the *inhA* promoter, and A1401G in the *rrs* gene. Mutations were detected either as a predominant mutant peak or as distinct double peaks corresponding to both wild-type and mutant T_m profiles. The assay was able to consistently detect as few as 20%, 20%, 30%, and 40% of the sequences with D94A, D94G, C(−15)T, and A1401G mutations, respectively (Fig. 6), whereas the results obtained by Sanger sequencing were 30%, 20%, 20%, and 30%, respectively, indicating the comparable performance of the two methods for the detection of heteroresistance.

Analytical specificity. We tested the specificity of the assay for *M. tuberculosis* detection against a large panel of nontuberculous mycobacteria (NTM) (Table 2) as well as common Gram-positive and Gram-negative respiratory pathogens and colonizers (listed in the Materials and Methods section) using 10^8 CFU of each species. Several of our assay's primers and SMBs were highly specific for *M. tuberculosis*. We chose the *inhA* assay, from among our specific SMBs, to definitively identify *M. tuberculosis* in each test sample because it generated particularly robust T_m peaks with the highest T_m (Fig. 4). All of the NTM samples tested negative for *M. tuberculosis* on the basis of the results obtained with the *inhA* SMB. Next, we examined the cross-reactivity of all of the other

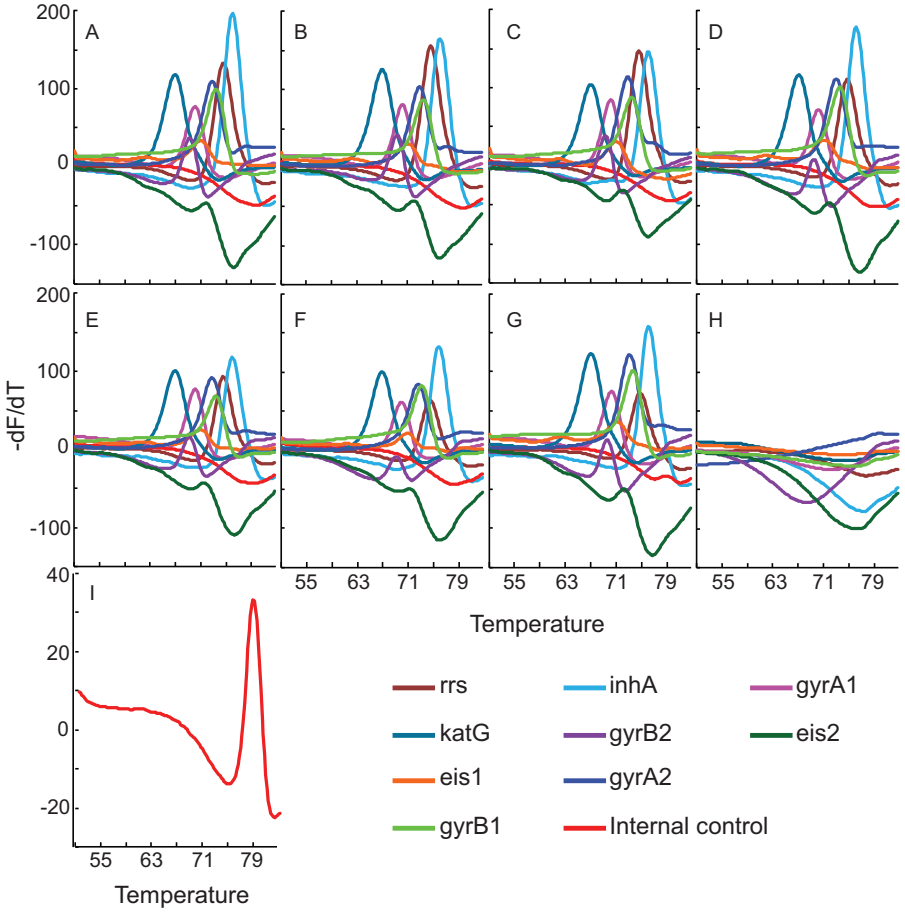

FIG 4 Assay dynamic range. One-milliliter sputum samples were spiked with 2.5×10^7 (A), 2.5×10^5 (B), 2.5×10^4 (C), 1,250 (D), 625 (E), 125 (F), and 62.5 (G) CFU/ml *M. tuberculosis* or were not spiked with *M. tuberculosis* (H and I). (A to H) Melt profiles of each of the nine *M. tuberculosis* detection probes; (I) melt profile of the internal control assay probe in the sputum sample into which *M. tuberculosis* was not spiked.

M. tuberculosis-specific SMBs in the assay. No isolate in the bacterial panel produced a T_m peak for any of the *M. tuberculosis* SMBs except for the *rrs* SMB, which generated a wild-type T_m peak from several of the Gram-positive and Gram-negative bacteria tested. Similarly, no NTM produced a T_m peak in the wild-type window for any of the *M. tuberculosis*-specific SMBs except *rrs*, which did generate a wild-type T_m for most of the NTMs (Table 2). This was expected, as the *rrs* region targeted by our assay is highly conserved among most *Mycobacterium* species as well as some Gram-positive and Gram-negative bacteria. Several NTMs also produced T_m peaks in the assay's mutant T_m windows for the *gyrA* probes. The gyrA1 probe generated a T_m of 65.6°C with *Mycobacterium xenopi* and T_ms of approximately 64°C with *M. scrofulaceum* and *M. avium* and 69.7°C with *M. gordonae*, and the gyrA2 probe generated T_ms of 66.7°C, 66.4°C 66.5°C, and 70.3°C with *M. scrofulaceum*, *M. malmoense*, *M. avium*, and *M. gordonae*, respectively (Table 2). Cross-reactivity with these NTMs would not normally be a concern, because none would be mistaken for *M. tuberculosis* due to the lack of an *inhA*-specific T_m. However, there remained the possibility that NTMs could produce a false-positive resistance designation in rare cases where patients had dual infections with both *M. tuberculosis* and a cross-reacting NTM. Fortunately, our assay primers only weakly amplified NTMs. When we mixed 10^6 CFU of any of the five cross-reacting NTMs with 1,000 CFU of *M. tuberculosis*, only *M. tuberculosis*-specific wild-type T_m values were produced (Table S3), indicating that our assay would be able to correctly determine the drug resistance genotype of even small quantities of *M. tuberculosis* against a large NTM background.

TABLE 1 T_m profile and mutations tested by the XDR assay

Target and mutation[a]	T_m (°C) of the indicated probes		SD[b] T_m (°C) for the indicated probes		dT_m (°C) of the indicated probes[c]	
gyrA QRDR	gyrA1	gyrA2	gyrA1	gyrA 2	gyrA1	gyrA2
WT (95T)	72.6	74.1	0.14	0.17		
WT (95S)	70.5	73.2	0.12	0.15		
A90V(95T)	68.9	69.8	0.17	0.20	0.7	4.3
S91P (95T)	68.6	69.4	0.21	0.18	4.0	4.7
D94A(95T)	72.8	71.2			−0.2	2.9
D94G(95T)	76.7	72.2	0.15	0.20	−4.1	1.9
D94N(95T)	71.9	69.4			0.7	4.7
D94Y(95T)	71.8	69.2			0.8	4.9
G88C + A90V (95T)	66.8	64.4			5.8	9.7
G88C + A90V + S91P (95T)	67.7	64.3			4.9	9.8
A90V + 95T[d]	69 + 72.7[d]	69.6 + 74.6[d]			3.6	5.0
gyrB	gyrB1	gyrB2	gyrB1	gyrB2	gyrB1	gyrB2
WT	73.8	69.4	0.21	0.24		
D500V	68.7	69.5			5.1	−0.1
D500N	67.3	69.4			6.5	0
N538T	73.7	66.7			0.1	2.7
E540V + WT[d]	73.8	66.1 + 69.6[d]			0	3.3
E540D	73.7	65.5			0.1	3.9
eis promoter	eis1	eis2	eis1	eis2	eis1	eis2
WT	71.4	71.9	0.12	0.27		
G(−37)T	66.7	72.0			4.7	−0.1
C(−14)T	71.5	64.3			−0.1	7.6
C(−12)T	71.5	65.0			−0.1	6.9
C (−8) deletion	71.5	65.3			−0.1	6.6
G(−10)A	71.4	66.3			0	5.6
rrs	rrs		rrs		rrs	
WT	74.9		0.13			
A1401G	70.8		0.19		4.1	
C1402T + WT[d]	69.1 + 75.0[d]				5.8	
inhA promoter	inhA		inhA		inhA	
WT	76.4		0.12			
C(−15)T	71.0		0.17		5.3	
T(−8)C	74.1				2.3	
T(−8)A	73.0				3.3	
katG	katG		katG		katG	
WT	67.2		0.11			
S315T	61.2		0.13		6.0	

[a]WT, wild type; other letters represent amino acids except for the promoter region and *rrs* gene targets, where they represent nucleotides.
[b]The SD values are shown only for the T_m values obtained from at least 10 different clinical samples.
[c]The shaded cells represent the difference in T_m (dT_m) values that enable the detection of the mutations. All the *gyrA* QRDR mutations in our study set contained the threonine polymorph at codon 95.
[d]Mixed DNA showing double T_ms from both the mutant and the wild-type sequences in the mixture.

Validation with clinical samples. We performed a limited clinical evaluation of our assay's performance using a panel of 24 sputum samples that were selected from patients at a clinical site with high MDR-TB and XDR-TB prevalences and for which the investigators were blind to the sequencing or culture results. *M. tuberculosis* cultured from a separate sputum aliquot of the same patient was used to generate a reference genotype by Sanger sequencing and a reference drug resistance phenotype by use of the MGIT culture system. Assay performance comparisons were performed on all samples for which either sequencing or culture results, or both, were available. Compared to the results of Sanger sequencing, our assay detected 3/3 (100%) *gyrA* mutants, 5/5 (100%) *katG* mutants, 3/3 (100%) *inhA* mutants, and 2/2 (100%) *rrs* mutants as mutants (Table S4). All *gyrA*, *katG*, and *inhA* sequences which were identified to be wild

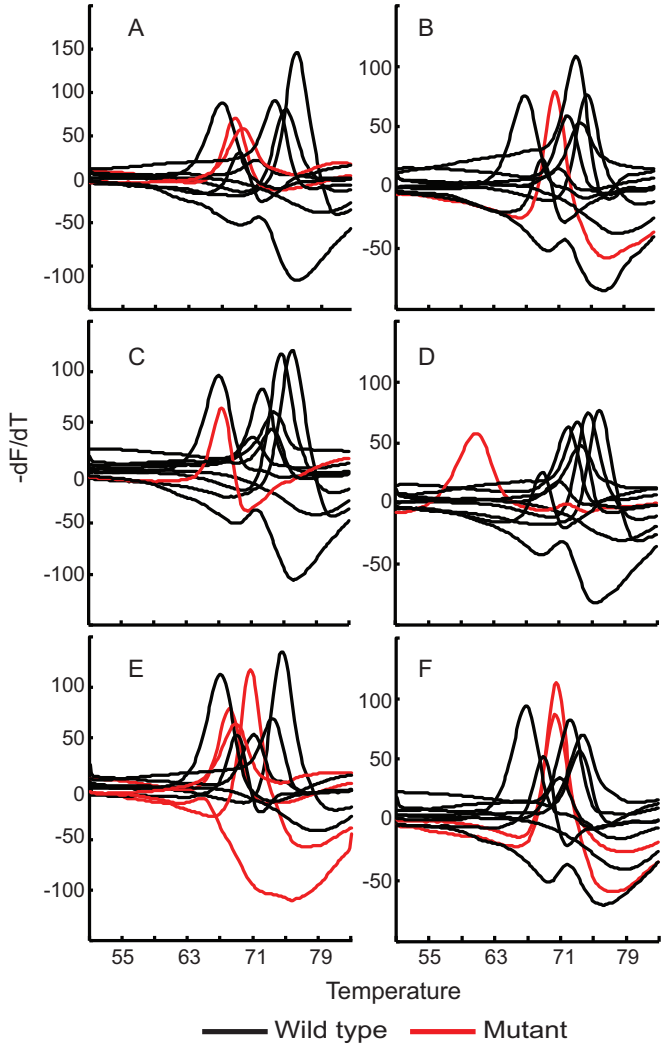

FIG 5 Resistance mutation detection in clinical *M. tuberculosis* isolates. Melt curves generated during tests of drug-resistant clinical *M. tuberculosis* isolates are shown for the *gyrA* A90V (A), *inhA* promoter C(−15)T (B), *gyrB* N538T (C), *katG* S315T (D), *gyrA* S91P, *inhA* promoter C(−15)T, and *eis* promoter C(−12)T (E), and *rrs* A1401G and *inhA* promoter C(−15)T (F) mutations. For the *gyrA* A90V and S91P mutants, both the *gyrA* probes showed distinct T_m peak shifts (red lines), as observed in panels A and E. For all other mutants, each individual mutation detection probe showed a T_m peak shift associated with the corresponding mutation (red lines in panels B to F). All other T_m peaks retain the wild-type profile (black lines).

type by Sanger sequencing were also identified to be wild type by our assay. None of the sputum samples in our study panel were shown to contain *gyrB* or *eis* promoter mutations by Sanger sequencing, and all of them were also identified to be wild type by our assay. The *rrs* assay detected 15/16 (93.8%) wild-type sequences as wild type. The single discordant sample for *rrs* was identified to be heteroresistant by our assay due to the presence of both the wild-type and mutant *rrs* peaks indicating the presence of mutant strains against a background of wild-type strains for that patient, which was not detected by Sanger sequencing. Compared to the results of phenotypic susceptibility testing, our assay identified 8/8 (100%) INH-resistant samples to be resistant, 2/2 (100%) AMK- and KAN-resistant samples to be resistant, and 3/4 (75%) FQ-resistant samples to be resistant (Table S5). The single discordant FQ-resistant sample did not show any mutations in either the *gyrA* or *gyrB* target upon Sanger sequencing, explaining why it was detected as susceptible by our assay. All phenotypically drug-susceptible samples were detected as wild type in their respective targets by our assay,

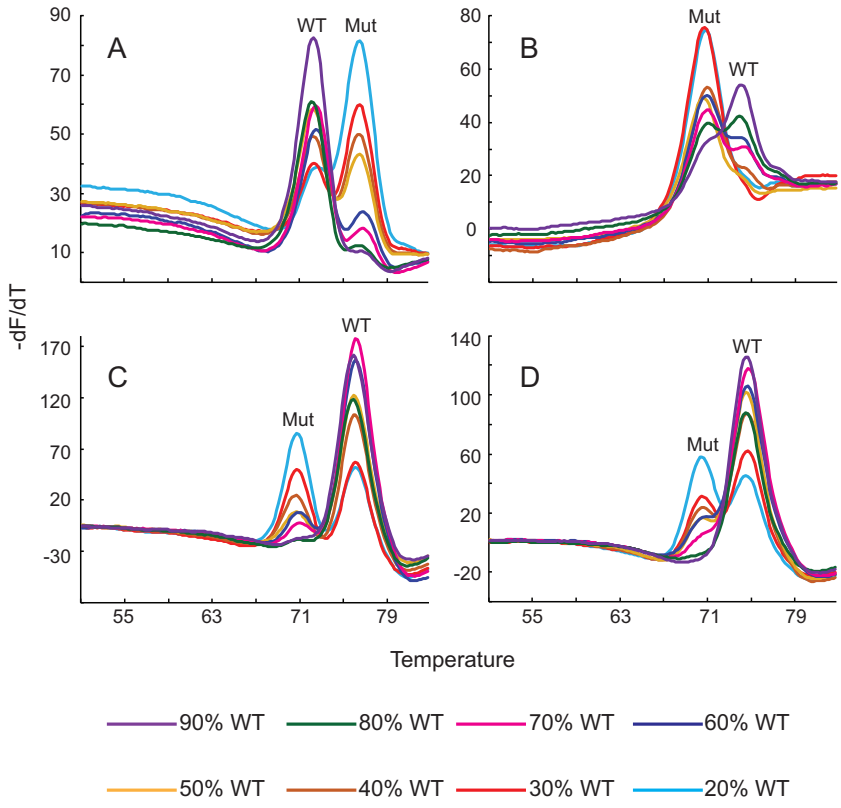

FIG 6 Detection of heteroresistance. Melting temperature profiles of four different probes obtained from assays using mixtures containing various concentrations of wild-type (WT) and mutant (Mut) DNA, as shown. The wild-type and mutant T_m peaks are indicated in each panel. (A) gyrA1 probe profile with *gyrA* D94G and wild-type *gyrA*; (B) gyrA2 probe profile with *gyrA* D94A and wild-type *gyrA*; (C) inhA probe profile with *inhA* promoter C(−15)T and wild-type *inhA* promoter; (D) rrs probe profile with *rrs* A1401G and wild-type *rrs*.

except for the single sample that was detected as having a mixed sequence for the *rrs* gene target but that was phenotypically susceptible to AMK and KAN.

DISCUSSION

We have leveraged new large-Stokes-shift fluorophores, innovative microfluidics enabling three sequential PCR phases, and SMB probes that are able to query relatively large stretches of DNA targets to create a highly sensitive multiplex assay which detects all of the important mutations currently thought to be associated with drug-resistant *M. tuberculosis*. The two manual steps of the assay (adding a buffer to the sputum sample and then placing the sample into an assay cartridge) are identical to those of the Xpert MTB/RIF test, and our assay is performed on the same six-color GeneXpert MTB/RIF system platform. The operation of the Xpert MTB/RIF assay is so simple that it can be easily performed near patients in settings such as primary care clinics, where assay implementation can shorten the time to TB diagnosis and treatment (51).

The rapid mortality associated with untreated XDR-TB among HIV-positive patients highlights the need for a rapid and simple assay such as ours (8). Our assay should detect resistance in approximately 90% of INH-resistant isolates, 90% of fluoroquinolone-resistant isolates, and 80% of kanamycin- and amikacin-resistant isolates, according to previous studies of the mutations detected in the XDR assay (28, 29, 31, 33, 35). Thus, relatively few cases of drug resistance should be missed. Given the very high level of agreement between our XDR assay and Sanger sequencing, our assay should be highly specific. The only likely cause of a false resistance call would be a heteroresistant sample (which should arguably be treated as a resistant TB case) or the rare presence of a synonymous mutation in a probe binding site, as is occasionally seen

TABLE 2 T_m profiles from *M. tuberculosis* complex and NTM

Species	T_m (°C) for the following probe:								
	gyrA1	gyrA2	gyrB1	gyrB2	inhA	katG	rrs	eis1	eis2
M. tuberculosis	70.4	73.2	73.6	69.4	76.4	67.3	74.9	71.3	72.0
M. bovis BCG	72.5	74.0	73.7	69.6	76.3	67.2	75.0	71.4	71.9
M. scrofulaceum	64.4	66.7					74.9		
M. flavescens							75.0		
M. vaccae							74.8		
M. triviale							74.9		
M. szulgai							74.9		
M. abscessus							75.0		
M. fortuitum							74.8		
M. smegmatis							74.9		
M. kansasii							75.0		
M. intracellulare							75.0		
M. simiae							74.9		
M. asiaticum							75.0		
M. marinum							74.9		
M. terrae							75.0		
M. celatum							74.9		
M. haemophilum							75.0		
M. malmoense		66.4					74.8		
M. avium	64.0	66.5					74.8		
M. xenopi	65.6								
M. gordonae	69.7	70.3					75.0		

with the Xpert MTB/RIF assay and rifampin resistance detection (52). The Xpert MTB/RIF assay and GeneXpert MTB/RIF instruments are already widely used in countries with a high burden of TB (26, 51), and our XDR assay can easily be added to sites that already detect TB using the GeneXpert MTB/RIF system. Thus, our new assay could be implemented with minimal additional costs, especially if it is used only as a reflex test on patients whose isolates are already identified to be RIF resistant by Xpert MTB/RIF assay.

The simple operation of our assay belies the actual complexity of its design. The assay can resolve 10 different fluorophores in a single PCR tube. The SMB probes that we used are products of multiple rounds of iterative design, which was needed to achieve reliable T_m separation between wild-type target sequences and all possible mutations. Finally, we were able to achieve a sensitivity equivalent to that of the duplex, heminested symmetric PCR assay used in the Xpert MTB/RIF test, even though our XDR assay amplified eight different amplicons and included an asymmetric amplification step. This was accomplished with a three-phase amplification scheme that took advantage of the exponential increase produced by the first two nested and heminested symmetrical amplification phases and the large amounts of single-stranded target produced by the final linear amplification phase. The linear amplification cycles in the final assay phase also eliminated any risk of exonuclease activity on the SMB probes, ensuring a low background and very robust T_m curves over the entire dynamic range of highly smear-positive to smear-negative samples. Our assay could theoretically accommodate the detection of additional mutations either by the development of fluorophores in another off-axis channel or by the use of two additional probes in the same channel that detect mutations over different temperature ranges.

The long probe length of the SMB probes and their mismatch tolerance capacity allowed us to design probes with programmable hybridization kinetics that could identify a wide range of mutations using a relatively small number of probes (34, 42, 53, 54). Our analytic studies demonstrated robust and reproducible T_m peaks that permitted error-free differentiation between wild-type and mutant target sequences, even at limiting concentrations of the target sequence. The mutation detection rate was 100% in all tests with clinical DNA samples, and the assay performed equally well in a small validation study performed on sputum. The explanation for the lack of detection of a single FQ-resistant isolate in a sputum sample by our assay may be the occurrence of a mutation somewhere outside the region with mutations that are known genetic

causes of FQ resistance. Indeed, although the *gyrA* and *gyrB* targets we selected have been used to identify approximately 60 to 90% of cases of FQ resistance (42), some cases will remain undetected until the additional genetic causes of FQ resistance are identified and incorporated into future assays. It is unclear whether the result for one sputum sample that we misidentified as AMK and KAN heteroresistant is truly a false-positive result. It is possible that this sample truly contained both resistant and susceptible clones and that the susceptible clones in this mixed infection would eventually also become resistant to AMK and KAN in a patient undergoing treatment with these drugs, resulting in absolute resistance to aminoglycoside treatment.

In summary, our assay provides a potential new tool to identify XDR-TB so that treatment may begin immediately. If the results are confirmed by larger clinical trials, this new test should contribute to enhanced diagnosis, more rapid treatment, and lower rates of mortality and XDR-TB propagation.

MATERIALS AND METHODS

Human subjects approvals. This study was approved by University of Medicine and Dentistry of New Jersey (now Rutgers University) Institutional Review Board protocol numbers 0120080314, 0120080291, and 0120090104, the Henan Provincial Chest Hospital (HPCH), and National Institute of Allergy and Infectious Diseases Institutional Review Board protocol NCT01071603. Subjects from HPCH provided written consent to participate in the study, including consent for the storage of sputum samples for future testing.

Sample selection. Sputum samples were collected as part of a study of the natural history of suspected tuberculosis in patients presenting to a tertiary respiratory treatment hospital (Henan Provincial Chest Hospital) in Zhengzhou, China. This population was previously known to be enriched with individuals with highly drug-resistant TB. The enrolled patients were excluded if they had received more than 2 weeks of treatment for TB in the past 3 months. Sputum samples were collected during the study and banked while conventional drug susceptibility testing was performed (see the supplemental material for additional details about the susceptibility testing). Twenty-four sputum samples from individual patients with MDR-TB, XDR-TB, as well as drug-susceptible TB were identified from the stored samples and tested. The results of the XDR assay were analyzed by individuals who were blind to the conventional drug susceptibility testing results and were not used in any way to decide the treatment of the patients.

Cartridge configuration, assay composition, and testing procedure. The assay used a filter-based sample processing/PCR cartridge (Cartridge A; Cepheid, Sunnyvale CA), which consisted of an integrated PCR tube, a multiposition fluidic valve, a filter to capture the bacteria, and 11 chambers that contained all the buffers needed for sample processing and PCR, as described previously (24). The complete details of the cartridge configuration and reagents are fully described in the supplemental material.

To perform a test, each sample (spiked sputum or cultured *M. tuberculosis* colonies) was first mixed at a 2:1 ratio with an NaOH- and isopropanol-containing sample reagent (SR) as described previously (24); the sample was then added to the sample loading chamber of the cartridge. The sample was then automatically processed as described fully in the supplemental material. The T_m values generated as output from the assay were then used to identify the presence of either wild-type or mutant targets through a look-up table of T_m windows (Table S6) for each wild-type sequence and each known mutant. A full description of the assay parameters is provided in the supplemental material.

Mutation panel challenge and reference T_m table. DNA was isolated from clinical *M. tuberculosis* isolates, including isolates with a wide range of mutations in the target genes, as shown in Table 1. Fifty DNA samples obtained from different pan-susceptible isolates with the wild-type sequence in the target regions were also tested as controls and were originally isolated from clinical *M. tuberculosis* cultures at the International Tuberculosis Research Center, Masan, South Korea. The XDR assay cartridge was preloaded with the DNA of interest, the loaded cartridge was placed into a GeneXpert MTB/RIF instrument bay, and the assay was performed by selecting the automated 10-color XDR *M. tuberculosis* detection protocol specific for genomic DNA PCR from the instrument software (research use only; Cepheid, Sunnyvale, CA). The different wild-type and mutant DNA samples were run over a period of 3 months in eight different modules in the GeneXpert MTB/RIF instrument using different cartridge lots to check for the T_m reproducibility. The reference T_m table was generated with the T_m values obtained from these runs.

Sputum sample spiking and GeneXpert MTB/RIF assay. The analytical sensitivity and limit of detection (LOD) of the XDR assay were determined by spiking an attenuated auxotrophic strain of *M. tuberculosis* H37Rv (mc²6030) (55) into sputum and testing each sample according to our standard protocol. This strain was grown as described previously (55) and quantified by the plate dilution method, and 100-μl aliquots were frozen at −80°C until use. The assay LOD was defined as the number of bacteria that could be spiked into 1 ml of sputum such that all the nine *M. tuberculosis*-specific SMB probes generated distinct melt curves with unequivocal T_m values for ≥95% of samples tested. The 10th SMB, the IC, was also included in all tests, but its T_m was relevant only for *M. tuberculosis*-negative samples. Additional details for the spiking and testing protocol are available in the supplemental material.

Evaluation of the analytical specificity of the assay. The analytical specificity of the assay was tested by using concentrated saturated bacterial liquid cultures (approximately 10^8 to 10^9 CFU/ml) or 10^8 genome equivalents of DNA (in the case of Gram-positive and Gram-negative bacteria for which cultures

were not available). The Gram-positive and Gram-negative bacteria included the most common microflora found in sputum and in the upper respiratory tract and mouth, namely, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Haemophilus influenzae*, *Neisseria* spp., *Nocardia* spp., *Corynebacterium* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Citrobacter freundii*, *Moraxella catarrhalis*, and *Enterobacter cloacae*. The NTM species included a laboratory strain of *Mycobacterium smegmatis* and 19 different 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. vaccae*, *M. simiae*, *M. triviale*, *M. malmoense*, and *M. gordonae*. The Gram-positive and Gram-negative bacteria were not incubated in the sample reagent for more than 2 min to prevent any lysis before they were loaded in the sample chamber. The NTM were incubated for the usual 15 min, as was done for *M. tuberculosis*. The assay was performed using the cartridges specific for the XDR assay as described above.

Evaluation of the assay with clinical samples. The evaluation of the assay with 24 clinical samples from patients with MDR-TB, XDR-TB, as well as pan-susceptible TB was performed at HPCH in China, and the results were analyzed in a blind manner at the New Jersey Medical School, Rutgers University. Two volumes of sample treatment reagent was added to 1 volume of sputum, the mixture was incubated for 15 min with occasional shaking, 2 ml was added to the investigational Xpert MTB/RIF cartridges for the XDR assay, and the assay was performed in the GeneXpert MTB/RIF instrument as described above. Details of the microbiological evaluation of the clinical samples are fully described in the supplemental material. For sequencing of the target genes, genomic DNA was isolated from the clinical isolates as described previously (34) and sequencing of the six target genes and promoter regions was performed as described previously (56, 57). The performance of the XDR assay was evaluated against that of the MGIT drug susceptibility testing assay and sequencing as the "gold standard."

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01771-16.

TEXT S1, PDF file, 0.09 MB.

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D.A. is one of a group of coinvestigators who invented the molecular beacon technology and who receive income from licensees, including a license to Cepheid for *M. tuberculosis* detection. To manage this conflict of interest, the income attributable to the GeneXpert MTB/RIF assay which he may receive has been irrevocably capped at $5,000 per year. D.A. also reports receiving two research contracts from Cepheid. D.A. and S.C. report the filing of patents for primers and probes for detecting drug resistance in *M. tuberculosis*.

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