A Rapid and Sensitive Chip-based Assay for Detection of *rpoB* Gene Mutations Conferring Rifampicin Resistance in *Mycobacterium tuberculosis*

Wanyuan Ao[1], Steve Aldous[1], Evelyn Woodruff[1], Brian Hicke[1], Larry Rea[1], Barry Kreiswirth[2] and Rob Jenison[1], [1]Great Basin Corporation, Salt Lake City, Utah and [2]Public Health Research Institute Tuberculosis Center, University of Medicine and Dentistry of New Jersey Newark, New Jersey

Abstract:

The objective of this study is to develop a rapid and cost-effective assay for detecting *Mycobacterium tuberculosis* (TB) infection in low-resource settings. Multi-drug resistant TB strains are widespread and present a challenge to effective treatment of this infection. The need for a low-cost and rapid detection method for clinically relevant mutations in TB that confer multi-drug resistance is urgent, particularly for developing countries with limited resources. We present here an assay that rapidly detects the majority of clinically relevant mutations in the beta-subunit of the RNA polymerase (*rpoB*) gene that confer resistance to rifampin (RIF), the treatment of choice for TB. The assay, termed TB ID/R, combines a novel target and temperature dependant RNase H2 mediated cleavage of blocked DNA primers to initiate isothermal helicase-dependent amplification of a *rpoB* gene target sequence. The use of this novel method with blocked primers allows efficient amplification that could detect as low as one copy of target template in 40 minutes. Amplified products are detected by probes arrayed onto a modified silicon chip that permits visible detection of both RIF-sensitive and RIF-resistant strains of TB. DNA templates of clinically relevant single nucleotide mutations in the *rpoB* gene were created to validate the performance of the TB ID/R assay. All mutations except one rare mutant were unambiguously detected. Additionally, 11 RIF-sensitive and 25 RIF-resistant clinical isolates were tested by TB ID/R and 35/36 samples were correctly classified (97.2%). This test is currently being configured into a low cost assay platform to provide rapid diagnosis and drug susceptibility information for TB. In summary, we have developed a rapid and sensitive TB assay that could be used for the point of care setting in the developing world where the need is acute.

Introduction:

The global incidence of drug resistant TB (*Mycobacterium tuberculosis)*, particularly multi-drug resistant (MDR) and extremely-drug resistant (XDR) strains, is a major worldwide issue. Rates of MDR TB have been estimated to be 4.8% of the estimated 9.8 million TB infections, but rates as high as 55% have been observed for previously treated patients. TB can be effectively treated if properly identified. However, delayed initiation of appropriate treatment in suspected MDR-TB cases is associated with excess morbidity and nosocomial transmission. It has been determined that the main contributor to delay in treatment is poor sensitivity of diagnostic tests; the average sensitivity of sputum microscopy is <60% in immunocompetent patients and is lower in HIV infected cases. Frequent smear-negative disease increases the difficulty with detecting HIV-associated TB as well. While mycobacterium culture is much more sensitive, it has a very slow turnaround time of 2-8 weeks and is technically complex. Nucleic acid amplification-based tests have improved detection sensitivity and time-to-result but historically have been difficult to effectively implement. A recently described real-time PCR approach brings ease-of-use but at high cost.

To address the needs of bringing sensitive and specific diagnostic testing closer to the patient in the developing world, we have designed a simple, low cost approach for the specific detection of *M. tuberculosis* and mutations within the *rpoB* gene that confer resistance to the front line drug, rifampin. Described herein is the performance of the bench top version of the assay, termed TB ID/R. Target DNA sequences within the *rpoB* gene are amplified using a novel method, bpHDA which utilizes the isothermal amplification method helicase-dependent amplification to exponentially amplify target DNA sequences coupled with blocked primer/ RNase H2 mediated target-specific "hot start". Resultant amplicons are detected by hybridization to a probe set arrayed onto a modified silicon chip surface that detects mutations in the *rpoB* gene amplified region such that intermolecular interactions trigger colorimetric intensity changes, permitting visual detection of attomole quantities of nucleic acids .

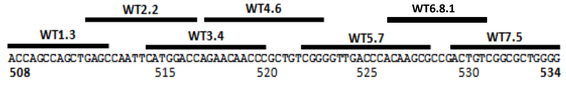

Figure 1. Alignment of Probes with the 81-bp Core Sequence of the *rpoB* Gene. The *rpoB* gene of *M. tuberculosis* has a 81-bp core sequence (including codons 508-534), which harbors the majority of those clinically relevant mutations that account for more than 95% of the reported drug-resistant TB cases. A 128 bp fragment containing this core region was amplified using bpHDA technology for the assay as described in the introduction.

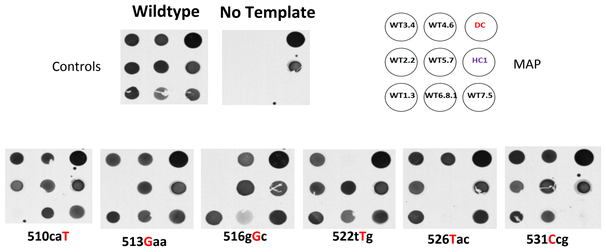

Figure 3. *rpoB* Gene SNP Discrimination. 30 synthetic 128 base pair templates were amplified using bpHDA as described, hybridized to the chip, and a CCD camera image was taken. (Top row, left) control chip data with wildtype or no template; (top row, right) array map. HC1 = hybridization control, DC = detect control; (bottom row) representative images of mutations that affect each probe are shown.

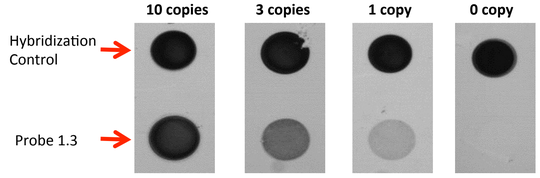

Figure 5: TB ID/R Assay Limit of Detection (LOD). The LOD was determined using wildtype (H37Ra) genomic template in the bpHDA reaction (40 ul) with an amplifcation time at 40 minutes. The assay could detect as low as one single copy of genomic template per reaction on the chip. The top row shows the hybridization control with a consistent strong signal and the bottom row indicates the decreased signal intensity of one probe (WT1.3) when the copy number reduced in the reaction.

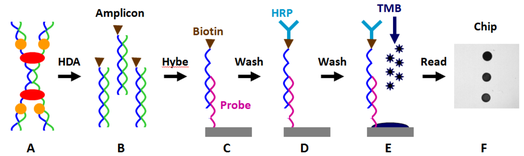

Figure 2: Amplification and Detection Process. The target is amplified using the bpHDA technology (A-B). After amplification, the biotin-labeled amplicon is hybridized to the probe on the chip surface (C). Then, HRP (horseradish peroxidase) - conjugated anti-biotin antibody binds to the biotin on the amplicon (D). Finally, the chromogenic TMB substrate reacts with HPR and produce blue color precipitates and forms spots on the chip surface for visualization (E-F).

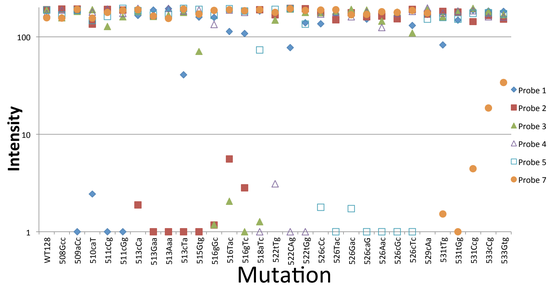

Figure 4. Performance of TB ID/R Assay for Detecting Synthetic Mutations. Synthetic templates with mutations indicated by uppercase letter were amplified, tested by TB ID/R, subject to CCD imaging, and image analysis using spot pixel intensity. Signals for each probe on each chip were plotted. The signals below 10 pixels are difficult to detect by the unaided eye.

Figure 6. TB ID/R Assay Automation. The assay is currently being automated on a cartridge-based compact device. (Left) A cartridge is shown with four blisters packaged with four different assay reagents at the bottom row and the amplification and detection chambers on the top row. (Right) A prototype device is shown with the cartridge loaded on the top right side (a soft drink aluminum can was photographed for easy comparison of the device dimensions).

		Sequence Result	
		Wildtype	***Mutant***
Tb ID/R Result	***Wildtype***	11	1
	Mutant	0	24

Table 1. Clinical Specimen Testing Results. 11 RIF-sensitive and 25 RIF-resistant clinical specimens were verified in this study. All the RIF-sensitive specimens (11/11 = 100%) were identified correctly as wildtype TB. 24/25 (96%) of the RIF-resistant specimens were classified correctly as mutants. The one that was mis-called as wildtype was verified to be a mixture of wildtype and mutant template population by sequencing. This kind of infection with both wildtype and mutant population could be a challenge for this assay.

Strain	Source	Hybhridization
Mycobacterium bovis-BCG	ATCC-#19015	Wildtype
Mycobacterium microti	ATCC-#11152	Wildtype
Mycobacterium africanum	ATCC-#35711	Wildtype
Mycobacterium africanum	ATCC-#25420	Wildtype
Mycobacterium abscessus	ATCC-#19977	No
Mycobacterium fortuitum	ATCC-#35754	No
Mycobacterium genavense	ATCC-#51234	weak cross-hybe
Mycobacterium chelonae	ATCC-#35749	No
Mycobacterium celatum	ATCC-#51131	No
C. difficle	ATCC-#BAA-1382D-5	No
B. subtilis	ATCC-#23857D-5	No
Staph. aures (MRSA)	ATCC-#1005-22-03	No
Staph. aures (MSSA)	ATCC-#3555D-5	No
H. sapiens	Roche (cat#-11691112001)	No
S. cerevisiae	Novagen (cat#-69240-3)	No

Table 2. Tb ID/R Assay Specificity. This assay only detects Tb and Tb-complex strains (*M. bovis-BCG, M. microti and M. africanum*) as positive. Other mycobacterium or non-mycobacterium strains will not be detected except M. genavense which generates some weak cross-hybridization signal for two probes. The table above shows a list of strains tested, including human, yeast and a few other bacterium strains.

Summary:

We have developed a rapid and low-cost TB assay that could detect wildtype TB and the majority of the clinically relevant drug-resistance mutations in the core region of *rpoB* gene, which covers more than 95% of the drug-resisitance Tb cases reported. The assay is very sensitive and could detect one single copy of genomic DNA in the reaction with an amplification time at 40 minutes. The assay also specifically detects TB and other Tb complex strains. Currently, it is being configured into a low-cost assay on a simple but automated device, which we believe will greatly benefit the Tb patients in the developing countries.