



Diagnostics

Highly sensitive sequence specific qPCR detection of *Mycobacterium tuberculosis* complex in respiratory specimens

Jennifer L. Reed^a, Zachary J. Walker^a, Debby Basu^a, Veronica Allen^b, Mark P. Nicol^b, David M. Kelso^a, Sally M. McFall^{a,*}

^a Center for Innovation in Global Health Technologies (CIGHT), Department of Biomedical Engineering, Northwestern University, Evanston, IL 60208, USA

^b Division of Medical Microbiology, Department of Pathology, University of Cape Town and National Health Laboratory Service, Cape Town, South Africa

ARTICLE INFO

Article history:

Received 24 July 2016

Received in revised form

25 August 2016

Accepted 3 September 2016

Keywords:

Mycobacterium tuberculosis

Quantitative PCR

Sequence specific capture

Diagnosis

Sputum

SUMMARY

Nucleic acid amplification tests for *Mycobacterium tuberculosis* (MTB) detection from sputum are highly sensitive and specific with smear microscopy positive specimens, but their sensitivity with smear-negative/culture-positive specimens is much lower; therefore, these tests cannot rule out a tuberculosis diagnosis. Co-extraction of PCR inhibitors may be a cause of decreased test sensitivity. Here the design and early validation of a MTB screening assay with sample preparation and qPCR methods designed to specifically address this diagnostic gap is reported. First, human genomic DNA is identified as a significant qPCR inhibitor. To circumvent this problem, a novel, streamlined sample preparation method utilizing detergent and proteolysis to thin the sputum and DNA sequence specific MTB DNA isolation was developed. Additionally, a multiplexed qPCR assay targeting two MTB complex-specific loci: the potentially multi-copy *IS6110* and the single-copy *senX3-regX3*, combined with the *cotJc* gene from *Bacillus atrophaeus* spores amplified as a process control was developed. The limit of detection of the test was estimated to be 20 cfu/ml which is significantly lower than the Xpert[®] MTB/RIF assay. In a preliminary field study of 60 de-identified blinded sputa, a test sensitivity of 96% and specificity of 100% was observed when compared to the Xpert[®] MTB/RIF assay.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB) complex species, is the deadliest infectious disease globally with an estimated 9.6 million people falling ill, and 1.5 million people dying from TB in 2014 [1]. HIV-1 positive populations are particularly susceptible to TB and are 29 times more likely to die from active TB disease than HIV-1 negative populations. HIV-TB coinfection contributes substantially to TB-associated deaths worldwide, as 1.2 million (12%) individuals who developed TB were HIV positive, and 0.4 million co-infected patients died in 2014, accounting for 27% of the estimated 1.5 million deaths from TB [1]. Accurate and timely diagnosis is the first step in providing care to patients and preventing transmission.

Mycobacterial culture has long been the conventional gold standard test for TB diagnosis in high resource settings. It has high

sensitivity (limit of detection ~10 cfu/ml) [2] but the time-to-result is lengthy (ranging from 2 to 8 weeks) [3] and the sample preparation is technically challenging, prone to contamination and requires a BSL-3 laboratory facility. In most low resource settings, bacterial culture is unavailable, leaving sputum smear microscopy as the only direct bacteriological test available [4]. The limit of detection (LOD) of the unconcentrated smear test is approximately 10,000 cfu/ml, and it has poor specificity in settings where non-tuberculosis mycobacteria are commonly isolated [3]. Smear microscopy's poor clinical performance is particularly troublesome in settings with high HIV-1 incidence. The HIV-1 epidemic has led to a disproportionate increase in the reported rate of smear-negative TB patients, and co-infection with HIV-1 changes the presentation of smear-negative TB to more rapidly progressive disease with a high mortality rate [5,6].

Nucleic acid amplification tests (NAT) have the potential to provide highly sensitive detection of paucibacillary forms of tuberculosis with rapid turn-around-time; however, the commercially available NATs are not yet meeting this need. To date, the analytical sensitivity of the most widely adopted NAT, the Xpert[®]

* Corresponding author. Fax: +1 847 491 4928.

E-mail address: s-mcfall@northwestern.edu (S.M. McFall).

MTB/RIF Assay (Cepheid; Sunnyvale, CA) is reported to be 131 cfu/ml sputum (95% CI) which is only slightly less sensitive than culture (10–100 cfu/ml), and the qPCR assay LOD is < 4.5 genomes per reaction [7]. The clinical sensitivity with smear-positive specimens is 98% when compared to culture; however, clinical sensitivity with smear-negative specimens is only 67% [8]. This sensitivity can be improved to greater than 90% by testing the same patient 3 times [7]. Suboptimal sensitivity is likely to lead to reduced test impact, as clinicians will continue to use empiric treatment in test-negative patients [9] or alternatively may miss the diagnosis of TB in patients with paucibacillary TB, such as children and people living with HIV.

The performance of MTB NATs is greatly impacted by the sample preparation, mycobacterial lysis, and DNA extraction methods utilized [10,11]. Leung et al. [12] reported up to a 12.5-fold difference in DNA yield simply due to different cell lysis and DNA extraction protocols. Additionally, biological factors in sputum can cause PCR inhibition with a reported 5-fold decrease in analytical sensitivity of an MTB-specific PCR assay when comparing extraction from buffer to sputum [13]. Indeed, sputum appeared to be one of the more difficult sample types for detecting MTB using the Xpert[®] MTB/RIF test because pulmonary specimens had a greater proportion of inhibited samples as measured by the Cq of internal process control of the than non-pulmonary sample types (6% vs. 1%) [14]. In a large study of 741 patients, 15.5% of the Xpert[®] MTB/RIF positive specimens were inaccurately quantified due to PCR inhibition observed by substantial delays in the internal control values [15], and in another smaller study, ~20% of false negatives were reported to be caused by the presence of *Taq* inhibitors from sputum [16]. The sensitivity gap between culture and NAT performance implies that an analysis of the different sample preparation and testing methods may reveal strategies that can improve NAT performance to approach that of culture.

MTB NAT sample preparation can be performed by concentrating and purifying intact MTB from sputum, or by directly lysing MTB bacteria in sputum followed by extraction of DNA. MTB in liquefied sputum can be concentrated through centrifugation followed by resuspension of the pellet in buffer, or MTB can be purified through filtration and washing as in the Xpert[®] MTB/RIF Assay [17]. However, logistical obstacles often thwart successful execution of these established sample processing methods. The sample volume of sputum is generally limited because the high concentration of PCR inhibitors and nucleases found in the specimen can cause assay failure. Sputum itself is a viscous, heterogeneous mixture that contains high levels of nucleic acid, and concentrating by filtration limits the test volume because the filter will readily clog. Tests that rely on centrifugation to concentrate the bacilli from the liquefied sputum also neglect the free MTB DNA present in the supernatant [18], which may reduce the overall sensitivity of the assay.

Here a MTB screening assay is described with a novel sample preparation method that achieves an LOD of 20 cfu/ml which is more sensitive than the current NATs and approaches that of culture. Human genomic DNA was identified as the key qPCR inhibitor present in sputum, and a sample preparation method was developed that substantially eliminates it from the amplification reaction. A multiplexed qPCR assay was also developed that targets two *M. tuberculosis* complex (MTBC)-specific loci to increase sensitivity and minimize the likelihood of false negatives due to target gene mutation or deletion. It includes the potentially multi-copy insertion sequence IS6110 [19], and the highly conserved single-copy backup target *senX3-regX3*, a mycobacterial two-component regulatory operon critical for MTB virulence [20]. Amplification of the *cotJc* gene in *Bacillus atrophaeus* spores, which are added to the raw

sputum, serves as a control for adequate processing of the target bacteria and to monitor qPCR inhibitors. The effectiveness of the sequence specific sample preparation method was verified with 60 sputum specimens collected from symptomatic TB patients displaying 96% sensitivity and 100% specificity compared to the GenExpert[®] MTB/RIF Assay.

2. Materials and methods

2.1. Bacterial strains, genomic and plasmid DNA and sample sources

Two MTBC strains, *M. tuberculosis* H37Ra and *Mycobacterium bovis* BCG were acquired from the American Type Culture Collection (ATCC; Manassas, VA) and used as positive controls for assay development. Mycobacterial samples were sonicated to break up cell clumps prior to contriving sputum specimens as described by Helb et al. [7]. *B. atrophaeus* spores (MesaLabs; Lakewood, CO) were used as the process control. *M. tuberculosis* H37Rv TMC 303 genomic DNA (ATCC; Manassas, VA) was used as the template for DNA-specific capture and real-time PCR assays. Genomic DNA from 6 *Mycobacterium* species: *Mycobacterium gordonae*, *Mycobacterium intracellulare*, *Mycobacterium terrae*, *Mycobacterium malmoeense*, *Mycobacterium celatum*, and *Mycobacterium abscessus* (ATCC; Manassas, VA) was used as the template for the specificity panel. The β -globulin plasmid used to generate a standard curve of the human single-copy β -globulin gene, *HBB* [21], was constructed by cloning 476 bp of nucleotide 76–552 of the *HBB* sequence (sequenceID:gbIKP309822.1) into the *EcoRI* site of pGEM(R)-T Easy Vector (Promega; Madison, WI). Residual sputum specimens were obtained from TriCore Reference Laboratories (Albuquerque, NM) in frozen 1 ml aliquots.

2.2. Quantitative PCR

PCR master mix for a 25 μ l total reaction volume (15 μ l master mix and 10 μ l of eluted DNA) consisted of: 0.2 mg/ml bovine serum albumin (BSA; Life Technologies Corporation; Grand Island, NY), 0.2% tween-20, 150 mM trehalose (Sigma; St. Louis, MO), 10% glycerol, 62.5 mM bicine pH 8 (Affymetrix; Santa Clara, CA), 135 mM potassium acetate pH 7.5 (Affymetrix; Santa Clara, CA), 1.5 mM manganese chloride (Sigma; St. Louis, MO), 0.325 mM each dNTP (Life Technologies Corporation; Grand Island, NY), 3.75 U RMS Z05 DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ), and sequence-specific oligonucleotides (Table 1). For the IS6110 single-plex assays, 200 nM IS6110 F7, 200 nM IS6110 R10, and 300 nM IS6110 probe were used. For the β -globulin assays, 100 nM bgF, 100 nM bgR and 150 nM β -globulin probe were used. For the multiplexed MTB assay, 200 nM IS6110 F7, 200 nM IS6110 R10, 300 nM IS6110 probe, 300 nM *senX3-regX3* F3, 200 nM *senX3-regX3* R2, 200 nM *senX3-regX3* probe 8, 100 nM *cotJc* F2, 100 nM *cotJc* R2 and 100 nM *cotJc* probe3. Freeze-dried qPCR master mix was used for the DNase I experiment and for the field testing study using clinical specimens. The freeze-dried master mix composition is the same as the liquid qPCR master mix except that the amount of qPCR enzyme stabilizers were modified as follows: 2.5 mg/ml BSA, 0.03% tween-20 and 138 mM trehalose, and the bicine buffer, potassium acetate and manganese were not freeze dried rather they were added as the resuspension buffer. Oligonucleotides were supplied by IDT (Corvallis, IA) with the exception of the *cotJc* probe3 which was supplied by Biosearch Technologies (Petaluma, CA).

Amplification was performed in a 5-plex Qiagen (Hilden, Germany) Rotor-Gene Q thermocycler, with the following cycling conditions: 1.95 °C 2:00, 2.95 °C 0:15, 3: 60 °C 0:45, 4. repeat steps

Table 1
Oligonucleotides Table. + indicates locked nucleic acid.

Gene	Oligo	Oligo sequence	Reference
Human β -globulin	bgF	5'-GGCAGGTTGGTATCAAGGTTAC-3'	[21]
Human β -globulin	bgR	5'-CCTAAGGTTGGGAAAATAGACC-3'	[21]
Human β -globulin	Bg Probe	5' Quasar 670– ACTGGGCATGTGGAGACAGA-BHQ2-dT 3'	[21]
MTB IS6110	IS6110 F7	5'-CGATGTGTACTGAGATCCCCTATCCG-3'	This study
MTB IS6110	IS6110 R10	5'-GGCCTTTGTCACCCAGGCC-3'	This study
MTB IS6110	IS6110 FAM	5'/56-FAM/AACGTCITT/ZEN/CAGGTCGAGTACGCCTT/3IABkFQ/-3'	This study
MTB <i>senX3-regX3</i>	<i>senX</i> F3	5'-CAGAGCGTAGCGATGAGGTGG-3'	This study
MTB <i>senX3-regX3</i>	<i>senX</i> R2	5'-GCCTCAAAGCCCTCCTTGGC-3'	This study
MTB <i>senX3-regX3</i>	<i>senX</i> probe 8	5'-/5HEX/GAGGA+CGAGGAGT+CGC+TGGC/3BHQ_1/-3'	This study
BA <i>cotJ</i> C	<i>cotJ</i> C F2	5'-GGCGGCTCTCTGTTACTTAAA-3'	This study
BA <i>cotJ</i> C	<i>cotJ</i> C R2	5'-GAACTCCTCGGTCCCTATATCA-3'	This study
BA <i>cotJ</i> C	<i>cotJ</i> C probe 3	5'-/d Quasar 670/TACCTGACAAAGTATCGGGCTGC/BHQ_2/-3'	This study
Gene	Capture probe	Oligo sequence	Reference
MTB IS6110	Cap-1	5'-Biotin-AAAAACGAACGGCTGATGACCAAATC-3'	[22]
MTB IS6110	Cap-2	5'-Biotin-AAAAAGGAGGTGGCCATCGTGAAG-3'	[22]
MTB <i>senX3-regX3</i>	SENX CAP 3	5'-Biotin- AAAAACCACTGTGTGATTTGTGGAGGACGAG-3'	This study
MTB <i>senX3-regX3</i>	SENX CAP 4	5'-Biotin-AAAAAGTGAATCAAAGCCCTCTGCG-3'	This study
BA <i>cotJ</i> C	<i>cotJ</i> C Cap 1	5'-Biotin-AAAAACAATACTTAATCGAGCAATATGGCGCGC-3'	This study
BA <i>cotJ</i> C	<i>cotJ</i> C Cap 2	5' Biotin-AAAAAGCTTATACACCATCGTCGCAATCATCTCC-3'	This study

2–3 44 times. The program was set to acquire in green, yellow and red channels, and copy number of the two MTB targets, relative to the H37Rv TMC 303 genomic DNA standard curve, was determined by using the Qiagen Rotor-Gene Q Series Software package.

2.3. Detection of qPCR inhibition

The presence of inhibitors which caused discordant qPCR results was examined by preparing and amplifying 10-fold dilutions of the eluates and comparing the quantification cycles (Cqs) of the diluted vs. the undiluted samples. Assuming a 100% efficient qPCR assay, the Cqs should be 3.3 cycles apart. A semi-quantitative inhibition rating (IR) was established based upon dCq, the difference between expected Cq and the actual Cq. A dCq less than 1 was represented by a score of IR = 0 (uninhibited); dCq between 1 and 3 was represented by a score of IR = 1 (moderately inhibited); and dCq greater than 3 or reaction failure was represented by a score of IR = 2 (severely inhibited).

2.4. DNase I treatment

A cocktail of sputum specimens was prepared by vortexing 15 ml residual sputum specimens with approximately 7.5 g acid-washed 5 mm glass beads (Sigma; St. Louis, MO) for 5 min. Five aliquots of 350 μ l each were extracted using the Dynabeads[®] SILANE Genomic DNA Kit (Life Technologies, Carlsbad, CA) as per manufacturer instructions, and the elutions were pooled. 11.5 μ l of the pooled eluate was treated with 1 μ l Turbo DNase I (Life Technologies, Carlsbad, CA), 1.5 μ l Turbo DNase I buffer, and 1 μ l molecular grade water in a total of 15 μ l and incubated for 30 min at 37 °C followed by 20 min at 95 °C to inactivate the DNase I. The controls for this study included heat inactivated DNase I treated eluate, untreated eluate, elution buffer and elution buffer plus DNase I buffer. For the heat inactivated DNase I control, Turbo DNase I was first inactivated for 20 min at 95 °C, and then combined with the eluted DNA and Turbo DNase I buffer as described above. Fifteen microliters of the untreated eluate, elution buffer and elution buffer plus DNase I buffer were added directly to the PCR reaction. Five thousand copies of H37Rv MTB genomic DNA was amplified PCR using the MTB IS6110 assay.

2.5. Sputum thinning and extraction method comparison

Three different sputum thinning and decontamination methods were compared with 16 individual sputum specimens (Figure 3A): N-acetyl-L-cysteine-sodium hydroxide (NALC), guanidinium hydrochloride (GuHCl) which is the first step of the Dynabeads[®] SILANE Genomic DNA Kit (Life Technologies, Carlsbad, CA), and sodium dodecyl sulfate with proteinase K (SDS/PK). The NALC-NaOH and GuHCl treated samples were subsequently extracted using the Dynabeads[®] SILANE Genomic DNA Kit, and the SDS/PK treated samples were extracted using DNA sequence specific capture. One million cfu of *M. bovis* BCG was added to each test.

Human DNA was quantified using a standard curve of 100 to 1,000,000 copies of β -globulin plasmid DNA. MTB yield was quantified via a standard curve of 100 to 100,000 copies of IS6110 *M. bovis* BCG genomic DNA. Samples were run at 10 μ l eluate, 10 μ l of 10⁻¹ dilution and 10 μ l of 10⁻² dilution in duplicate to insure that the Cq would be on the standard curve and to use the dilutions to determine if amplification was inhibited. For the GuHCl samples, the β -globulin gene was quantified using the 10⁻² dilution and determined the inhibition by comparing the Cqs of the 10⁻¹ and 10⁻² dilutions. For the NALC treated samples, the β -globulin gene was quantified using the 10⁻¹ dilutions and determined inhibition by comparing the Cqs of the neat samples vs. the 10⁻¹ dilutions. For the SDS/PK treated samples, the β -globulin gene was quantified using the neat eluates and determined inhibition by comparing the Cqs of the neat samples vs. the 10⁻¹ dilutions. For IS6110, all 3 conditions, GuHCl, NALC and SDS/PK were quantified using the 10⁻¹ dilutions, and inhibition was identified by comparing the Cqs of the neat eluates vs. the 10⁻¹ dilutions.

2.5.1. Sequence specific capture

A protocol for thinning and decontaminating sputum with proteinase K, SDS and heat was developed to be compatible with a DNA sequence specific capture method adapted from the protocol published by Mangiapan et al. [22]. The reactions were performed with dried sputum thinning and oligo hybridization reagents.

2.5.2. Dried reagent tubes

Two tubes per reaction were prepared with dried reagents: one for sputum thinning and the second for specific capture probe (oligo) binding. The sputum thinning buffer was prepared by combining 50 μ l of 20% SDS, 30 μ l of 1 M Tris, pH 8.0; 20 μ l of 0.5 M

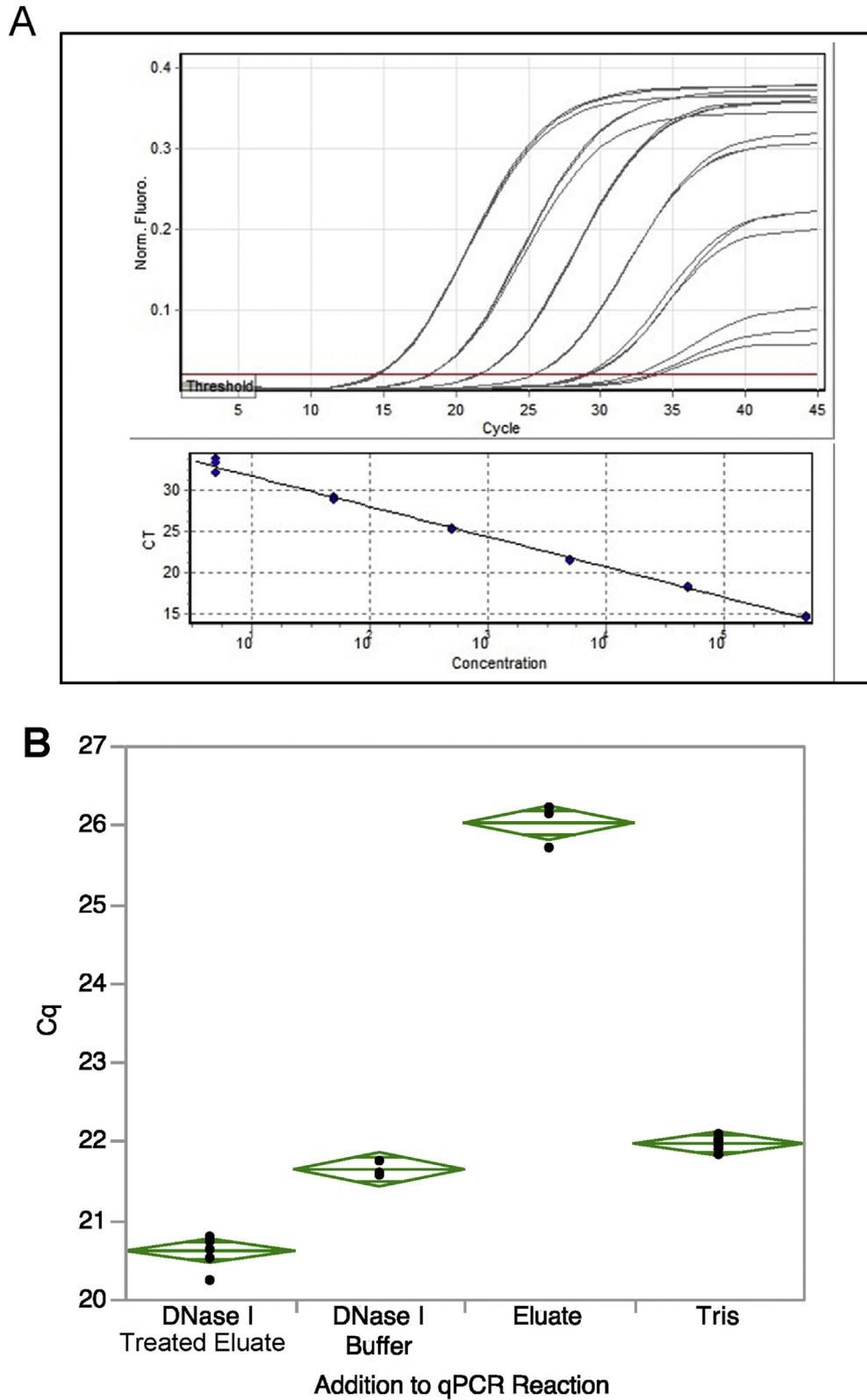


Figure 1. Development of qPCR assay for sample prep development and DNase I treatment of samples removes qPCR inhibition of *IS6110* assay. (A) *IS6110* qPCR assay. Standard curve of 500,000; 50,000; 5000, 500, 50, and 5 MTB H37Rv genomic DNA copies in triplicate. The equation of the line is $y = -3.7X + 35.5$; $R^2 = 0.997$. qPCR efficiency = 89%. (B) Means diamonds plot of DNase I treated samples. Eluates from bulk sputum extractions were spiked into control qPCR assay to measure PCR inhibition. The center line of the diamond is the mean. The tips of the diamond are the 95% confidence interval and the lines near the tips indicate significant differences if they don't overlap with other diamonds. n = 6 DNase I treated eluates, 3 DNase I buffer controls, 3 Tris buffer controls, and 6 untreated eluates. Heat-killed DNase I data not shown. 4 of 6 replicates failed to amplify.

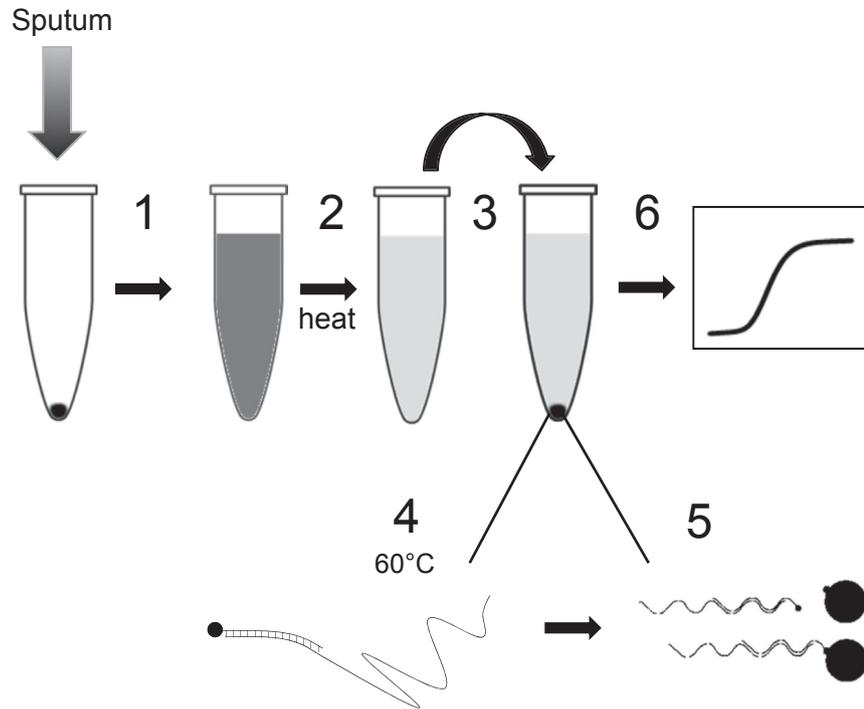


Figure 2. SDS/Proteinase K sputum thinning and sequence specific capture work flow. 1. One ml sputum is added to sputum thinning tube containing dried reagents represented as a black dot at bottom of tube. 2. Sputum is thinned at 55 °C, organisms are lysed, and double-stranded DNA is melted at 95 °C. 3. Thinned sputum is transferred to hybridization tube containing dried reagents represented as a black dot at bottom of tube; 4. Within the hybridization tube, the oligos are hybridized to denatured MTB DNA target at 60 °C; 5. Streptavidin-coated PMPs capture biotinylated oligo-DNA complex; 6. The MTB DNA is eluted from the PMPs and subsequently amplified and detected using qPCR.

EDTA, pH 8.0 per reaction for a final concentration in 1 ml of 1% SDS, 30 mM Tris pH 8.0 and 10 mM EDTA, pH 8.0. One hundred microliters of this SDS solution was pipetted on to the side of sterile 1.5 ml conical-bottom tubes; the tubes were kept in a horizontal position and dried in a 55 °C oven overnight. The oligo binding buffer was prepared by combining 50 µl of 5 M NaCl, 10 µl of 1 M Tris, pH 8.0, 2 µl of 0.5 M EDTA, pH 8.0 and 0.5 µl of 10% Tween-20 per reaction for a final concentration of 250 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA and 0.005% Tween-20. Sixty-two and one half microliters of oligo binding buffer was pipetted on to the side of a 1.5 ml sterile conical tube and kept in a horizontal position while dried in a 55 °C oven overnight. After drying, the tubes were capped and stored at room temperature in aluminum moisture-barrier pouches (Ted Pella; Redding, CA), with silica gel desiccant (McMaster Carr; Elmhurst, IL) to maintain dryness and a humidity indicator card (Static Control Components; San Diego, CA) to monitor moisture.

A cocktail of biotin-labeled oligonucleotides was prepared in advance in which 5 µl contained a total of 5 or 5.5 pmol of probe: for the singleplex IS6110 assay, 2.5 pmol of IS6110 capture probes 1 and 2 were used, and for the multiplexed assay, 1.25 pmol each of IS6110 capture probes 1 and 2, 1.25 pmol each of *senX3-regX3* capture probes 3 and 4, and 0.25 pmol each of *cotJc* capture probe 1 and capture probe 2 were used. All capture oligonucleotides contained a 5' end biotin moiety, included a spacer of 5 adenine residues prior to the specific sequences described above, and were HPLC-purified. Probes were obtained from Integrated DNA Technologies (IDT; Coralville, IA), were diluted in 10 mM Tris pH 8, and stored at -20 °C until time of use.

2.5.3. Sputum liquefaction, decontamination and lysis

To initiate sputum thinning, 950 µl sputum, 50 µl of Proteinase K solution containing 30 U Proteinase K, and 1 µl 1 M CaCl₂ were

added to a 1.5 ml tube containing dried thinning reagent. The samples were heated to 55 °C for 8 min with mixing at 1000 rpm (Benchmark Scientific, Inc; South Plainfield, NJ). The temperature was then ramped to 95 °C, and the specimens were incubated for 10 min with 1000 rpm mixing.

2.5.4. Specific capture based MTB DNA extraction

Samples were removed from the thermal mixer, spun briefly, and the entire volume was transferred to the tube containing dried oligo binding buffer. Five microliters of biotin-labeled oligonucleotide cocktail mix (5 pmol in total) was added, and the samples were incubated at 60 °C and 1000 rpm for 20 min. Twenty microliters of pre-washed Dynal Streptavidin M-270 paramagnetic particles (PMPs) (Thermo Fisher Scientific; Waltham, MA) were added and samples were incubated for 10 min at room temperature with end-over-end rotation. The liquid was collected to the bottom of the tube by a short centrifugation step and the PMPs were pelleted on a magnetic rack (DynaMag2™) for 2 min. Supernatants were aspirated, the PMP pellet was resuspended in 1 ml wash buffer (10 mM Tris, pH 8.0 & 0.01% Tween 20), and the entire volume was transferred to a new 1.5 ml tube. Again, PMPs were collected on a magnetic rack, followed by supernatant aspiration and resuspension of PMPs in 1 ml wash buffer. Following this second wash, PMPs were collected on the magnetic rack and the supernatant was aspirated, taking care to remove all remaining wash buffer volume. The pellet was carefully resuspended in 10 µl elution buffer (10 mM Tris, pH 8.0 0.01% Tween 20, and 10% glycerol) and heated at 75 °C with 1500 rpm mixing, for 3 min. It is important that PMPs are resuspended in the elution buffer during the heated elution step. The liquid was collected at the bottom of the tube by a brief spin, and the samples were placed on the magnetic rack to collect PMPs. The 10 µl elution was carefully transferred to clean tube ready for qPCR.

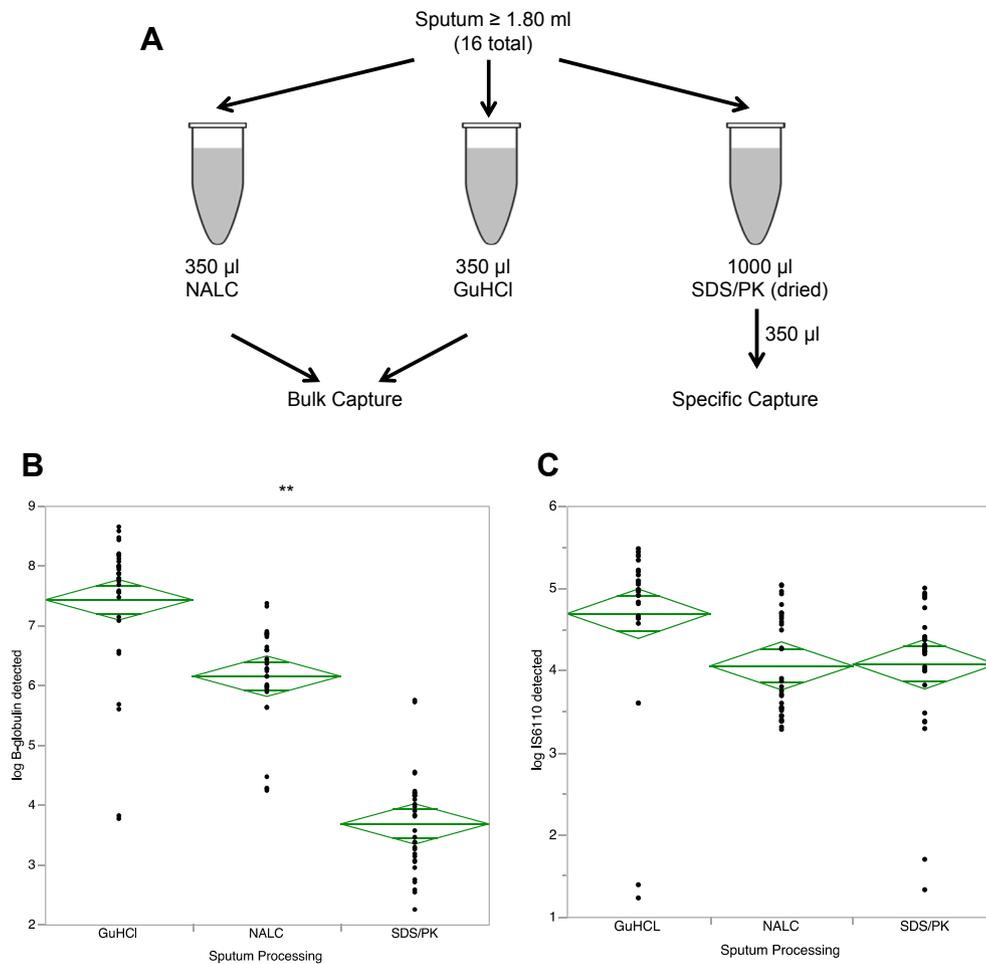


Figure 3. Impact of sputum thinning and DNA extraction methods on MTB IS6110 yield and the amount of co-extracted human genomic DNA. (A) Experimental design. 16 individual sputum specimens were tested. (B) Means diamonds plot of human genomic DNA co-extracted with MTB DNA quantified via the HBB (β -globulin) gene. (C) Means diamonds plot of IS6110 DNA captured. The center line of the diamond is the mean. The tips of the diamond are the 95% confidence interval and the lines near the tips indicate significant differences if they don't overlap with other diamonds. Each group is represented by $n = 16$.

2.5.5. Bulk DNA capture

Prior to bulk DNA extraction using the Dynabeads[®] SILANE Genomic DNA Kit, the sputum specimens were either thinned by NALC treatment or by the addition of GuHCl, as specified in the kit's instructions. Fresh NALC reagent was prepared by adding equal volumes of 4% NaOH and 2.9% sodium citrate with 0.5 g NALC per 100 ml NaOH/citrate solution [23]. To each sputum specimen, an equal volume of the NALC reagent was added and mixed by vortexing, then incubated for 15 min at room temperature with intermittent shaking. The samples were neutralized by the addition of phosphate buffer [pH 6.8] at a ratio of 1: 1.5 (sample: phosphate buffer) and mixed by vortexing. The pellet was collected via centrifugation for 15 min at $3000 \times g$, resuspended with 350 μ l phosphate buffer and immediately processed using the Dynabeads[®] SILANE Genomic DNA Kit according to the manufacturer's instructions. The GuHCl thinned sputa were immediately processed using the Dynabeads[®] SILANE Genomic DNA Kit according to the manufacturer's instructions.

2.6. Laboratory validation study

The Xpert[®] MTB/RIF Assay was used as the clinical standard since South Africa has adopted Xpert[®] MTB/RIF Assay (Cepheid; Sunnyvale, CA) testing on sputum samples as the standard first-line

test for diagnosis of MTB, with MTB culture only performed on selected samples. Sixty de-identified blinded sputum specimens were tested; 27 were Xpert[®] MTB/RIF positive and 33 were Xpert[®] MTB/RIF negative. One of the Xpert negative specimens was invalid (no Cq in *cot1C* test).

2.7. Statistical methods

JMP[®] software (SDS Institute; Cary, NC) was used to calculate differences between means via one-way analysis of variance for independent samples, as well as to generate means diamonds plots. *P*-values of less than 0.01 were considered significantly different. Pearson correlation tests were used to generate R^2 values to evaluate correlations between sputum-specific factors and MTB assay inhibition.

3. Results

3.1. IS6110 qPCR assay

The MTBC-specific potentially multi-copy insertion sequence IS6110 [19] was selected as the genetic target for a qPCR assay to determine the efficacy of MTB DNA extraction protocols from sputum. The primers and probes were designed with 100%

sequence homology to all complete MTBC species sequences available in the NCBI database. The assay was demonstrated to be linear across at least 6 logs of concentration from 500,000 to 5 copies (Figure 1A) and could detect as few as 1 MTB H37Rv genome 6 out of 6 times (data not shown).

3.2. MTB extraction from sputum

Multiple investigators have reported that silica-based extraction methods such as the Boom method [24] yield MTB DNA with less PCR inhibition than alternative methods [12,25,26]. The Dynabeads® SILANE Genomic DNA Kit (Life Technologies, Carlsbad, CA) protocol is designed to extract genomic DNA from 350 µl whole blood using 50 µl paramagnetic particles (PMP), but input volume can be adjusted to suit specific experimental needs. In order to evaluate bulk DNA extraction, initial studies were performed with 350 µl contrived TB-positive sputum specimens with the intention of ultimately scaling up the sample volume to 1 ml. However, in extracting MTB DNA using this system, the qPCR results differed markedly between independent sputum samples. By amplifying 10-fold dilutions of the eluted DNA and quantifying the yield using a standard curve derived from serial dilutions of MTB H37Rv genomic DNA, it was determined that the MTB DNA yield from the Dynabeads® extraction was similar between samples, but qPCR inhibition varied dramatically (data not shown). In order to identify the potential source of inhibition, 7 sputum samples were evaluated for: appearance (bloody, mucopurulent, etc.), density, viscosity, protein levels and human genomic DNA levels and compared these results to qPCR inhibition. Of these variables, only human genomic DNA (gDNA) content correlated with inhibition ($R^2 = 0.985$, Pearson correlation test). Three of the 7 samples displayed qPCR inhibition of greater than 1 Cq, and these 3 samples also had high levels of co-extracted human gDNA as measured by copy number of the single copy gene for β -globulin (data not shown). The other four sputa tested had much lower β -globulin copy number in the eluted sample and no evidence of qPCR inhibition.

Co-extraction of high amounts of human gDNA was confirmed to be the source of inhibition by treating sputum-derived DNA eluates with Turbo DNase I for 1 h at 37 °C, heat-inactivating the enzyme and then adding the eluates or equal volume of controls to qPCR assays containing 5000 genomic copies of H37Rv genomic DNA. Relief of inhibition was observed in the specimens that were treated with active DNase I (Figure 1B). The Tris buffer control had an average Cq of 22.0 ± 0.1 , and the untreated eluate from sputum extraction had an average Cq of 26.0 ± 0.3 . This four Cq delay would lead to more than a 10-fold under-quantification of the MTB bacterial load. Treatment of eluate with DNase I allowed a mean recovery of 5.4 Cq. DNase I activity was required for this relief of inhibition because heat-inactivated DNase I did not improve amplification (data not shown). In fact, 4 of the 6 replicates incubated with inactivated DNase I failed to amplify while the remaining two samples had Cqs of 24.6 and 28.5, similar to the untreated eluate. Eluates from 3 additional sputum extractions gave analogous results with DNase I treatment (data not shown) indicating that human genomic DNA in sputum is a frequent and potent qPCR inhibitor.

3.3. Development of sequence specific capture method

To limit the co-extraction of human DNA from sputum specimens as observed with bulk DNA extraction, a protocol to selectively purify mycobacterial DNA was developed using sequence specific capture. The protocol is divided into two phases (Figure 2): 1) sputum thinning and MTB lysis (steps 1–3) and 2) MTB DNA

capture (steps 4–6). The sputum is thinned to a pipettable consistency and simultaneously sterilized with minimum operator steps. The process involves treating with Proteinase K (PK) and SDS with a subsequent incubation at 95 °C for 10 min. The SDS acts as an emulsifier breaking up mucoid complexes by unfolding proteins, and PK facilitates sputum thinning by digesting the unfolded proteins. Heating to 95 °C decontaminates the specimen by killing MTB and other flora present, while simultaneously denaturing the PK and initiating the specific capture reaction through melting the double stranded DNA in the specimen. The thinned sputum is then transferred to a tube containing dried NaCl and MTB-specific biotinylated oligonucleotides (capture probes) where a two-step capture process using streptavidin-coated paramagnetic particles (PMP) is performed. The high affinity of biotin and streptavidin allows for the biotinylated oligo-DNA complex to be isolated from the thinned sputum solution for washing. The eluted DNA is subsequently amplified and detected using real-time PCR.

3.4. Sequence specific capture versus silica bulk DNA capture

Sixteen MTB-negative sputum specimens with sufficient volume (≥ 1.8 ml) were selected for the study. MTB-spiked sputum specimen were thinned in one of 3 ways: 1) NALC, 2) GuHCl, or 3) SDS/PK, and 350 µl of the thinned sputa were extracted by either Dynabeads® SILANE Genomic DNA Kit (NALC & GuHCl) or sequence specific capture (SDS/PK) (Figure 3A). Human gDNA yield measured in β -globulin copies (Figure 3B), MTB DNA yield measured in IS6110 copies (Figure 3C), and qPCR inhibition rating (IR) was determined by comparing the amplification of the straight eluate versus the 1:10 dilution of the eluate. By using the SDS/PK thinning technique with MTB specific capture, the average amount of human genomic DNA co-extracted was reduced 2.5 to 3.7 logs. Mean β -globulin copy number co-extracted from 350 µl of sputum was 6.2 ± 0.7 log for NALC treatment, 7.4 ± 1.4 log for GuHCl treatment, and 3.7 ± 0.7 log for SDS/PK treatment. The IR was zero for all 16 of the NALC-treated or SDS/PK-treated samples indicating no qPCR inhibition. However, 75% (12/16) of the GuHCl treated specimens exhibited qPCR inhibition with the amount of β -globulin co-extracted corresponding to qPCR inhibition (Figure 4). The mean β -globulin log copy yield of GuHCl treated samples with an IR of 0 (5.9 ± 1.6) was significantly different ($p < 0.01$) from IR1 (7.8 ± 0.5) and IR2 (8.1 ± 0.4), but the mean yields of IR1 and IR2 were not significantly different.

The yield of MTB DNA was measured using 10^{-1} dilutions of the eluate to bypass the effect of qPCR inhibition discussed above. There were no significant differences between mean MTB yield of the GuHCl treated specimens (4.7 ± 1.0 log copies), the NALC treated specimens (4.1 ± 0.6 log copies), or the SDS/PK treated specimens (4.1 ± 0.8 log copies). It was estimated that at least 1 ml of sputum must be extracted and amplified in order to achieve equivalent sensitivity to culture (10 cfu/ml) [2]. Because only 10% of the eluate from a 350 µl specimen was added, roughly 30-fold less specimen volume was used in this experiment than the target 1 ml. If the average genomic DNA yield were multiplied by a factor of 30, the average yield of β -globulin copies would increase to 7.7 logs for NALC, 8.9 logs for GuHCl, and 5.2 logs for SDS/PK thinning with specific capture extraction. The amount of co-extracted β -globulin DNA in the NALC treated specimens would then be equivalent to the average amount of DNA found in the inhibited GuHCl specimens, suggesting that 1 ml NALC-treated samples would also likely be inhibited. The SDS/PK treated specimens average gDNA yield for 1 ml of sputum would still be below the likely inhibited level. Due to the specific capture based DNA extraction resulting in equivalent IS6110 copies (Figure 3B), but

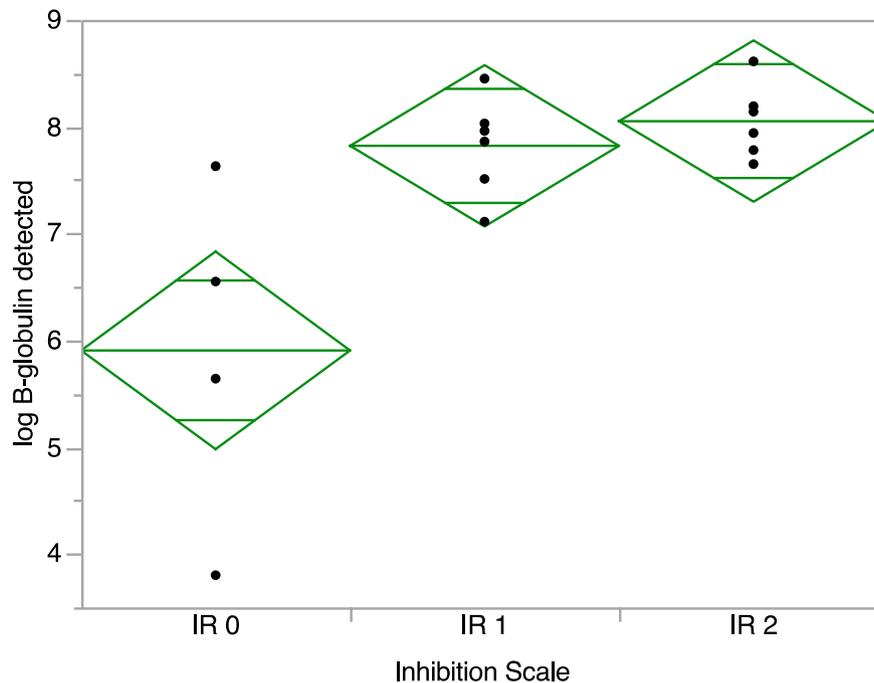


Figure 4. Sputum processing using guanidinium hydrochloride results in high levels of co-extracted human DNA leading to MTB qPCR inhibition. Nominal/Ordinal Scatterplot with Means Diamonds of qPCR inhibition ratings vs. co-extracted human DNA is shown, the tips of the diamond are the 95% confidence interval, the lines near the tips indicate significant differences if they don't overlap with other diamonds, and the center line is the mean. $n = 4$ IR = 0, 6 IR = 1, 6 IR = 2.

significantly lower human gDNA (Figure 3C), it was selected as the preferred nucleic acid extraction strategy for the MTB diagnostic assay.

3.5. Multiplexed qPCR assay

Since IS6110 is not present in some clinical strains [27], the IS6110 assay was multiplexed with primers and probes specific to an intergenic region in the two-component regulatory operon *senX3-regX3* [20]. An amplicon of the *B. atrophaeus cotJC* gene that encodes a spore coat composition polypeptide [28] was also included to monitor the test for extraction, qPCR inhibitors, and amplification efficiency. The *B. atrophaeus* spores were added to the sputum prior to sample processing and were thinned and lysed along with the MTB. Capture probes were designed for each specific target, tested in isolation and then in combination for optimal specific MTB DNA extraction (Figure 5). Contrived sputum specimens of 5000 cfu of MTB H37Ra and 500 *B. atrophaeus* spores in triplicate were extracted using the specific capture MTB DNA extraction method, and the multiplexed PCR assay was performed to confirm capture of multiple targets and assay performance. The IS6110 amplicon had an average Cq of 24.8 ± 0.4 , the *senX3-regX3* had an average Cq of 28.7 ± 0.5 and the *cotJC* amplicon had an average Cq of 32.1 ± 0.6 (Figure 5).

To estimate the limit of detection, 1 ml sputum specimens were contrived containing 100, 50 or 20 cfu/ml, and six replicates of each concentration were assayed. All 6 of the 50 and 100 cfu/ml specimens were detected by both the IS6110 and *senX3-regX3* assays. Five of the 20 cfu/ml specimens were detected by the IS6110 assay, and none of these samples were detected by the *senX3-regX3* assay. Therefore, the LOD of the combined assay was estimated to be 20 cfu/ml. One of the 20 cfu/ml samples was excluded from analysis because the Cqs in both MTB assays were anomalously low. MTB has a tendency to clump, and despite the fact that the bacterial

preparation was sonicated to break up clumps before it was serially diluted for contriving the sputum specimens; this sample may have had a clump of bacteria. Six different *Mycobacterium* species were also tested for potential cross reactivity. Genomic DNA extracted from *M. gordonae*, *M. intracellulare*, *M. terrae*, *M. malmoense*, *M. celatum*, and *M. abscessus* was added to qPCR reactions at 50,000 copies/PCR reaction, and no amplification was observed for any of these species.

3.6. Laboratory validation study of sputum processing and work flow

To determine if the SDS/PK sputum pre-treatment combined with sequence specific capture is effective in processing clinical specimens, a small-scale laboratory validation study was performed at the routine TB diagnostic laboratory at Groote Schuur Hospital, Cape Town, South Africa. A convenient sample of 60 blinded routinely collected specimens that had ≥ 1 ml of sputum left over after GeneXpert[®] testing were assayed using the multiplexed MTB assay protocol described here; 27 Xpert[®] MTB/RIF positive specimens and 33 negative specimens were analyzed. All 60 distinct sputum samples thinned to a pipetable liquid in the sputum thinning step. Of the 27 positive specimens, three samples were negative in *senX3-regX3* assay and one of these same samples was also negative in the IS6110 assay yielding a sensitivity of 88.9% (24/27) and 96.3% (26/27), respectively (Table 2). One of the Xpert[®] MTB/RIF negative specimens failed to amplify the *cotJC* gene and was therefore considered an invalid result. The sample that was a false negative, having failed in both the IS6110 and *senX3-regX3* assays, was reported as a very low positive in the Xpert[®] MTB/RIF assay. The 32 negative specimens with valid test results were also negative in the IS6110 and *senX3-regX3* assays, yielding 100% specificity in both assays.

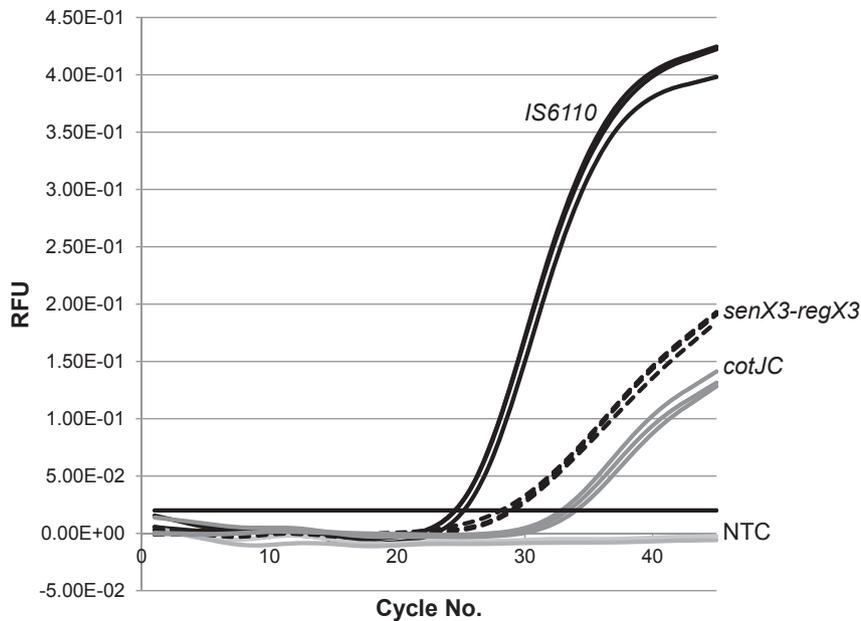


Figure 5. Multiplexed *IS6110*, *senX3-regX3* and *cotJC* assay of contrived sputum specimens containing 5000 cfu/ml H37Ra MTB and 500 *B. atropaueus* spores. All 3 targets are efficiently captured and amplified from sputum. Horizontal black line is threshold. Solid black line is *IS6110*. Dashed black line is *senX3-regX3*. Dark gray line is *cotJC*, and light gray line is no template control (NTC).

Table 2

Summary of testing results of CIGHT MTB assay and Xpert[®] MTB/RIF assay. *senX* = *senX3-regX3* intragenic region. One of the Xpert[®] MTB/RIF negative specimens had an invalid test result yielding valid results for 59 specimens.

	GeneXpert [®] MTB/RIF assay		Sensitivity or specificity (CI)
	Positive	Negative	
<i>IS6110</i> positive	26	0	96.3% (79.1–99.8)
<i>IS6110</i> negative	1	32	100% (86.7–100)
totals	27	32	59 valid results
<i>senX</i> positive	24	0	88.9% (69.7–99.1)
<i>senX</i> negative	3	32	100%
totals	27	32	59 valid results

4. Discussion

In this report, novel methods of sputum pre-treatment, extraction and amplification were combined to address the many of the challenges of MTB diagnosis. The pre-treatment method liquefies sputum and lyses cells without the dilution associated with liquid solutions. It also results in a homogeneous distribution of MTB DNA that eliminates potential sampling errors due to the bacilli's buoyancy and tendency to clump and chain. The specific capture extraction method minimizes co-extraction of human DNA which is a major inhibitor of amplification. By combining *IS6110*, a potentially multi-copy target that is not present in all MTB strains, and *senX3-regX3*, a single-copy target in a genetic locus that is essential for virulence [29], an assay was created that will detect all MTBC strains with high sensitivity. A preliminary estimate of the LOD is 20 cfu/ml for the combined assay which is more than 6-times lower than the LOD of the leading commercial test, Xpert[®]MTB/RIF which is reported as 131 cfu/ml [7].

In order to achieve this high sensitivity, potential sputum-derived factors that could contribute to assay inhibition were methodically evaluated and human genomic DNA was identified as a major contributor to MTBC-specific qPCR assay inhibition.

Furthermore, high amounts of human genomic DNA could have a secondary technical effect of making the PMPs sticky, leading to greater co-extraction of inhibitors from either the biological specimen or DNA extraction solvents such as guanidinium or alcohols. Guanidinium hydrochloride treated sputum yielded the highest levels of co-extracted human DNA among the three DNA extraction methods tested, as reflected by the single-copy human gene β -globulin, as well as strongest assay inhibition.

In this study, one ml sputum specimen was introduced into the tube containing dried sputum thinning reagent for sputum liquefaction and decontamination. In this regard, the sample prep strategy mimics the processing of sputum for mycobacterial culture growth, but the cumbersome sample neutralization and centrifugation steps have been eliminated. This strategy also addresses the biohazard risk associated with inadequate management of infectious samples and the limitations of sample preparation methods to enhance the overall sensitivity of the assay. Sputum collection and manipulation from putative TB patients puts healthcare workers at risk for infection [30,31], and, for NAT testing in settings without biosafety precautions, it is necessary to completely sterilize the sample. This MTB screening assay is able to use a larger volume of sputum (1 ml) which is fully homogenized by treatment with SDS and proteinase K thus ameliorating sampling error. Although it was not explicitly demonstrated in this study, the 95 °C incubation step reduces the biosafety hazard to the test operator by fully lysing the bacteria and melting the double stranded DNA to prepared for the subsequent sequence specific capture step. This method is not reliant on a BSL-3 facility thereby expanding its potential to be applied in limited-resource settings or even at the patient point-of-care, nor is it reliant on centrifugation, therefore improving sensitivity by using both intact and lysed mycobacterial DNA [18] that would otherwise be discarded in supernatants of centrifugation-based extraction methods. By using paramagnetic particles (PMP), the MTB DNA can be extracted from a larger specimen volume, and the sputum-derived inhibitors can be removed with simple wash steps. This sputum processing protocol is currently being adapted

so that it may be performed directly in the sputum collection cup, in order to minimize operator handling and potential for cross-contamination.

It was recently reported that in patients who have been previously treated for TB, 1 in 7 Xpert[®] MTB/RIF-positive results were false-positive relative to mycobacterial culture in a high incidence setting [32]. False positives are correlated with a previous infection that occurred in the last 3 years [33]. The test described here is designed to lyse all the bacteria before an aliquot is removed to reduce sampling error and promote safety of the operator. Therefore, testing the specificity of this assay with specimens from recently recovered TB patients must be carefully evaluated. The test was designed to have a high negative predictive value so that it can be used to rule out a TB diagnosis. Additional algorithms designed to determine if a patient has been treated for TB in the past 3 years could be used to increase the specificity of the test in previously infected patients [32,33].

This study also lends merit to the use of specific capture based DNA extraction in other applications of clinical diagnostics and in other clinical sample types where target DNA may be underrepresented relative to a large pool of human DNA or other potential assay inhibitors. Compared to the two other common sputum processing and DNA extraction methods discussed here (NALC or GuHCl treatment with bulk DNA extraction), while specific capture based DNA extraction resulted in an equivalent level of MTB-specific IS6110 copies, there was a significant reduction (2.5–3.7 log copies β -globulin DNA) in co-eluted human genomic DNA. When tested on a panel of TB positive and negative clinical sputa, showed a sensitivity of 88.9–96.3% for *senX3-regX3* and IS6110, respectively relative to Xpert[®] MTB/RIF, with 100% specificity in both targets. The assay now requires further validation using samples that test culture-positive, Xpert[®] MTB/RIF negative to determine whether the clinical accuracy exceeds that of Xpert[®] MTB/RIF.

The WHO Millennium Development Goal of stopping the increase of TB incidence and halving the TB-associated mortality by 2015 was achieved globally, but not in Africa [1,34]. The next goal set by WHO is to eliminate TB by 2050. This will require a reduction of TB of 16% per year compared to the current rate of 1% per year. In order to achieve these goals, major advances in diagnostics, vaccines and social policy are required [35]. Increasing diagnostic sensitivity is capable of dramatically reducing delays in diagnosis and treatment, and it has been estimated that every 10% increase in test sensitivity can reduce diagnostic delays experienced by patients by 3–5 days [36]. However, without a combinatorial approach, the success of even a promising TB diagnostic test is not likely to impart a significant impact in high burden settings. In particular, a highly sensitive assay is required to reduce reliance on empiric treatment and improve detection in highly vulnerable populations such as children and people living with HIV. Specifically, this will require targeted development of diagnostic tests designed to address the challenges of MTB including the intrinsic properties of difficult clinical sample types that may limit diagnostic assay performance, and barriers of implementation in settings with high HIV and TB incidence.

Acknowledgements

This work was funded by the Northwestern Global Health Foundation and Quidel Corporation. The study sponsors played no role in the design of any of the studies, in the collection, analysis and interpretation of data; in the writing of the manuscript; nor in the decision to submit the manuscript for publication.

Funding: None.

Competing interests: None declared.

Ethical approval: Not required.

References

- [1] WHO. Global Tuberculosis Report 2015. Geneva, Switzerland: World Health Organization; 2015.
- [2] van Zyl-Smit RN, Binder A, Meldau R, Mishra H, Semple PL, Theron G, Peter J, Whitelaw A, Sharma SK, Warren R, Bateman ED, Dheda K. Comparison of quantitative techniques including Xpert MTB/RIF to evaluate mycobacterial burden. *PLoS One* 2011;6(12):e28815.
- [3] The American Thoracic Society. Diagnostic standards and classification of tuberculosis in adults and children. This official statement of the American thoracic society and the centers for disease control and prevention was adopted by the ATS board of directors, July 1999. This statement was endorsed by the council of the infectious disease society of America, September 1999. *Epub* 2000/04/14 *Am J Respir Crit Care Med* 2000;161(4 Pt 1):1376–95. <http://dx.doi.org/10.1164/ajrccm.161.4.16141>. PubMed PMID: 10764337.
- [4] Wejse C. Point-of-care diagnostics for tuberculosis elimination?. *Epub* 2013/11/02 *Lancet* 2014;383(9915):388–90. [http://dx.doi.org/10.1016/s0140-6736\(13\)62003-6](http://dx.doi.org/10.1016/s0140-6736(13)62003-6). PubMed PMID: 24176145.
- [5] Colebunders R, Bastian I. A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2000;4(2):97–107. *Epub* 2000/02/29. PubMed PMID: 10694086.
- [6] Getahun H, Harrington M, O'Brien R, Nunn P. Diagnosis of smear-negative pulmonary tuberculosis in people with HIV infection or AIDS in resource-constrained settings: informing urgent policy changes. *Epub* 2007/06/19 *Lancet* 2007;369(9578):2042–9. [http://dx.doi.org/10.1016/s0140-6736\(07\)60284-0](http://dx.doi.org/10.1016/s0140-6736(07)60284-0). PubMed PMID: 17574096.
- [7] Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, Kop J, Owens MR, Rodgers R, Banada P, Safi H, Blakemore R, Lan NTN, Jones-Lopez EC, Levi M, Burday M, Ayakaka I, Muggerwa RD, McMillan B, Winn-Deen E, Christel L, Dailey P, Perkins MD, Persing DH, Alland D. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. *J Clin Microbiol* 2010;48(1):229–37. <http://dx.doi.org/10.1128/jcm.01463-09>.
- [8] Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Epub* 2014/01/23 *Cochrane database Syst Rev* 2014;1:CD009593. <http://dx.doi.org/10.1002/14651858.CD009593.pub3>. PubMed PMID: 24448973; PubMed Central PMCID: PMCPCMC4470349.
- [9] Theron G, Zijenah L, Chanda D, Clowes P, Rachow A, Lesosky M, Bara W, Mungofa S, Pai M, Hoelscher M, Dowdy D, Pym A, Mwaba P, Mason P, Peter J, Dheda K. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. *Epub* 2013/11/02 *Lancet* 2014;383(9915):424–35. [http://dx.doi.org/10.1016/s0140-6736\(13\)62073-5](http://dx.doi.org/10.1016/s0140-6736(13)62073-5). PubMed PMID: 24176144.
- [10] Aldous WK, Pounder JL, Cloud JL, Woods GL. Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative real-time PCR. *Epub* 2005/05/06 *J Clin Microbiol* 2005;43(5):2471–3. <http://dx.doi.org/10.1128/jcm.43.5.2471-2473.2005>. PubMed PMID: 15872286; PubMed Central PMCID: PMCPCMC1153782.
- [11] Boddinhaus B, Wichelhaus TA, Brade V, Bittner T. Removal of PCR inhibitors by silica membranes: evaluating the Amplicor *Mycobacterium tuberculosis* kit. *Epub* 2001/09/28 *J Clin Microbiol* 2001;39(10):3750–2. <http://dx.doi.org/10.1128/jcm.39.10.3750-3752.2001>. PubMed PMID: 11574609; PubMed Central PMCID: PMCPCMC88425.
- [12] Leung ETY, Zheng L, Wong RYK, Chan EWC, Au TK, Chan RCY, Lui G, Lee N, Ip M. Rapid and simultaneous detection of *Mycobacterium tuberculosis* complex and Beijing/W genotype in sputum by an optimized DNA extraction protocol and a novel multiplex real-time PCR. *J Clin Microbiol* 2011;49(7):2509–15. <http://dx.doi.org/10.1128/jcm.00108-11>.
- [13] Shawar RM, el-Zaatari FA, Nataraj A, Clarridge JE. Detection of *Mycobacterium tuberculosis* in clinical samples by two-step polymerase chain reaction and nonisotopic hybridization methods. *J Clin Microbiol* 1993;31(1):61–5. *Epub* 1993/01/01. PubMed PMID: 8417034; PubMed Central PMCID: PMCPCMC262622.
- [14] Theron G, Peter J, Calligaro G, Meldau R, Hanrahan C, Khalfey H, Matinyanya B, Muchinga T, Smith L, Pandie S, Lenders L, Patel V, Mayosi BM, Dheda K. Determinants of PCR performance (Xpert MTB/RIF), including bacterial load and inhibition, for TB diagnosis using specimens from different body compartments. *Epub* 2014/07/12 *Sci Rep* 2014;4:5658. <http://dx.doi.org/10.1038/srep05658>. PubMed PMID: 25014250.
- [15] Blakemore R, Nabeta P, Davidow AL, Vadwai V, Tahirli R, Munsamy V, Nicol M, Jones M, Persing DH, Hillemann D, Ruesch-Gerdes S, Leisegang F, Zamudio C, Rodrigues C, Boehme CC, Perkins MD, Alland D. A multisite assessment of the quantitative capabilities of the Xpert MTB/RIF assay. *Am J Respir Crit Care Med* 2011;184(9):1076–84. <http://dx.doi.org/10.1164/rccm.201103-0536OC>.

- [16] Amicosante M, Richeldi L, Trenti G, Paone G, Campa M, Bisetti A, Saltini C. Inactivation of polymerase inhibitors for *Mycobacterium tuberculosis* DNA amplification in sputum by using capture resin. *J Clin Microbiol* 1995;33(3): 629–30. Epub 1995/03/01. PubMed PMID: 7751368; PubMed Central PMCID: PMCPMC228003.
- [17] Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, Allen J, Tahirl R, Blakemore R, Rustomjee R, Milovic A, Jones M, O'Brien SM, Persing DH, Ruesch-Gerdes S, Gotuzzo E, Rodrigues C, Alland D, Perkins MD. Rapid molecular detection of tuberculosis and rifampin resistance. *N. Engl J Med* 2010;363(11):1005–15. <http://dx.doi.org/10.1056/NEJMoa0907847>.
- [18] Pathak D, Chakravorty S, Hanif M, Tyagi J. Lysis of tubercle bacilli in fresh and stored sputum specimens: implications for diagnosing tuberculosis in stored and paucibacillary specimens by PCR. *BMC Microbiol* 2007;7(1):83. <http://dx.doi.org/10.1186/1471-2180-7-83>. PubMed PMID.
- [19] Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon JL, Gicquel B. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. *J Clin Microbiol* 1990;28(12):2668–73. Epub 1990/12/01. PubMed PMID: 2177747; PubMed Central PMCID: PMCPMC268253.
- [20] Rifat D, Karakousis PC. Differential regulation of the two-component regulatory system *senX3-regX3* in *Mycobacterium tuberculosis*. *Epub* 2014/04/12 *Microbiol Read Engl* 2014;160(Pt 6):1125–33. <http://dx.doi.org/10.1099/mic.0.077180-0>. PubMed PMID: 24722908; PubMed Central PMCID: PMCPMC4039243.
- [21] Abravaya K, Erickson BJ, Huang SX, Mak WBX, Salituro JA, Tang N. Primers and probes for detecting human papillomavirus and human beta globin sequences in test samples. Google Pat 2010. US9090948 B2.
- [22] Mangiapan G, Vokurka M, Schouls L, Cadranet J, Lecossier D, van Embden J, Hance AJ. Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *J Clin Microbiol* 1996;34(5):1209–15.
- [23] Kent PT. Public health mycobacteriology: a guide for the level III laboratory. In: Kubica GP, editor. Atlanta, Ga.: U.S. Dept. of Health and Human Services. Public Health Service, Centers for Disease Control; 1985.
- [24] Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990;28(3):495–503. Epub 1990/03/01. PubMed PMID: 1691208; PubMed Central PMCID: PMCPMC269651.
- [25] Suffys P, Vanderborcht PR, Santos PB, Correa IA, Bravin Y, Kritski AL. Inhibition of the polymerase chain reaction by sputum samples from tuberculosis patients after processing using a silica-guanidiniumthiocyanate DNA isolation procedure. *Memorias do Inst Oswaldo Cruz* 2001;96(8):1137–9. Epub 2002/01/11. PubMed PMID: 11784936.
- [26] van Doorn HR, Bruijnesteijn van Coppenraet ES, Duim B, Vandenbroucke-Grauls CM, Weel JF, Dankert J, Kuijper EJ, de Jong MD. Silica-guanidinium thiocyanate-based nucleic acid isolation protocol does not improve sensitivity of two commercial tests for detection of *Mycobacterium tuberculosis* in respiratory samples. *Epub* 2006/09/12 *Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol* 2006;25(10):673–5. <http://dx.doi.org/10.1007/s10096-006-0194-z>. PubMed PMID: 16964511.
- [27] Lok KH, Benjamin Jr WH, Kimerling ME, Pruitt V, Lathan M, Razeq J, Hooper N, Cronin W, Dunlap NE. Molecular differentiation of *Mycobacterium tuberculosis* strains without IS6110 insertions. *Epub* 2002/11/28 *Emerg Infect Dis* 2002;8(11):1310–3. <http://dx.doi.org/10.3201/eid0811.020291>. PubMed PMID: 12453362; PubMed Central PMCID: PMCPMC2738562.
- [28] Seyler Jr RW, Henriques AO, Ozin AJ, Moran Jr CP. Assembly and interactions of *cotJ*-encoded proteins, constituents of the inner layers of the *Bacillus subtilis* spore coat. *Mol Microbiol* 1997;25(5):955–66. Epub 1997/11/19. PubMed PMID: 9364920.
- [29] Parish T. Two-component regulatory systems of mycobacteria. *MGM2-0010-2013*. Epub 2014/02/01 *Microbiol Spectr* 2014;2(1). <http://dx.doi.org/10.1128/microbiolspec.MGM2-0010-2013>. PubMed PMID: 26082118.
- [30] Jensen PA, Lambert LA, Iademarco MF, Ridzon R. Guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health-care settings, 2005. *MMWR Recommendations and reports : morbidity and mortality weekly report Recommendations and reports*. Centers Dis Control 2005;54(RR-17): 1–141. Epub 2005/12/31. PubMed PMID: 16382216.
- [31] Niemz A, Boyle DS. Nucleic acid testing for tuberculosis at the point-of-care in high-burden countries. *Epub* 2012/11/17 *Expert Rev Mol Diagn* 2012;12(7): 687–701. <http://dx.doi.org/10.1586/erm.12.71>. PubMed PMID: 23153237; PubMed Central PMCID: PMCPMC3740172.
- [32] Theron G, Venter R, Calligaro G, Smith L, Limberis J, Meldau R, Chanda D, Esmail A, Peter J, Dheda K. Xpert MTB/RIF results in patients with previous tuberculosis: can we distinguish true from false positive results?. *Epub* 2016/02/26 *Clin Infect Dis Off Publ Infect Dis Soc Am* 2016;62(8):995–1001. <http://dx.doi.org/10.1093/cid/civ1223>. PubMed PMID: 26908793.
- [33] Denkinger CM, Kik SV, Cirillo DM, Casenghi M, Shinnick T, Weyer K, Gilpin C, Boehme CC, Schito M, Kimerling M, Pai M. Defining the needs for next generation assays for tuberculosis. *Epub* 2015/03/15 *J Infect Dis* 2015;211(Suppl 2):S29–38. <http://dx.doi.org/10.1093/infdis/jiu821>. PubMed PMID: 25765104; PubMed Central PMCID: PMCPMC4447829.
- [34] Raviglione M, Marais B, Floyd K, Lonnroth K, Getahun H, Migliori GB, Harries AD, Nunn P, Lienhardt C, Graham S, Chakaya J, Weyer K, Cole S, Kaufmann SH, Zumla A. Scaling up interventions to achieve global tuberculosis control: progress and new developments. *Epub* 2012/05/23 *Lancet* 2012;379(9829):1902–13. [http://dx.doi.org/10.1016/s0140-6736\(12\)60727-2](http://dx.doi.org/10.1016/s0140-6736(12)60727-2). PubMed PMID: 22608339.
- [35] Wejse C. Tuberculosis elimination in the post Millennium development goals era. *Epub* 2015/03/27 *Int J Infect Dis IJID Off Publ Int Soc Infect Dis* 2015;32: 152–5. <http://dx.doi.org/10.1016/j.ijid.2014.11.020>. PubMed PMID: 25809772.
- [36] Millen SJ, Uys PW, Hargrove J, van Helden PD, Williams BG. The effect of diagnostic delays on the drop-out rate and the total delay to diagnosis of tuberculosis. *Epub* 2008/04/10 *PLoS One* 2008;3(4):e1933. <http://dx.doi.org/10.1371/journal.pone.0001933>. PubMed PMID: 18398459; PubMed Central PMCID: PMCPMC2276686.