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Multiplex Detection of Extensively Drug Resistant Tuberculosis using Binary Deoxyribozyme Sensors

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Abstract

Current diagnostic tools for *Mycobacterium tuberculosis* (*Mtb*) have many disadvantages including low sensitivity, slow turnaround times, or high cost. Accurate, easy to use, and inexpensive point of care molecular diagnostic tests are urgently needed for the analysis of multidrug resistant (MDR) and extensively drug resistant (XDR) *Mtb* strains that emerge globally as a public health threat. In this study, we established proof-of-concept for a novel diagnostic platform (TB-DzT) for *Mtb* detection and the identification of drug resistant mutants using binary deoxyribozyme sensors (BiDz). TB-DzT combines a multiplex PCR with single nucleotide polymorphism (SNP) detection using highly selective BiDz sensors targeting loci associated with species typing and resistance to rifampin, isoniazid and fluoroquinolone antibiotics. Using the TB-DzT assay, we demonstrated accurate detection of *Mtb* and 5 mutations associated with resistance to three anti-TB drugs in clinical isolates. The assay also enables detection of a minority population of drug resistant *Mtb*, a clinically relevant scenario referred to as heteroresistance. Additionally, we show that TB-DzT can detect the presence of unknown mutations at target loci using combinatorial BiDz sensors. This diagnostic platform provides the foundation for the development of cost-effective, accurate and sensitive alternatives for molecular diagnostics of MDR- and XDR-TB.

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Keywords

Binary deoxyribozyme; Multiplex PCR; Diagnostics; Tuberculosis; Drug resistance; Nucleic acid sensors

1. INTRODUCTION

Mycobacterium tuberculosis (*Mtb*), the causative agent for tuberculosis (TB), is responsible for the highest mortality rates worldwide due to a single infectious agent (WHO 2015). According to the World Health Organization (WHO), in 2014 approximately 3.3% of new infections and 20% of previously treated TB cases were multidrug resistant (MDR) (WHO 2015). MDR-TB is resistant to the two first-line antibiotics rifampin (RIF) and isoniazid (INH) and requires the administration of second line drugs, which are more expensive and less effective (CDC 2009). Strains which acquire additional resistance to fluoroquinolones (FQ) and one of the injectable drugs (amikacin, capreomycin, or kanamycin) are considered extensively drug resistant (XDR) and were responsible for 9.7% of MDR infections in 2014 (WHO 2015). To prevent ongoing transmission, high mortality rates, and acquisition of additional drug resistance, there is an urgent need for accurate, easy to use, and inexpensive point-of-care (POC) diagnostics capable of detecting *Mtb* and drug resistance.

The predominant cause of drug resistance in *Mtb* is single nucleotide polymorphisms (SNPs) in genomic loci that result in impaired antibiotic efficacy. For example, mutations in the 81-base pair “hot spot” region of *rpoB* that prevent drug-target interactions are responsible for 95–98% of RIF resistance (Dominguez et al. 2016; Heep et al. 2001). Mutations in the *katG* gene encoding catalase-peroxidase, on the other hand, lead to reduced activation of the front-line antibiotic prodrug INH (Zhang et al. 1992). INH resistance can also arise due to SNPs in the promoter or coding region of *inhA*, a target of INH which encodes enoyl-ACP reductase involved in mycolic acid synthesis (Banerjee et al. 1994; Dessen et al. 1995; Morlock et al. 2003). Additionally, the most common SNPs for FQ resistance are found in one of the subunits of the DNA gyrase (*gyrA*) (Takiff et al. 1994). Knowledge of the genetics underlying drug resistance in *Mtb* may be exploited for genotypic drug susceptibility testing (DST) using molecular diagnostic tools.

Current diagnostic tools routinely used for the detection of *Mtb* suffer from various shortcomings such as inadequate sensitivity, slow turnaround times, and/or the requirement for high-infrastructure laboratories (Lemaire and Casenghi 2010). The gold standards for TB diagnosis are sputum smear microscopy (SSM) and culture-based detection methods. SSM fails to detect over 50% of all cases and exhibits even lower sensitivity in HIV-positive patients and children (Shingadia and Novelli 2003; Steingart et al. 2007). Although culture based detection is highly sensitive, it is limited by turnaround time (2–6 weeks), increased biosafety risks, need for trained personnel, and risk of contamination (Dunlap et al. 1995). Subsequent phenotypic drug susceptibility testing (DST) requires additional processing and time (weeks to months) preventing the initiation of appropriate anti-TB treatment in a timely manner (Cruciani et al. 2004).

Recent advances in molecular diagnostics, including the development of nucleic acid amplification tests (NAAT), have enabled faster detection and drug susceptibility testing of *Mtb* (<1 day), resulting in improved time to treatment (Dominguez et al. 2016; Kwak et al. 2013). The current state of the art molecular diagnostic technique, Cepheid's GeneXpert MTB/RIF (Cepheid, Sunnyvale, California) is WHO-endorsed for detection of RIF resistant *Mtb*. This fluorescent real-time quantitative-PCR based platform is capable of sensitive TB detection and identification of RIF resistance in ~2 hrs (Boehme et al. 2010). While this technology can have a positive impact by improving the time to treatment it is prohibitively expensive (Helb et al. 2010) and requires a 6-color fluorescent readout to answer only two questions. Additional commercial DST such as line probe assays (LiPAs) can detect *Mtb* and drug resistance but are limited by detection of only first line antibiotics (RIF and INH), are expensive and labor intensive (Brossier et al. 2006). Other groups have published new molecular methods for the diagnosis of *Mtb* which can enable the detection of *Mtb* and/or identification of drug resistance but have not been validated for clinical use (Ajmani et al. 2011; Campbell et al. 2011; Dauendorffer et al. 2003; Feng et al. 2013; Feuerriegel et al. 2009; Luo et al. 2011; Mokrousov et al. 2003; Shenai et al. 2009).

In this study, we developed a novel diagnostic platform for simultaneous detection of *Mtb* and resistance to multiple antibiotics using binary deoxyribozyme (BiDz) sensors (TB-DzT) (Fig. 1). First, we developed a multiplex PCR (mPCR) for simultaneous amplification of 5 fragments of the *Mtb* genome including a fragment of *23S rRNA* for *Mtb* identification and four genes (*rpoB*, *katG*, *inhA* and *gyrA*) which account for the majority of SNPs associated with drug resistance to the main anti-TB drugs (RIF, INH and FQs) (Almeida Da Silva and Palomino 2011). We then detected the presence of drug resistance conferring SNPs in mPCR products using highly selective BiDz sensors. BiDz sensors utilize two DNA strands (Dz_a and Dz_b, Fig. 1A) each of which consists of a substrate binding arm, half of the deoxyribozyme catalytic core, and sequences complementary to the target nucleic acid (analyte binding arm) (Gerasimova and Kolpashchikov 2010; Kolpashchikov 2007, 2010; Mokany et al. 2010). Dz_a and Dz_b hybridize to a target analyte and reform the Dz catalytic core, which is now capable of cleaving the fluorophore and a quencher-labeled substrate resulting in an increase in fluorescence (F-sub in Fig. 1A). All BiDz sensors used in the TB-DzT assay utilize a universal substrate, which significantly decreases the cost of sensor design and optimization. Additionally, a single BiDz sensor cleaves approximately 80 F-sub molecules per minute (16) resulting in a lower limit-of-detection compared to detection methods utilizing molecular beacon probes. Another important advantage of the BiDz approach is the ability to detect and differentiate both DNA and RNA analytes that differ by a single nucleotide (Gerasimova and Kolpashchikov 2010; Gerasimova et al. 2015; Kolpashchikov 2007; Mokany et al. 2010). To enable high selectivity of the sensors, the analyte binding arm of Dz_b is designed short enough to enable formation of the catalytically active complex only in the presence of the fully matched analyte (Fig. 1B). In this study, we demonstrate that TB-DzT represents an accurate and potentially low cost platform for multiplex analysis of MDR- and XDR- TB.

2. MATERIALS AND METHODS

2.1. Mycobacterial DNA used for Multiplex PCR

Mtb DNA for multiplex PCR was isolated from either *Mycobacterium tuberculosis* CDC1551 (BEI) or clinical isolates (Fleischmann et al. 2002). DNA from clinical isolates of *Mtb* were obtained from archived stocks of the Mycobacteria Laboratory of the Federal University of Rio Grande Brazil or the National Reference Center for Mycobacteria, Borstel, Germany. The DNA of *Mtb* isolates was extracted using a standardized method (cetyltrimethylammonium bromide-NaCl (CTAB)/NaCl) and stored at -20°C , as previously described (van Soolingen et al. 1994).

2.2. Multiplex PCR

The multiplex assay was designed to produce small amplicons (177–437 base pairs) containing either regions associated with drug resistance (*rpoB*, *katG*, *inhA*, and *gyrA*) or a hypervariable region of 23S rRNA used for the specific detection of *Mtb* (Table S1). Nucleotide sequences of *Mtb* CDC1551 genes were used to design primers. The primers were designed to produce amplicons differing in length by at least 40 base pairs to enable unambiguous separation in a 2% agarose gel. Reverse primers contained a 5' phosphate to allow for digestion of the antisense strand by lambda exonuclease for optimal detection of ssDNA products by BiDz sensors. Multiplex PCR samples contained 5 units PhilisaFAST DNA Polymerase (Streck), 0.6 μL dsDNase (PCR Decontamination kit, Articzymes), 100 nM of each primer, 0.4 mM of each dNTP, 1 $\times$ FastBuffer I, and water to a final volume of 25 μL . Samples were then decontaminated by incubation at 37°C for 20 min followed by 60°C for 20 min to inactivate the dsDNase. *Mtb* DNA (5 fg – 20 ng) was added to the samples and PCR amplified using the following cycling conditions: initial denaturation 98°C for 30 sec, followed by 50 cycles of denaturation at 98°C for 10 sec, annealing at 65°C for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 2 min. Following PCR, samples were treated with 1 unit of Lambda Exonuclease (New England Biolabs) and incubated at 37°C for 20 min followed by enzyme inactivation at 75°C for 10 min. After treatment with lambda exonuclease, ssPCR products were used in fluorescent DNAzyme assays (described in 2.4). All experiments for Fig. 3 used 20 ng of DNA. For heteroresistance experiments (Fig. 4) 20 ng of *Mtb* CDC1551 DNA (WT) and 2 ng of MT4 DNA was used. For limit-of-detection (LoD) experiments (Table 1) 5 fg – 15.6 pg of *Mtb* CDC1551 DNA (WT) was used.

2.3. Gel Validation

Multiplex PCR samples were analyzed in a 2% agarose gel using a BioRad horizontal gel electrophoresis system. The samples were run at 120 V for 2 hr followed by staining in GelRed (Biotium, Inc., Fremont, CA) for 30 min.

2.4. Fluorescent DNAzyme Assays

All oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). The fluorogenic substrate (F-sub) was synthesized and HPLC purified by TriLink BioTechnologies, Inc. (San Diego, CA) (Mokany et al. 2013). All fluorescent assays

contained the F-sub (200 nM), two adaptor strands (Dz_a and Dz_b) at 0.15–150 nM each, (Table S2), and either synthetic analyte (1 nM) or 3.5 μ L multiplex PCR product in reaction buffer (50 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 20 mM KCl, 120 mM NaCl, 0.03% Triton X-100, and 1% DMSO) in a final volume of 30 μ L. The assay is comprised of a panel of 11 sensors targeting either *23S rRNA* for the species-specific identification of *Mtb* (*Mtb control*) or genes containing single nucleotide polymorphisms (SNP) associated with drug resistance. A range of sensor concentrations was used to enable sensitive and specific detection of analytes and is dependent on the number of mutations in the target sequence. The *23S rRNA* sensor was used at high concentrations without a loss of specificity due to the presence of many mismatches in the target sequence (nt 1967–2016) between *Mtb* and nontuberculous mycobacteria (Fig. S2). However, sensors used for discrimination of single mismatch analytes were used at lower concentrations to maintain specificity. Note, the mutant (mt) specific sensors were designed to specifically detect the most prevalent SNP associated with clinically relevant drug resistance in each selected gene (Sandgren et al. 2009). Sensors targeting *rpoB* codons 526 and 531 were designed since mutations in each of these codons account for the majority of resistance to RIF. Samples were placed in a 384-well plate (PerkinElmer OptiPlate), sealed (ThermalSeal 2) and incubated at 55 °C for 30–60 mins. Fluorescence of the samples was measured on a Synergy 4 plate reader (Biotek) with a xenon flash lamp (λ_{ex} =485 nm, λ_{em} =517 nm).

2.5. Statistical Analysis

The data were analyzed using Microsoft Excel. Data are presented as signal to background (S/B) ratio. Signal is the absolute fluorescence from samples containing analyte, F-sub, and sensor strands (Dz_a and Dz_b). To account for signal produced by spontaneous cleavage in the absence of analyte, background controls included only F-sub and sensor strands (Dz_a and Dz_b). Additional controls with matched (positive control) and mismatched (negative control) synthetic analytes were used in all experiments. S/B ratios are used to standardize data based on background controls to enable comparison of all sensors. The average and standard deviations of 3–6 independent experiments are presented. Additional statistical analysis (Student's t-test, paired, CI 95%) were completed using GraphPad Prism version 5. For each sensor the S/B ratio produced by complementary (match) analytes was compared to the analyte containing a SNP (mismatch). LoD was calculated using raw data from 6 independent multiplex PCR experiments in the presence of 0, 1, 5, 25, 125, 635, and 3125 genome equivalents (1 genome equivalent = 5 fg of DNA). The data was plotted and the slope-intercept equation ($y = mx + b$) was used to calculate LoD with y equal to the average background signal from negative control samples (six replicates) plus three standard deviations. The resulting value was then divided by 7.14 to account for only 3.5 μ L of the 25 μ L multiplex PCR sample being used with each BiDz sensor. The R^2 values the *23S rRNA* and *rpoB* 531 WT sensors were at least 0.949 and 0.873, respectively (Refer to supplemental data for more details.)

3. RESULTS AND DISCUSSION

3.1. Design and Validation of Binary Deoxyribozyme (BiDz) Sensors

To enable the identification of *Mtb* and drug resistance conferring SNPs, we first designed and optimized a panel of BiDz sensors for the specific and sensitive detection of either wild-type (wt) or mutant (mt) sequence (Table S2). All sensors were initially optimized using synthetic single-stranded DNA analytes listed in Table S2. Sensors are optimized to allow for discrimination of SNPs (specificity) while maintaining high signal-to-background (S/B) ratios (sensitivity). This is achieved by optimizing the length and concentration of Dz_a and Dz_b and the position of the SNP. The panel of BiDz sensors demonstrated in Fig. 2 produced low background in the presence of nonspecific analytes (S/B ratio <2) and high signal (S/B ratio >3) in the presence of specific analytes (Fig. 2) in a 45 min assay and were selected for inclusion in the TB-DzT assay. Our data demonstrate the flexible utility of the TB-DzT platform as the basis for either a gain-of-signal (positive detection of MT analytes) or loss-of-signal (loss of WT detection) assay. Many current commercial molecular diagnostic tools for *Mtb* detect only WT sequences associated with drug susceptibility, with loss of signal due to SNPs interpreted as drug resistance. A loss-of-signal assay design is not ideal since it can result in false positives due to silent mutations or polymorphisms which interfere with probe hybridization (Williamson et al. 2012). In future applications of this technology for molecular detection of drug resistance, we envision a gain-of-signal assay using only MT-specific BiDz sensors for primary testing, since in this case the signal will be generated only in the presence of drug resistance conferring SNPs (Seifert et al. 2016). A combinatorial sensor approach introduced below could facilitate the detection of many SNPs associated with a particular resistance phenotype. If the WT sequence needs to be confirmed for conclusive genotype determination, WT sensors could be used as a secondary test.

3.2. Detection of MDR- and XDR-TB using the TB-DzT assay

To validate the TB-DzT assay, we used multiplex PCR to amplify select chromosomal loci from wild type (WT) CDC1551 and 5 drug-resistant *Mtb* clinical isolates (MT1-5) with different patterns of drug resistance mutations in *rpoB*, *katG*, *inhA*, and *gyrA* (Fig. S1). Using the panel of optimized sensors, we identified drug resistance conferring SNPs in all 5 clinical isolates (MT1-5). In all strains the *Mtb* specific sensor (*23S rRNA*) produced very high response (S/B >16) and all other sensors accurately reported genotypes corresponding to their known SNP profile (Fig. 3). For example, in the presence of wild-type mPCR amplicons, only wild-type-specific sensors produced high signal (at least 2 times above background) (Fig. 3A). Alternatively, in the presence of mPCR amplicons containing drug-resistance conferring SNPs (MT1-5), corresponding mutant specific sensors produced high signal. The presence of particular mutations and/or resistance profiles in the clinical isolates were verified by whole genome sequencing or phenotypic DST and sequencing of specific loci (Table S3). The TB-DzT assay demonstrated the ability to detect the presence of *Mtb* and drug resistance associated SNPs in all cases. We did note a range in sensor performance (S/B ratios) in the presence of specific analytes indicating room for further optimization of some sensors to achieve more uniform performance and robust detection of analytes. Future development of sensors for mutations leading to second line drug resistance and further optimization of combinatorial sensors (refer to Fig. 5) will improve the coverage of our

assay for the detection of clinically relevant SNPs. Subsequent preclinical validation of the TB-DzT assay will include testing of unknown clinical samples to determine the sensitivity and specificity of our assay in a clinical setting.

3.3. Use of BiDz sensors for the detection of mixed infections

Initially, the selection of drug resistant mutants within patients during clinical treatment of TB results in a minority resistant subpopulation of *Mtb* within a larger population of susceptible bacilli. It was reported that such heteroresistance occurs in up to 30% of TB cases (Cohen et al. 2012; Folkvardsen et al. 2013; Tolani et al. 2012; Zhang et al. 2016; Zhang et al. 2012), perhaps even more frequently in the early stages of treatment. Current molecular diagnostic tools fail to identify such minor populations of drug resistant bacteria (Cho and Brennan 2007). Therefore, it is important to develop a diagnostic assay that can detect heteroresistance to enable proper diagnosis and treatment. To establish the ability of the TB-DzT assay to detect heteroresistance, we conducted a multiplex PCR using a mixture of wild-type CDC1551 and MT4 DNA (XDR strain containing mutations in *rpoB*, *katG*, and *gyrA*) in a 10:1 ratio. Since the drug resistance profile was known, only BiDz sensors targeting these mutations were included in the assay. As seen in Fig. 4 (light grey bars), our assay enables the detection of drug resistance SNPs even when present in only ~10% of the population. As expected, the 23S rRNA sensor (*Mtb* control) detected all samples as positive, and wild-type and mutant sensors targeting drug resistance SNPs detected corresponding samples with matching pure DNA. In the case of a mixed infection (WT + MT4, light grey bars), all sensors produced high signal (S/B ratio >3) due to the presence of both WT and MT4 analytes. This is a key advantage of our TB-DzT diagnostic platform. In contrast, the GeneXpert MTB-RIF assay (Cepheid) could only detect rifampin resistance when >90% of the bacterial population were resistant (Zetola et al. 2014). With further optimization, it may be possible to identify resistance earlier after emergence when a mutant is present in even lower proportions.

3.4. Limit of Detection (LoD) of TB-DzT assay

To determine the impact of parameters such as sensor concentration and assay duration on the LoD, we evaluated assay kinetics using sensors that yielded optimal sensitivity and specificity when used at 1000-fold different concentrations. We amplified 0, 1, 5, 25, 125, 625, and 3125 genome equivalents using mPCR followed by detection with either the 23S rRNA or *rpoB* 531 WT sensor after 30 min, 45 min, or 60 min. Note, all data shown in other figures represent 45 min incubations, which was found to be the earliest timepoint with sensitive (high signal, S/B ratio >3) and specific (differentiation of analytes) detection with all sensors. The LoD was calculated for the two sensors at each timepoint (refer to Table 1 and Figure S3) to assess the overall sensitivity range of our assay. In each case, we noted improved LoD over time, due to the catalytic turnover of F-sub by activated BiDz sensors. The *rpoB* 531 WT sensors exhibited slower kinetics with an LoD ~1.7-fold higher than the 23S rRNA sensor at 30 min, presumably due to the lower *rpoB* sensor concentrations required to maintain SNP specificity. However, by 60 min both sensors reached a similar LoD. Detection by BiDz sensors was only measured up to 60 mins since the assay is intended to be rapid for the use at the POC. Using our proof-of-concept TB-DzT assay we established a LoD of 10–15 genomes (depending on the sensor), which is only ~2–3 times

higher than the GeneXpert MTB/RIF assay (Helb et al. 2010). We believe it is possible to achieve even lower LoD by further optimization of the mPCR conditions and BiDz sensors.

3.5. Detection of alternative SNPs using a combinatorial approach

In the clinical setting, drug resistance can often result from several substitutions at one codon or multiple codons in the same gene. With this scenario in mind, we sought to determine if our TB-DzT assay could be used to detect *Mtb* single base-pair mutations without *a priori* knowledge of specific nucleotide substitutions present. To do this, we used a combinatorial sensor approach that utilized a universal Dz_a strand and a mixture of Dz_b (mt1-3) strands, each complementary to the sequence containing one of the three possible nucleotide substitutions. In the presence of any non-wildtype sequence indicative of drug resistance, the matching Dz_b and universal Dz_a assemble and produce fluorescent signal. To establish proof-of-principle, we designed sensors for the detection of 3 mutations in *rpoB* codon 531 (Kapur et al. 1994). Data shown in Fig. 5 demonstrate the successful use of the combinatorial BiDz sensor assay for the specific detection of WT or MT analytes. Each sensor alone produced signal only in the presence of the corresponding complementary analyte (wt, mt1, mt2, and mt3). However, in the presence of any of the 3 mutant analytes (mt1, mt2, mt3) the combinatorial sensor produced high signal (Fig. 5, mt1-3). This data provides support for the use of a combinatorial sensor approach coupled with a universal substrate for the detection of multiple mutations. In future applications, a “cocktail of sensors” can be designed for the detection of a set of clinically relevant SNPs within a gene associated with drug resistance (i.e. mutations in *rpoB*). This technology will enable the use of a single color substrate for the detection of several mutations in a gene(s) enabling the use of alternative substrates for the detection of resistance to additional drugs.

4. CONCLUSIONS

Although NAATs cannot replace phenotypic drug testing they provide a quick alternative for the identification of drug resistance at the POC (Yakrus et al. 2014). In addition to traditional PCR and hybridization based methods, there have been numerous reports of alternative technologies for ultrasensitive detection of nucleic acids (Lapitan et al. 2015; Sun et al. 2014). These have exploited innovative DNA nanotechnology schemes and a variety of readouts including electrochemical detection, quantum dots, and derivatized nanoparticles (Bernacka-Wojcik et al. 2013; Hwang et al. 2014; Torati et al. 2016; Yan et al. 2015). However, the clinical utility of these methods has not been established and few of these studies focus on drug resistance detection in *Mtb*. Work from our group and others has established binary deoxyribozyme (BiDz) sensors as a potentially transformative technology for the sensitive, sequence specific detection of nucleic acids (Bone et al. 2014; Gerasimova et al. 2010; Kolpashchikov 2006, 2007, 2010). Here we report a novel methodology combining multiplex PCR and BiDZ sensors for the detection of *Mtb* and mutations related to drug resistance to first-line drugs (RIF and INH) and second line drugs (FQs). The simple reagent requirements of TB-DzT, a universal substrate and two short unmodified oligonucleotide BiDz sensors, offers an inexpensive, flexible molecular diagnostic platform. The advantages of BiDz sensors include simple design, chemical stability, high selectivity and high sensitivity due to catalytic turnover of substrate. Our results indicate that BiDz

sensors have the potential to enable the rapid (~4 hrs), accurate and affordable diagnosis of *Mtb* and drug resistance even when the mutant is only 10% of the total *Mtb* present in a sample.

Subsequent development of the TB-DzT technology will include testing on a wider selection of clinical samples, shortening of the assay time, and eliminating the requirement of PCR instrument by replacing the PCR amplification step with isothermal amplification methods. In recent studies, we have demonstrated innovative approaches to further improve the sensitivity of BiDz assays via multicomponent nucleic acid assemblies that “trap” substrate molecules near sites of BiDz assembly and unwind RNA analytes (Cox et al. 2016a; Cox et al. 2016b). We have also recently reported a proof-of-principle study for an alternative visual, colorimetric readout which would be ideal for point-of-care implementation (Gerasimova et al. 2013). Future studies will also focus on the incorporation of the TB-DzT assay into a self-contained microfluidics device. Given the flexibility and simple design of BiDz sensors, this platform could be applied to diagnostics of any disease from microbial infections to cancer for which specific detection of clinically relevant nucleic acid sequences or mutations is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Detection of drug resistant tuberculosis using binary deoxyribozyme (Dz) sensors
- Show utility of Dz sensors for detection of heteroresistance
- Combinatorial Dz sensors for detection of multiple possible mutations
- Limit of detection is ~15 bacteria in a one hour deoxyribozyme sensor assay

Fig. 1. Schematic diagram of binary deoxyribozyme sensors (Dz)

A. Principle of BiDz sensor: Dz_a and Dz_b DNA strands hybridize to adjacent positions of the DNA/RNA analyte and reform a deoxyribozyme catalytic core, which cleaves the fluorescent substrate (F-sub). F-sub contains a 5' fluorescein (FAM) label and 3' black hole quencher which are in close proximity to ensure low fluorescence of the intact substrate. When the F-sub is cleaved, the fluorophore is separated from the quencher allowing for the increase of fluorescence. **B.** A panel of BiDz sensors was designed for the detection of SNPs in multiplex PCR amplicons. In the presence of the complementary (specific) analyte, Dz_a and Dz_b bind to the analyte and F-sub forming the sensor with catalytic activity, which cleaves F-sub and produce a fluorescent signal. Alternatively, in the presence of a mismatched (nonspecific) analyte the catalytic core of the sensor cannot be formed and the fluorescence of F-sub remains quenched.

Fig. 2. Validation of BiDz sensors for the detection of *Mtb* and drug-resistance conferring SNPs
A. BiDz sensors designed for specific detection of WT sequences. **B.** BiDz sensors designed for specific detection of mutations (MT) responsible for drug resistance. This data represents the signal to background (S/B) ratios obtained after 45 min of incubation in the presence of synthetic DNA analytes. The *rpoB* 531 mutant sensor shown corresponds to MT1 (Table S1). A student's t-test was completed for each sensor. All comparisons (detection of WT vs MT analyte) resulted in p-values < 0.05.

Fig. 3. Use of the TB-DzT assay for the detection of drug resistant *Mtb* strains

Each panel (A–F) represents the drug resistance profiles produced by the TB-DzT assay in the presence of CDC1551 (WT) *Mtb* DNA and five strains containing drug resistant SNPs (MT1–5). The inset box indicates the phenotype of each strain which was confirmed by sequencing.

Fig. 4. Detection of heteroresistance via TB-DzT assay

This data represents the average S/B ratios and standard deviations for 5 independent experiments. Each sensor was tested in the presence of WT (dark grey bars), WT+10% MT4 (light grey bars), or 100% MT4 (white bars) multiplex PCR analytes. A student's t-test was completed to establish statistical significance in the presence of specific analytes. All SNP specific sensors yield statistically significant S/B ratios in the presence of specific analytes compared to nonspecific analytes ($p < 0.05$).

Fig. 5. Combinatorial sensor assay for the detection of multiple drug resistant SNPs

This data demonstrates the detection of synthetic DNA analytes using BiDz sensors in 30 min. In these experiments Dz_a and Dz_b strands were at 15 nM. All sensors were tested in the presence of 1nM synthetic analytes wt (dark grey), mt1 (medium grey), mt2 (light grey), mt3 (white).

TABLE 1

Limit of Detection with mPCR products, values indicate genome equivalents

Sensor	30 min	45 min	60 min
<i>23S rRNA</i>	128	31	10
<i>rpoB 531 WT</i>	221	43	15

^aThis data represents the LOD (average background + three standard deviations) in genome equivalents calculated from 6 independent experiments.