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Integrated Nucleic Acid Testing System to Enable TB Diagnosis in Peripheral Settings

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Abstract

To facilitate treatment and limit transmission of tuberculosis (TB), new methods are needed to enable rapid and affordable diagnosis of the disease in high-burden low-resource settings. We have developed a prototype integrated nucleic acid testing device to detect *Mycobacterium tuberculosis* (*M.tb*) in sputum. The device consists of a disposable cartridge and compact, inexpensive instrument that automates pathogen lysis, nucleic acid extraction, isothermal DNA amplification and lateral flow detection. A liquefied and disinfected sputum sample is manually injected into the cartridge, and all other steps are automated, with a result provided in <1.5 h. Cell disruption and DNA extraction is executed within a four-port active valve containing a miniature bead blender (based on PureLyse® technology, Claremont BioSolutions LLC). The DNA-containing eluate is combined with dry master-mix reagents and target DNA is isothermally amplified. Amplified master-mix is then pumped into a lateral flow strip chamber for detection. The entire process is performed in a single-use closed-system cartridge to prevent amplicon carryover. For testing of *M.tb*-spiked sputum the system provided a limit of detection of 5×10^3 colony forming units (CFU)/mL. None of the negative sputum-only controls yielded a false-positive result. Testing of 45 clinical sputum specimens from TB cases and controls relative to a validated manual qPCR-based comparator method revealed a preliminary sensitivity of 90% and specificity of 96%. With further

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Author contributions

Conceptualization: HWL, RS, PK, TMF, RWD, KDH, RPT, DSB, AN; Investigation: HWL, RS, PK, TMF, KDH, RPT; Validation: HWL, KMW, FKN; Formal analysis: HWL, KMW, FKN, AN; Visualization: HWL, AN; Resources: MN; Writing - Original Draft: HWL, KMW, AN, Writing - Review & Editing: HWL, TMF, RWD, KMW, GAC, MN, DSB, AN; Supervision: RWD, GAC, MN, AN.

Conflicts of interest

RWD, TMF, KDH, and RPT each own a minority interest in Claremont BioSolutions, LLC as a result of their past or current employment at the company.

development, the herein described integrated nucleic acid testing device can enable TB diagnosis and treatment initiation in the same clinical encounter in near-patient low-resource settings of high TB burden countries.

Table of Contents Entry:

We developed a nucleic acid testing device that automates pathogen lysis, DNA extraction, isothermal DNA amplification and lateral flow detection.

Introduction

Tuberculosis (TB) is a major global public health concern, with an estimated 10 million new cases and 1.45 million deaths in 2018.¹ Approximately 1/3 of the world's population is infected with latent TB,² which can progress to active TB, most commonly as pulmonary TB³ that becomes the source of TB transmission to others. Successfully interrupting TB transmission depends on accurate and timely diagnosis, yet an estimated 3 million incident TB cases were not diagnosed and reported in 2018, and of the reported cases, only ~55% were bacteriologically confirmed.¹ When diagnosis and treatment initiation do occur, it is often long after onset of symptoms and infectiousness, which compromises the ability to effectively treat patients and curb transmission.^{4,5} Therefore, the World Health Organization (WHO) identified early detection of TB and the development of robust, accurate, and affordable point-of-care diagnostic tests as high priorities in the End TB Strategy.⁶

Diagnosis of active pulmonary TB relies mainly on sputum smear microscopy,^{7,8} which has limited sensitivity, especially in peripheral sites with limited infrastructure.^{5,9} Microbiological culture, the gold standard for TB diagnosis, requires 2–8 weeks to generate results and can only be performed in reference laboratories.¹⁰ Nucleic acid testing (NAT) enables sensitive and specific TB diagnosis and can facilitate testing and treatment initiation in the same visit.^{11,12} NAT has become an integral part of the TB diagnostic portfolio since the WHO endorsed global roll-out of the Xpert MTB/RIF assay.^{13–15} The Xpert system is a fully automated qPCR-based platform (Polymerase Chain Reaction with real time fluorescence detection) that enables TB diagnosis and rifampin resistance testing in less than two hours performed with minimal user input.¹⁴ The new version, the Xpert MTB/RIF Ultra,

features a limit of detection similar to liquid culture, and improved identification of rifampin resistance.¹⁶ However, the Xpert system is expensive despite substantial subsidies (\$17,500 for a four-module instrument, \$9.98 per cartridge, plus service contract), requires an external computer, stable uninterrupted line power, and operating temperatures $\sim 30^{\circ}\text{C}$.¹¹ The Xpert MTB/RIF test is recommended for use in district and sub-district level laboratories, but is not suitable for primary care settings that serve the majority of the affected patient population.^{5,17,18} Cepheid developed the Xpert Omni platform to enable near-patient testing, but the Omni's launch was delayed due to technical challenges and the current status of the system is unclear.^{19,20} Other NAT systems in late-stage development or on the market for TB diagnosis^{12,19,20} include smaller battery-operated qPCR-based systems such as the Truelab Uno (Molbio Diagnostics, India)^{21,22} and the Genedrive (Epistem, UK).^{23,24} A rigorous independent evaluation of the GeneDrive TB test revealed sub-optimal performance.²⁴ Furthermore, both systems are still relatively expensive ($\sim \$4,000$ – $6,000$ for a single module instrument, and $\sim \$10$ – 17 per test), and sample preparation has to be performed manually (GeneDrive) or in a separate device (TrueLab), which limits usability in peripheral sites. The instrument cost can be lowered through the use of manual assays based on isothermal nucleic acid amplification with endpoint detection, such as the Pure-LAMP assay (Eiken)^{25,26} and the EasyNAT™ TB kit (Ustar).^{27–29} However, this cost reduction comes at the expense of process integration and ease of use, which poses implementation and biosafety challenges especially in settings with low-skilled users and limited or no laboratory infrastructure. Furthermore, false positives in manual assays have been attributed to carry-over contamination.¹² These challenges can be mitigated by integrated and automated closed-system tests.

Several microfluidic NAT devices,³⁰ including paper based devices,^{31,32} have been developed for infectious disease testing. For TB diagnosis, systems have been developed that perform nucleic acid amplification and detection,³³ but not sample preparation. However, sample preparation is a significant challenge for a complex sample matrix such as sputum and a hard to lyse organism such as *M.tb*. Sample preparation coupled to electrochemical detection has been reported for the detection of HCV from whole blood,³⁴ but without nucleic acid amplification the sensitivity is limited. Systems have been reported that perform sample preparation, nucleic acid amplification, and detection, either in a semi-integrated or fully integrated form.³¹ Semi-integrated systems^{35,36} require multiple manual pipetting and other manipulation steps during process execution, and open handling of amplified master-mix poses a significant risk for carry over contamination. Some fully integrated systems^{37,38} likewise involve a series of manual steps during process execution to move liquids or the sample to different reaction zones. In one system the process is fully automated after sample addition.³⁹ However, this system only processes nasal swab samples, therefore involves minimal sample preparation, i.e. only enzymatic lysis without nucleic acid purification. Some systems are non-instrumented single use devices,^{38,39} which significantly increases the consumables cost since the instrument is integrated into the disposable. In addition, manufacturability is a challenge since early prototypes are often made via non-scalable methods.

We herein report the development and initial testing of an integrated NAT device for detecting *M.tb* in sputum, consisting of a disposable cartridge and compact, inexpensive

instrument. The cartridge is produced via industry standard scalable manufacturing methods. This system automates and integrates pathogen lysis, nucleic acid extraction, isothermal DNA amplification and lateral flow detection with no user intervention after the sample is introduced into the cartridge.

Experimental

General Reagents and Materials

LAMP oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA) while the reference qPCR method primers were purchased from Eurofins Scientific (Louisville KY). As target for the isothermal amplification reactions, we used *M.tb* genomic DNA (H37Ra) commercially available from ATCC (Manassas, VA). Betaine, bovine serum albumin, dNTPs and trehalose were obtained from Sigma Aldrich (St. Louis, MO, USA). Bst 2.0 warmstart glycerol free polymerase was obtained from New England Biolabs (Ipswich, MA, USA). *M. tuberculosis* strains H37Ra and H37Rv were obtained from American Type Culture Collection (ATCC) stocks (Manassas, VA). Sputum specimen pre-processing involved disinfection using Povidone Iodine (PVI; Rite-Aid) and liquefaction via trisodium phosphate (TSP; Sigma-Aldrich). *M.tb* DNA extraction from spiked sputum and clinical specimens was performed using slightly modified reagents contained within the commercially available PureLyse® Kit from Claremont BioSolutions (Upland, CA).

Sputum Sample Sources

Analytical studies were performed using as matrix pooled human sputum purchased from BioIVT (Westbury, NY), confirmed by us to be negative for *M.tb* complex genomic DNA via the qPCR comparator method of Halse *et al.*⁴⁰ Clinical sputum specimens were collected at the Public Health – Seattle & King County TB Clinic (Seattle, WA) from de-identified male and female subjects 18 years or older able to provide spontaneous sputum specimens. Protocols and consent forms were approved by Institutional Review Boards at the University of Washington (HSD# 48114) and Claremont Graduate University (IRB# 1435). Informed consent was obtained from all subjects, and sample collection was carried out in accordance with the approved protocol. Up to ten specimens were collected per subject, with at least eight hours between each sample. For subjects with active TB, samples were collected within seven days of treatment initiation. Of the 47 specimens included in this study, 23 were obtained from 15 subjects who were subsequently diagnosed by microbiological culture and clinical criteria as active pulmonary TB cases. Another 24 specimens came from 13 patients who visited the clinic with suspected TB but were subsequently determined by microbiological and clinical criteria to not have TB, and were not put on treatment. Individual samples with ~2 mL volume were well mixed and divided equally into paired 1 mL aliquots and then retained at -80 °C for later testing with both the integrated cartridge system and the comparator method. Some low volume specimens <2 mL (n=4 from TB+ patients and n=3 from TB- patients) were first pooled with other specimens from the same patient to exceed 2 mL, prior to division and aliquoting. All aliquots were stored in 5 mL screw-top tubes.

qPCR – Based Comparator Method:

A 1-mL aliquot of each sputum sample was first subjected to the comparative nucleic acid testing method, consisting of sputum sample preparation, followed by qPCR-based amplification and detection, as described by Halse *et al.*⁴⁰ Briefly, an equal volume of 3.5% NaOH was added to each 1 mL sputum sample. Samples were vortexed and incubated for 15 min at room temperature to liquefy and decontaminate the sputum. Decontamination was stopped by diluting the samples with PBS (pH 6.8) to a final volume of 50 mL, followed by inversion mixing. The contents were pelleted by centrifugation (15 min, 3000 x g), and supernatants were carefully removed by aspiration. Pellets were resuspended in 1 mL PBS, heated at 80 °C for 1 hr to lyse the mycobacteria, and stored on ice until qPCR was performed. We then performed qPCR-based detection of *M. tuberculosis* complex (MTBC) DNA, with internal amplification control, as described by Halse *et al.*,⁴⁰ with 0.8 mg/mL BSA added to the master-mix. This qPCR method targets the MTBC-specific insertion element IS6110 and a sequence within an engineered internal control plasmid (pIC) flanked with identical primer binding sites. The IS6110 and pIC amplicons can be differentiated with FAM and VIC labeled sequence-specific hydrolysis probes. Each sample was analyzed in two separate reactions: one with only the IS6110-FAM probe, the other with the IS6110-FAM probe, the pIC-VIC probe, and 1 fg/μL pIC. The degree of qPCR inhibition was assessed by comparing Cq values for the internal amplification control (pIC-VIC probe) relative to those in uninhibited reactions receiving water or purified genomic DNA as template. qPCR amplification and detection was executed using a StepOnePlus qPCR instrument (Applied Biosystems). Specimens from subjects with pulmonary TB were iteratively tested with the comparator method until 23 qPCR-positive samples had been identified. Samples were deemed qPCR-positive when both reactions yielded Cq values ≤ 37 (Supplementary Information Figure S6). All 24 specimens tested from Tb-negative subjects were qPCR-negative (Supplementary Information Figure S7). Significant qPCR inhibition (defined as pIC – Cq >3) was observed in three of the PCR-positive samples and none of the qPCR-negative samples.

Preparation of quantified M.tb H37Ra stock solution:

Quantified M.tb H37Ra stock solution that was used for the analytical spiking experiments was prepared as follows: from the primary glycerol stock, single colonies of H37Ra were grown on 7H9 agar media enriched with OADC (7H9 Agar). Next, 5mL 7H9 broth media supplemented with OADC (7H9 broth) was inoculated with a single colony of H37Ra and the culture was incubated at 37°C until the growth reached an optical density (OD) at 600nm of 0.6 – 0.8. Then 100μl of the culture was inoculated into 10mL 7H9 broth for a second passage. After the culture reached 0.6 – 0.8 OD, it was centrifuged for 15 min at 3500 rpm at 4°C and re-suspended in 5mL 7H9 broth. To de-clump the mycobacterial cells, this resuspension step was performed by vortexing for 1min and then placing the culture on ice for 6 min as described by Blakemore *et al.*⁴¹ To quantify the mycobacteria, 1mL of the culture suspension was serially diluted and 100μl of the serial dilutions were plated on 7H9 agar. The remaining culture suspension in 100μl aliquots were stored in cryovials at –80°C until further use. The concentration in CFU/mL of the culture suspension was determined by the number of colonies obtained on the 7H9 agar plates from serial dilutions. Before each

spiking experiment, an aliquot of the stock culture suspension was thawed and serially diluted in 7H9 broth to obtain the required dilution for spiking into the sputum sample.

Cartridge Fabrication

The cartridge consists of three key components: i) A cartridge body made of polycarbonate contains the majority of fluidic channels and reagent reservoirs. ii) The key component for sample preparation is a four-port valve (termed the OmniValve) made of HDPE, coupled to a micromotor-based bead blender with the same internal geometry as Claremont BioSolutions' commercial OmniLyse device. iii) A polypropylene reaction insert is the key component for DNA amplification coupled to downstream lateral flow detection. The cartridge body, reaction insert, and OmniValve were produced via injection molding by Leardon Solutions (San Diego, CA). The OmniValve body, which contained 420mg of specialty ceramic beads obtained from Claremont BioSolutions for pathogen lysis and DNA binding, was inserted and secured into the cartridge body. Four hydrophobic barriers were then press-fitted onto the four electrolytic pump (ePump) chambers, and a layer of 125 μ m thick polycarbonate film was thermally bonded on the top and bottom surfaces of the cartridge body to close the fluidic chambers and channels. A PTFE membrane with polypropylene backing was thermally bonded onto a small chamber of the cartridge body upstream of the inlet boss to the reaction insert to facilitate venting of air in the fluidic channels,⁴² and a female luer fitting was cemented to the sample input port using epoxy. A lateral flow strip was inserted in a chamber within the cartridge body downstream of the reaction insert outlet boss, pressed down by two 1mm x 1mm polystyrene strips for pressure points, and sealed in the chamber through a thermally bonded 125 μ m thick polycarbonate film.

Next, the reagents were added into the cartridge: 80 μ L 1M sodium sulfate electrolyte was pipetted into three of the four ePump chambers, while the larger ePump chamber connected to the elution buffer reservoir received 200 μ L. The ePump chambers were then closed via custom silicone septa into which two 13 mm long nickel-plated pins were inserted to act as electrodes. Four mL of a 1:5 dilution of the supplied 1X PureLyse® wash buffer was injected into the wash chamber, while 275 μ L of the PureLyse® elution buffer supplied with the PureLyse® Kit (Claremont BioSolutions) were injected into the elution chamber, in each case by piercing through the polycarbonate film covering the chambers and then resealing the hole with epoxy. The polypropylene reaction insert was assembled by inserting custom check valves⁴³ into the inlet and outlet conduits, placing two reagent pads containing lyophilized master mix components (described below) into the dome-shaped reaction chamber on the underside of the insert body, and then sealing the circular reaction chamber by thermally bonding a polypropylene film to the bottom of the insert body. A tear shaped pump pouch covering the reaction chamber and the bottom opening of the pump conduit was formed by thermally bonding another polypropylene film to the bottom of the reaction insert, and then filling the pump pouch with silicone oil (viscosity 5cSt). The fully assembled reaction insert was then attached to the cartridge body using three Plastite screws, with a polyurethane insulation foam in-between the reaction insert and cartridge body.

Instrument Fabrication and Characterization

Three identical instruments were fabricated and tested to ensure equivalent performance during the studies reported herein. The instruments contained a spring-loaded holder that pivots open to receive the cartridge. Upon cartridge insertion, a coupler interfaces the OmniValve in the cartridge to a stepper motor in the instrument, the electrodes in the cartridge connect with corresponding instrument connectors, and the bottom of the reaction insert interfaces intimately with the heater within the instrument. An Arduino Mega microcontroller board plus additional custom printed circuit board assemblies were used to control stepper motor rotation, assay timing, the current levels for the ePumps, the voltage level for the blender motor, the heater temperature, and the user interface which consisted of an alpha-numeric LCD display and two buttons.

The thermal control subsystem of each instrument was calibrated by inserting a thermocouple into the reaction pouch of a cartridge filled with reaction buffer. Another thermocouple was attached to the heater to simultaneously measure the heater temperature. The instrument was then turned on to allow the heater to warm to the set-point of the thermal control circuit. The heater set-point was adjusted via on-board electronics to achieve a temperature of 62.0 ± 1.0 °C within the reaction pouch. The procedure was repeated with 3 different cartridges to ensure consistent thermal control across different test runs. The instrument achieved an initial ramp rate of 12.2 °C/min. Starting from room temperature, it reached the required temperature range within 6.1 min, with no overshoot. The system maintained a temperature of 62.2 ± 0.3 °C for the duration of the run (Supplementary Information Figure S3).

To verify that the bead blender inside the OmniValve had the necessary power to achieve similar lysis performance as the PureLyse device used in prior studies,^{44,45} the OmniValve chamber was filled with water or synthetic sputum to simulate different loading conditions. Power was applied to the blender motor via the instrument electronics, and we then measured the attainable voltage drop across the motor. Independently, we determined the motor's speed of revolution based on the acoustic signature of the motor operating at different voltages.

We empirically determined the current settings that had to be applied by the instrument to specific ePumps inside the cartridge to control the required flow rates at each steps of the assay execution. Initial optimization was performed using synthetic sputum as input samples. We then verified proper fluidic functionality of the final execution protocol with multiple replicate cartridges using disinfected and liquefied human sputum.

Upstream Sputum Disinfection and Liquefaction

The sample preparation approach incorporated into the cartridge entails a modified version of our previously reported semi-automated process for DNA extraction from sputum to enable *M.tb* detection.^{44,45} In brief, sputum is disinfected and liquefied outside of the cartridge, followed by mechanical lysis and solid-phase nucleic acid extraction inside the cartridge. Prior to performing the sputum disinfection and liquefaction steps, the standard 8X PureLyse® Binding Buffer supplied within the PureLyse® Kit was diluted using a

solution containing EDTA and tris(2-carboxyethyl)phosphine (TCEP) to yield a final concentration of 20 mM EDTA and 50 mM TCEP in 3X binding buffer, which was then adjusted to pH 2.65 using concentrated HCl. For sputum disinfection and liquefaction, a 1 mL sputum specimen was treated with 329 μ L PVI for 7.5 minutes, followed by liquefaction through adding and mixing in 40 mg TSP. After adding 1.429 mL of the modified 3X PureLyse® Binding Buffer to the sputum mixture, 80 mg of glycine HCL was added and mixed thoroughly in order to adjust the pH to 3.5 – 4.0. The entire volume (~2.8 mL) was then pulled through a custom pre-column (~15 mm x 6 mm diameter) with luer fittings and loosely packed with 670 mg of a dry clean-up bead matrix (Claremont BioSolutions) into a 3 mL syringe, along with extra headspace above the liquid phase. This pre-processed sample was then manually introduced into the cartridge as described below.

Lysis and DNA Extraction in the Cartridge

The user turns on the instrument, inserts a cartridge, and then presses a button to initiate the cartridge check, wherein the instrument verifies that the ePump connections and blender motor are functional. See Supplementary Information Figure S1 for a visualization of the following process. The user attaches the syringe containing the liquefied and disinfected sputum sample to the cartridge luer adapter, and injects ~250 μ L of the processed sample to fill the OmniValve. Next, the user initiates the assay execution protocol and injects the rest of the sample at <0.5 mL/minute into the cartridge. The sample passes through the OmniValve to the waste chamber, while the blender motor is activated at 6.5 V, which lyses the bacteria and allows for DNA binding to the beads inside the OmniValve. After the entire content of the syringe is injected into the cartridge, the user presses a button to signify completion of sample input and the instrument takes over the process. The OmniValve is rotated to connect the PureLyse® device with the wash and waste chambers. The ePump connected to the wash chamber then pumps the wash buffer through the PureLyse® device at ~1 mL/minute, with intermittent blender motor activation at 1.5 V. After the electrolytic pump empties the wash chamber and purges the PureLyse® chamber of wash buffer, the OmniValve rotates again to connect the elution chamber to the holding chamber #1. The ePump connected to the elution chamber pumps the elution buffer to fill the PureLyse® device, the blender motor is activated intermittently at 6.5 V to expel trapped bubbles from the chamber, and then the cartridge incubates the beads with elution buffer for 35 seconds with no pumping or blending. After incubation, the ePump and blender are activated again to pump all eluate to holding chamber #1. The OmniValve turns again to connect the holding chamber #1 to holding chamber #2, and the ePump connected to holding chamber #1 then pushes all eluate into holding chamber #2. To allow eluate to enter the reaction insert for isothermal amplification, this ePump generates sufficient pressure to reach the opening pressure of the check valve inserted into the reaction chamber inlet port downstream of the holding chamber #2.

The venting chamber is covered by a thermally bonded porous PTFE membrane (0.3 μ m pore size) with PP laminated backing (Sterlitech, PTFE23005). Thermal bonding is performed with the PP laminated membrane facing outward from the chamber, providing structural support against internal pressure. As the buffer advances toward the inlet of the reaction chamber, the air in the channel vents out through the hydrophobic membrane until the buffer

fills the chamber. The hydrophobic pores contain the aqueous buffer in the chamber to build up internal pressure until the inlet valve opens and allow the buffer to enter the reaction chamber. The gas generated by the ePump chases the remaining buffer stream into the reaction chamber until the gas reaches the PTFE membrane and vents out of the hydrophobic pores to relieve the internal pressure, which closes the inlet valve.

Isothermal Amplification in the Cartridge

Isothermal nucleic acid amplification inside the cartridge was based on Loop Mediated Amplification (LAMP) targeting a conserved region in the *M.tb gyrB* gene, using primer sequences reported by Iwamoto *et al.*⁴⁶ To enable the detection of LAMP amplicons via lateral flow, the backward inner primer (BIP) was 5'-labeled with digoxigenin, and the forward loop primer (FLP) was biotinylated at the 5' end.

To facilitate thermostable master-mix reagent storage within the cartridge, we freeze dried concentrated master-mix with suitable excipients onto pre-treated paper pads. CF1 100% cotton linter sample pads (176 μm thick, GE Lifesciences, Marlborough, MA) were cut into 9.6 mm diameter circles, then pre-treated with 15 μL of a solution containing 70 mM Tris-HCl, pH 8.0, 2.33 M betaine, 2.67 mg/mL bovine serum albumin (BSA) and 20 % trehalose, and allowed to air-dry in a desiccator for 4h. Concentrated *Mtb* LAMP mastermix was prepared in two parts to prepare primer papers and enzyme papers, respectively. In each case, 12.5 μL of concentrated part 1 or part 2 master-mix was dispensed onto pre-treated paper pads, which were then frozen at -80°C for 1 hr before freeze drying using a Labconco 6 Liter Console Freeze Dry System for 4 hr at < 200 mbar. The freeze-dried reagent papers were stored at -20°C in airtight aluminium pouches with desiccant and oxygen scavenger bags until further use.

Part 1 master-mix was prepared such that after reconstitution in 160 μL it yielded final 1x concentrations of 400 μM of each dNTP, 2.4 μM FIP, 1.6 μM DIG-labeled BIP, 0.2 μM of F3 and B3, 0.4 μM biotinylated-LF, and 0.8 μM LB. The Part 2 master-mix was prepared such that after reconstitution in 160 μL it yielded a final 1x concentration of 0.3 U/ μL of Bst 2.0 warmstart glycerol free DNA polymerase. Of the 10x isothermal amplification buffer (IAB), 1/3 was included in the primer paper, while 2/3 was included in the enzyme paper. The final 1x IAB concentration was 20 mM Tris-HCl pH 9.2, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM KCl, 2 mM MgSO_4 , and 0.1.% Triton X-100.

One primer and one enzyme pad were incorporated into the reaction pouch of the cartridge during assembly. Following sample preparation in the cartridge, the eluate was pumped into the reaction pouch where it reconstituted the master mix from the dried reagent pads. The heater below the reaction insert then heated the liquid to $62 \pm 1^{\circ}\text{C}$ and maintained that constant temperature for 60 minutes to carry out the isothermal amplification reaction. After the incubation, the heater turned off and the electrolytic pump connected to the pump pouch was activated. This inflated the pump pouch, and the outlet check valve opened when the pressure within the reaction insert reached 7–9 PSI, allowing the amplified product to enter the lateral flow strip chamber.

Lateral Flow Based Detection in the Cartridge

Nucleic acid lateral flow (NALF) strips (Supplementary Information Figure S2) for detecting *M.tb* LAMP amplicons were fabricated in-house according to Roskos *et al.*⁴⁷ using an ALFRD lateral flow reagent dispenser (Claremont BioSolutions). In brief, oligo d(T)90 and neutravidin were conjugated to red-dyed carboxylate modified polystyrene microspheres (Bangs Laboratories Inc., Fishers, IN), which were then dispensed and dried onto a pre-treated NALF conjugate pad. To detect the LAMP amplicon, an anti-DIG antibody was dispensed onto a nitrocellulose membrane as the test line. To detect proper conjugate release and LF strip performance, a biotinylated d(A)30 oligonucleotide coupled to neutravidin was dispensed onto the nitrocellulose membrane as a control line. This nitrocellulose membrane, the treated conjugate pad, and a cellulose fiber absorbent pad were then mounted onto an adhesive plastic backing, with a 1–2 mm overlap of the conjugate and absorbent pads with the nitrocellulose membrane. In this study, an additional glass fiber overhang was added to the bottom of the lateral flow strip to promote the introduction of amplified master-mix onto the strip.

In the cartridge, amplified master-mix was pumped from the reaction insert into a chamber containing the lateral flow strip, which then absorbed the amplified product and produced the visual readout after 10-minutes of development. Images of the developed lateral flow strip in the cartridge were taken with a Panasonic Lumix® DMC-FZ47 camera (12.1MP) under uniform lighting.

The signal intensity of the test line was then analysed using the software suite imageJ.⁴⁸ Briefly, each image was cropped to obtain consistent registration features and sizes. Two regions of interest were defined: one region at the test line to signify presence or absence of *M.tb*, and a second separate region just upstream of the test line to obtain the background intensity. Presence of a red test line relative to background was determined based on a decrease in the imageJ green channel intensity, (i.e. less green light was reflected at the test line due to absorbance by the micro-particles on the test strip, which makes the line appear red). A common cut-off value was used to differentiate positive from negative results.

Limit of Detection in the Integrated Cartridge and Instrument

Using our system, we tested sputum spiked with *M.tb* H37Ra at concentrations ranging from 10⁵ CFU/mL to 10³ CFU/mL with 6–11 replicates per concentration plus negative sputum (10 replicates). H37Ra quantified stock was diluted such that the desired concentration of H37Ra was obtained by introducing 5 µL of the diluted stock to 1mL of pooled *M.tb* DNA negative human sputum from BioIVT. Negative sputum samples were spiked with 5 µL 7H9 broth. One mL of the respective samples was processed in the cartridge and device as previously discussed. Result were interpreted as positive or negative based on the lateral flow strip readout, analysed via ImageJ. The Limit of Detection (LOD) was determined via Probit analysis of the data using Microsoft Excel.

Evaluation of Clinical Sputum Samples in the Integrated Cartridge and Instrument

Aliquots (1 mL) from the 23 qPCR-positive and 24 qPCR-negative clinical sputum specimens collected at the Seattle King County TB clinic were tested with the cartridge and

device as described above. Two qPCR-positive samples could not be drawn through the pre-column and were therefore not analysed and excluded from the comparison. Both of these samples were among the TB qPCR-positive samples that exhibited qPCR inhibition in the reference method internal control reaction. One qPCR-positive sample that was analysed required the use of a second pre-column to pass the entire sample. Otherwise, the process execution and result analysis was identical to the protocol used for the LOD study.

Results and Discussion

We developed a prototype integrated cartridge (Figure 1A-C), approximately the size of a mobile phone, with an associated compact instrument (Figure 1D). In < 1.5h, this system can execute sputum sample preparation, isothermal DNA amplification and lateral flow-based detection in a fully automated manner, following introduction of a liquefied and disinfected sputum sample into the cartridge.

System Design and Operating Concept

The cartridge (Figure 1A-C) consists of three main components: (A) the cartridge body, (B) the OmniValve, and (C) the reaction insert. The cartridge body contains wells and channels at the top and bottom surfaces, in addition to the lateral flow strip chamber and four miniaturized electrolytic pumps (ePumps).^{42,47} Each ePump consists of a small chamber filled with electrolyte, contains electrode contacts at the bottom, and is capped with a hydrophobic barrier to separate the electrolyte from downstream reaction fluids. The OmniValve / blender assembly that is inserted into the body automates PureLyse® sample preparation.⁴⁵ The reaction insert attached to the cartridge body enables isothermal amplification coupled to lateral flow detection.^{42,47}

The process execution and operating principle of the cartridge is illustrated in Figure 2. The manual sputum pre-processing steps (Figure 2A) entail sample disinfection and liquefaction, followed by removal of debris and some inhibitors by pulling the sample through a pre-column. This sample is then injected into the cartridge inside the instrument. The rest of the process is automated as described in Figure 2B and C, with further information provided in Supplementary Information Figure S1. Following the manual sample input, all liquid handling steps are executed via ePumps connected to the wash and elution buffer reservoirs, to holding chamber #1, and to the pump pouch. These ePumps enable fluidic control in a simple miniaturized format, based on water electrolysis. Applying a constant current to electrodes inserted into electrolyte generates gas, which displaces liquid downstream at a constant flow rate proportional to the current setting. This enables pumping with no moving parts.

Pathogen lysis and DNA extraction is performed in the OmniValve, which contains ceramic beads retained by filters in the 6 ports inside the valve. These ports are uniquely staggered, with sets of holes spaced 90° apart, to direct flow through 3 channels each on the top and bottom surfaces of the cartridge. This enables flow through the OmniValve from the sample inlet port to waste, with concomitant lysis of bacteria and binding of DNA to the beads. Next, wash buffer is pumped through the OmniValve to waste, to remove impurities and inhibitors. Finally, elution buffer is pumped through the OmniValve to holding chamber #1,

and then from holding chamber #1 through the OmniValve to holding chamber #2, to elute the purified DNA.

Isothermal amplification occurs in the polypropylene reaction insert, which is attached to the cartridge body, with insulation foam sandwiched between the two pieces. A reaction pouch enclosed by a slightly larger pump pouch is formed at the bottom of the insert, through thermally bonding two layers of polypropylene film to the insert.^{42,47} The reaction pouch contains the dry LAMP master-mix reagent pads. Implementing the LAMP master-mix in dry reagent form on pads facilitates reagent integration into the cartridge as well as reagent storage without refrigeration. In a five-week thermo-stability study we have demonstrated that the master-mix pads remain stable upon storage at room temperature and at 40 °C (manuscript in preparation).

The reaction pouch can be accessed through an inlet and an outlet port, which contain miniature, inexpensive check valves⁴³ in anti-parallel orientation to control unidirectional flow. The pump pouch that overlays the reaction pouch is filled with thermally conductive fluid to facilitate heat transfer to the reaction chamber,⁴⁷ and is connected to the fourth ePump. During process execution, eluate containing target DNA is pushed by ePump 3 from holding chamber #2, past a venting barrier to remove dead air, and through the inlet valve into the reaction pouch. At this point, the heater in the instrument ramps up to the specified temperature (62 ± 1 °C), which initiates isothermal DNA amplification. Once the reaction is completed, current is applied to the fourth ePump, which pressurizes and inflates the pump pouch, pumping the amplified master-mix within the reaction pouch through the outlet valve and onto the lateral flow strip in an enclosed chamber at the top of the cartridge.

The lateral flow strip was ready for interpretation 10 minutes after introducing the amplified master-mix into the lateral flow strip chamber. The front line indicates the presence of LAMP amplicon, while the back line determines proper functioning of the lateral flow strip. To minimize risk of subjective sample interpretation, we quantified the test line intensity using the image processing software ImageJ,⁴⁸ with a common threshold intensity above which the result was considered to be positive.

Limit of Detection for Spiked Sputum Samples

We measured the limit of detection (LOD) for the integrated cartridge and instrument using sputum samples that were determined to be *M.tb* DNA negative, and that were spiked with known amounts of *M.tb* H37Ra cells. Samples with spiked *M.tb* concentrations ranging from 10^5 CFU/mL to 10^3 CFU/mL resulted in hit rates (percent positives) from 100% to 73%, based on the lateral flow strip readout (Table 1). None of the negative control sputum samples yielded a positive result. We then calculated the limit of detection (LOD), i.e. the concentration expected to provide a 95% hit rate, via nonlinear Probit regression analysis (Figure 3). The LOD was determined to be $\sim 5 \times 10^3$ CFU/mL.

Testing of Clinical Sputum Samples

We tested 45 clinical sputum specimens from TB cases and controls using the integrated cartridge, relative to a validated qPCR based reference method,⁴⁰ which has been used by the Wadsworth Center / New York Department of Public Health. From this study we

obtained a positive percent agreement (preliminary sensitivity) of 90.5% (19/21, 95% CI: 68.2–98.3%) and a negative percent agreement (preliminary specificity) of 95.8% (23/24, 95% CI: 76.9–99.8%) (Table 2).

We did not perform sputum smear microscopy and mycobacterial culture as reference methods, and therefore cannot disaggregate based on smear / culture positive or negative. However, for the samples considered positive, the qPCR-based comparator method resulted in Cq values ranging from 22.1 to 36.2. Therefore, this study included samples with a very low bacterial load. The two false negative results obtained via our new method had Cq values >36 in the comparator method. The cut-off for being considered positive in the comparator method is 37, therefore these two samples likely had very low bacterial load. The single false positive result (**FP** in Figure 4A) showed a test line of lower intensity than all true positive samples, but fell above our arbitrarily pre-selected threshold intensity (Figure 4B). However, based on visual interpretation this would likely be considered a positive result, and thus we left it as a false positive. In future work we plan to incorporate a lateral flow strip reader into the device to ensure consistent objective result interpretation.

Two TB-positive clinical samples were excluded from this study because of difficulties in the upstream sputum liquefaction and disinfection step. Viscous and particulate-laden sputum samples were difficult to process through the pre-column, and may lead to clogging, therefore further optimization of the pre-processing step is required.

Conclusions

The herein reported prototype device enables detection of *M.tb* in sputum via integrated and automated sample preparation, amplification, and detection. The current LOD of the system for detecting *M.tb* H37Ra spiked into sputum is 5×10^3 CFU/mL. Sputum smear microscopy has an LOD of $10^4 - 10^5$ bacilli/ mL,⁴⁹ thus our current system is expected to be more sensitive than sputum smear microscopy. However, further optimization of the LOD is required, since our device is not as sensitive as the Xpert MTB/RIF, which has an LOD of 131 CFU/mL for the initial version,⁵⁰ and 15.6 CFU/mL for the Ultra version.¹⁶ If similar sensitivities are achieved with our device, it will have advantages compared to the Xpert with respect to instrument cost, robustness, and power consumption. The cost of all components for the current prototype instrument is ~\$500 (Supplementary information Table 1), and we estimate that upon further refinement and scale up the instrument's cost of goods and manufacturing will be ~\$200. The cost of the cartridge at the current production volume is ~\$25, including the master-mix, lateral flow strip, and other reagents. We anticipate that the cartridge cost will be reduced to <\$10 through component and manufacturing refinements, and upon scale-up. We will improve system robustness e.g. by refining the reagent integration into the cartridge. The instrument does not require an external computer to execute the process. Pumping, heating, and detection is accomplished with simple, robust components (e.g. no thermo-cycler or fluorescence optics) and the system is expected to be maintenance-free. The instrument uses low-power components to allow for eventual battery-powered operation in the field. To ensure unambiguous result interpretation we plan to incorporate a lateral flow strip reader into the device, and we can include wireless connectivity to support the requirements for connected diagnostics.

To facilitate ease of use and further mitigate biohazard risk, we aim to develop a closed-system sample pre-processing device that streamlines the manual sputum disinfection and liquefaction steps. In addition, we anticipate that future iterations of the cartridge and instrument will be able to accommodate different sample matrices such as blood, saliva, nasopharyngeal, oral^{51,52} and urogenital swab samples, and urine samples. We can incorporate RT-LAMP into the system to detect viruses such as SARS-CoV2. Likewise, different isothermal DNA amplification assays coupled to NALF detection can be accommodated in this device, which may be faster than LAMP⁵³ and can therefore shorten the time to result. We envision that the system will evolve into a low-cost and robust platform technology that can detect different pathogens using the same device but different pathogen-specific test cartridges in a format suitable for use in near-patient low-resource settings. In addition, this system has the potential to facilitate rapid on-site detection of COVID-19.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Integrated cartridge, displaying the readout of the final test result after process execution. Cartridge body with (1) sample input port, (2) waste chamber, (3) wash buffer chamber, (4) elution buffer chamber, (5) holding chamber 1, (6) holding chamber 2, (7) venting chamber, (8) lateral flow strip chamber, and (9) electrolytic pumps (ePumps). (B) OmniValve (1) body and (2) sleeve in the cartridge, (C) (1) reaction insert with (2) inlet and (3) outlet ports, which contain (4) passive check valves. The reaction chamber (5) at the bottom contains the dry LAMP master-mix reagent pads, and is enclosed by an inflatable

pump pouch. (6) Insulation foam. (D) Integrated instrument: (1) Hinged lid (partially open) for cartridge insertion, with rectangular viewing window of the lateral flow strip, (2) cartridge inserted into the lid, (3) instrument case containing electronics, heater, etc., (4) stepper motor and coupler connecting to the OmniValve, (5) LCD screen and (6) push buttons providing the user interface.

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Figure 2.

(A) Overview of pre-processing steps: sputum sample is disinfected with Povidone Iodine (PVI) for 7.5 min, followed by addition of Tri-Sodium Phosphate (TSP) to liquefy the sample, which is diluted in 3X PureLyse® Binding Buffer plus glycine HCL. The sample is then pulled through a pre-column and injected into the cartridge. (B) Diagram of the integrated cartridge. (C) Process execution steps performed inside the cartridge.

Figure 3. Non-linear Probit analysis to determine the LOD. Open squares: Experimental data points, solid circle: calculated log(LOD) (i.e. log(conc) yielding a 95% hit rate).

Figure 4.

Clinical study results: (A) Images of lateral flow strips in the cartridge with test (T) and control (C) lines, showing the results for three representative true positives (TP), two true negative (TN) and the single false positive (FP) that was obtained in this study. The numbers below the lateral flow strips correspond to the line intensities determined via ImageJ. (B) Line intensities for all positives (P) and negatives (N), showing two false negative and one false positive result, given a threshold intensity of 20.

Table 1:Detection of *M.tb* H37Ra in spiked sputum

<i>M.tb</i> concentration [CFU/mL sputum]	10 ⁵	10 ⁴	3*10 ³	10 ³	0
positives / total tested	6/6	10/10	9/10	8/11	0/10
Hit Rate	100%	100%	90%	73%	0%

Table 2:

Testing of clinical sputum samples from TB patients and controls using the integrated cartridge and instrument as new method, and as gold standard a validated qPCR-based method.⁴⁰

		Validated qPCR based method		
		Total	Positive	Negative
		45	21	24
New Method	Positive	20	19	1
	Negative	25	2	23