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Solid-Phase Microextraction of DNA from Mycobacteria in Artificial Sputum Samples To Enable Visual Detection Using Isothermal Amplification

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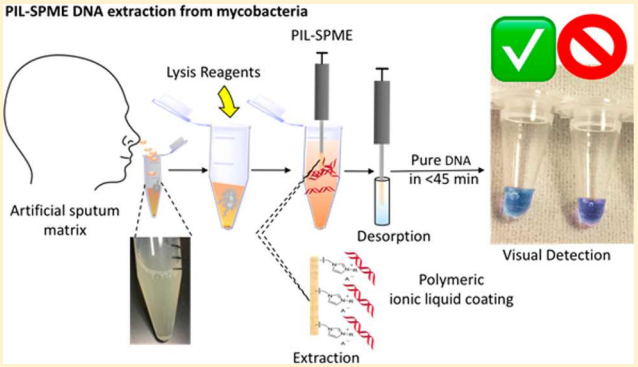
Supporting Information

ABSTRACT: Point-of-care (POC) technologies for the detection of pathogens in clinical samples are highly valued due to their speed, ease of use, and cost-effectiveness. Furthermore, they are ideally suited for resource-limited settings where expensive and sophisticated laboratory equipment may not be readily available. In this study, a rapid method based on solid-phase microextraction (SPME) of mycobacterial DNA with subsequent isothermal amplification and visual detection was developed. Direct coupling of the SPME desorption solution (1 M NaCl) to the isothermal reaction system was achieved to circumvent dilution steps and improve detection limits. Using this method, DNA was preconcentrated from lysed mycobacteria in just 2 min, subjected to isothermal multiple-self-matching-initiated amplification (IMSA), and the amplicons were detected visually. With a total analysis times of less than 2 h, the optimized method was capable of extracting and visually detecting mycobacterial DNA from artificial sputum samples containing clinically relevant concentrations of mycobacteria (10^7 colony forming units/mL), demonstrating its potential for future POC applications.



The rapid and sensitive detection of pathogens in clinical samples is important for prompt diagnosis and administration of the most effective treatment.[1] Traditional microbiological methods for pathogen identification include selective culture, immunoassays,[2,3] and nucleic acid amplification-based methods.[4,5] While culture-based methods are often considered the gold standard owing to their high degree of accuracy, they are incompatible with point-of-care (POC) applications due to the extensive time that is often required for cultures to proliferate (e.g., 2 weeks for *Mycobacterium tuberculosis*).[1,6] POC methods for the detection of pathogens are highly valuable as they provide rapid results and can be used without extensive training.[7] Although immunoassays are a promising alternative to culture-based approaches, the development of pathogen-specific antibodies is a time-consuming (up to 3 months)[8] and expensive process. Nucleic acid amplification-based methods are less expensive alternatives that still maintain high sensitivity and specificity for their targets, making them ideally suited for POC applications.

Popular techniques that exploit the rich information in nucleic acids through the amplification of pathogen specific sequences include polymerase chain reaction (PCR)[9] and real-time quantitative PCR (qPCR).[10] Recently developed isothermal nucleic acid amplification (INAA) techniques such as loop-mediated isothermal amplification (LAMP),[11] isothermal multiple-self-matching-initiated amplification (IMSA),[12] and recombinase polymerase amplification (RPA)[13] show incredible promise for POC applications due to their lack of dependence on sophisticated thermal cycling equipment and their compatibility with visual detection methods. However, one core challenge shared by these methods is the need to isolate highly pure nucleic acid samples that are devoid of polymerase inhibitors in order to achieve optimal performance.[14] This need is emphasized when DNA must be isolated from complex sample matrixes such as blood or sputum that contain a variety of components that hinder enzymatic amplification. Therefore, sample preparation methods that can rapidly extract and purify nucleic acids from interfering agents and be subsequently interfaced with existing nucleic acid amplification technologies are highly desirable for POC applications.

Conventional methods for nucleic acid purification include phenol–chloroform liquid–liquid extraction (LLE)[15] and silica-based solid-phase extraction (SPE).[16] LLE is a time-consuming process that relies on the partitioning of nucleic acids between two immiscible phases. However, the requisite multiple centrifugation steps and the use of organic solvents make this technique unsuitable for POC applications. Silica-based SPE is another commonly used method for the extraction and purification of nucleic acids and relies on the reversible binding of the nucleic acid to the sorbent phase. Using a

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chaotropic salt such as guanidine hydrochloride, the nucleic acid is dehydrated and subsequently adsorbed onto silica.[17] Following multiple washing steps to remove unwanted compounds such as proteins and lipids, the nucleic acid is eluted with a low ionic strength solution. This technique is faster and uses less organic solvents than LLE. However, SPE requires significant user intervention, multiple centrifugation steps, and its reuse is not recommended making it incompatible with POC diagnostics.

Solid-phase microextraction (SPME), developed by Arthur and Pawliszyn in 1990,[18] circumvents many of the aforementioned issues associated with both LLE and SPE. SPME involves the immobilization of a thin sorbent layer on a solid support and has been exploited in the extraction of a wide range of compounds including acrylamide in coffee,[19] antifungal medication in plasma,[20] and metabolites in vivo.[21] This solventless technique does not require centrifugation and overcomes many shortcomings of traditional LLE and SPE approaches.

Recently, our group[22,23] and others[24] have explored the use of polymeric ionic liquids (PILs) as selective phases for the extraction of nucleic acids. PILs are a subclass of ionic liquids that contain polymerizable moieties in the cation and/or anion structures. Our group first applied PILs as SPME coatings for the extraction of nucleic acids from aqueous samples by copolymerizing the PIL monomer and cross-linker with the fiber support, thereby creating a robust and selective extraction phase. Electrostatic interactions of the cationic imidazolium moiety of the PIL with the negatively charged phosphate groups of nucleic acids and ion exchange were identified as the two main forces driving the extraction of nucleic acids by the PIL.[22,23] These methods showed that PILs were suitable for the extraction of nucleic acids from aqueous samples. The reusability, robustness, and effectiveness of PIL-SPME make it an attractive sample preparation tool for the purification of nucleic acids in POC applications.

There currently exists one World Health Organization (WHO)-endorsed method for the detection of *M. tuberculosis* from sputum samples at the POC. This utilizes PCR and molecular beacon probes to detect tuberculosis and test for drug resistance.[25] Although this method has been used extensively for the detection of mycobacteria from infected patients, the need for an uninterrupted power supply, low ambient temperatures (<30 °C), and annual calibration demonstrate a need for more robust methods for the detection of mycobacteria in resource-limited settings.[26,27]

In this study, a rapid and sensitive method was developed for the isolation and visual detection of genomic DNA from mycobacteria inoculated in artificial sputum media (ASM) by PIL-SPME coupled with IMSA. This is the first reported use of IMSA for the amplification of mycobacterial genomic DNA. By using vortex agitation instead of stirring, the optimized method provided a 15-fold shorter extraction time compared to a previously reported DNA extraction method using PIL-SPME.[22] Furthermore, an IMSA reaction using hydroxynaphthol blue (HNB) for visual detection was optimized and directly coupled to PIL-SPME in an approach that circumvents dilution of the desorption solution to maximize sensitivity. We demonstrate the potential applicability of the method at the POC by extracting genomic DNA from *Mycobacterium smegmatis* spiked in artificial sputum media (ASM). The concentration of *M. smegmatis* was selected to simulate clinically relevant concentrations of mycobacteria in the sputum of individuals with active infections (10^7 cfu/mL).[28] The method is capable of extracting, amplifying, and subsequently visually detecting mycobacterial DNA in less than 2 h. Other commonly used sample preparation methods for the extraction of mycobacterial DNA were compared with the PIL-SPME method and found to be slower while also requiring multiple centrifugation steps that are typically incompatible for POC applications.

EXPERIMENTAL SECTION

Preparation of DNA. A modified 3.9 kbp plasmid from Eurofin Genomics (Louisville, KY, U.S.A.) containing a 280 bp insert was amplified by PCR using the following primers: 5′-GGA TGT GTC TGC GGC GTT TT-3′ and 5′-GAG GCC CAC TCC CAT AGG TT-3′. Following amplification, agarose gel electrophoresis was performed using a Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.) horizontal gel electrophoresis system H4 chamber with a Neo/Sci (Rochester, NY, U.S.A.) dual-output power supply. The amplicon band was excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To quantify the amount of DNA recovered, a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.) was used. A standard solution of 1 ng μL^{-1} (3.48×10^9 copies) was then serially diluted, and the dilutions were stored at −20.0 °C. For the extraction of DNA from *M. smegmatis* MC^2 155 (ATCC, Manassas, VA, U.S.A.), a QiaAmp DNA mini kit was used according to the manufacturer's instructions. The recovered DNA was quantified with a Nanodrop spectrophotometer. A standard solution of 1.3 ng μL^{-1} was serially diluted (from 130 to 0.13 pg μL^{-1}) and stored in −20.0 °C.

qPCR Assays and Conditions. Quantification of DNA during the method development process was performed using qPCR on a CFX96 Touch real-time PCR detection system from Bio-Rad Laboratories (Hercules, CA). The following amplification conditions were used: an initial denaturation step of 3 min at 95.0 °C, followed by 40 cycles of 10 s at 95.0 °C and 30 s at 58.0 °C. All reactions were performed in triplicate. The following volumes of reagents were used for each reaction: 1 μL of DNA template solution, 10 μL (2×) SsoAdvanced Universal SYBR green supermix (Bio-Rad laboratories), 8.2 μL of deionized water, and 0.8 μL of 10 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA, U.S.A.). To quantify the amount of DNA extracted by the SPME fibers, an external five-point calibration curve was prepared (10-fold dilutions, from 1 to 1×10^{-4} pg) with 10 mM NaCl in the reaction mixture, as shown in Figure S1 of the Supporting Information. The amplification efficiency was calculated using eq 1 and found to be 110%, within the acceptable range according to The Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines.[29] For reference, an amplification efficiency of 100% indicates that the amount of PCR product generated doubles with each cycle.

$$\text{efficiency} = [10^{-1/\text{slope}} - 1] \times 100 \quad (1)$$

HNB-IMSA Assays. HNB-IMSA assays for 16S rRNA targets were performed for 90 min at 70 °C in a 10 μL mixture containing the following components: 1.0 μL of 10× reaction buffer (500 mM Tris−HCl and 10 mM DTT), 0.2 μM each of DsF and DsR, 0.8 μM each of FIT and RIT, 1.6 μM each of SteF and SteR, 1.4 mM each of dNTP, 3.2 U of Bst 2.0

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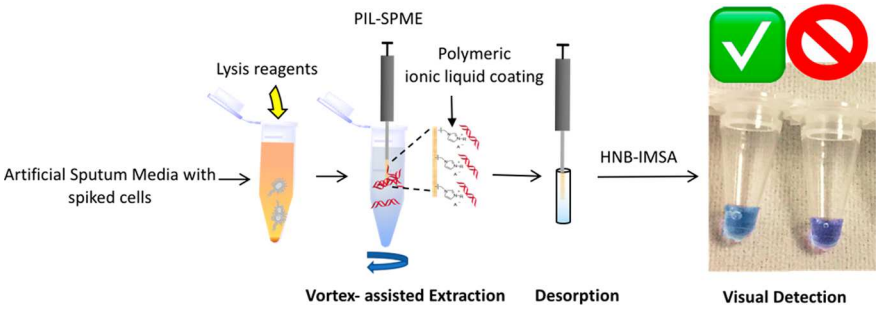

Figure 1. Schematic representation of the developed method for rapid extraction of mycobacterial DNA and subsequent visual detection by isothermal amplification (HNB-IMSA).

WarmStart DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.), 0.8 M betaine (Sigma-Aldrich), 8 mM $MgSO_4$ (NEB), 240 μM HNB (Sigma-Aldrich), and 1.0 μL of template solution. The sequences of all primers used for IMSA can be found in Table S1 of the Supporting Information. A general schematic of the IMSA amplification process as well as primer binding sites can be found in Figures S2 and S3.

***M. smegmatis* Culture Conditions.** *M. smegmatis* was initially cultured in Middlebrook 7H9 broth supplemented with 30% glycerol, 0.05% Tween 80, ADC enrichment medium (NaCl, bovine serum albumin, and dextrose), carbenicillin (50 μg mL^{-1}), and cycloheximide (10 μg mL^{-1}) for 72 h in an incubator shaker (37.0 °C, 250 rpm). Following the incubation period, a 100 μL aliquot of the culture was inoculated into a second culture of equal volume (5 mL) containing the same components as the initial culture, with the exception of Tween. The secondary culture was subsequently incubated for 2 days. To accurately determine the number of cells corresponding to an OD_{600} value of 1, serial 10-fold dilutions were performed once this culture reached an OD_{600} = 1. After the dilutions were made, 100 μL was taken from each of the dilutions and plated on 7H10 agar. Colonies were counted after incubating the cells for 3 days from the lowest plated dilution, which corresponded to 1.9×10^7 colony forming units (cfu)/mL in the original solution. Prior to all extractions, cells were pelleted, washed, and resuspended in phosphate-buffered saline (PBS) or artificial sputum.

DNA Extraction Using SPME Fibers. A general schematic illustrating the workflow for the PIL-SPME-based DNA extraction method is shown in Figure 1. A 10 pg mL^{-1} solution of DNA in 1.5 mL TE buffer at pH 8 was prepared in a LoBind tube (Eppendorf, Hamburg, Germany) immediately prior to extraction. The tube was modified prior to extraction by piercing the cap with a syringe needle and inserting the SPME fiber through the resulting opening. The lid was closed, ensuring that the sorbent coating was immersed in the sample solution, and subjected to vortex agitation with a Fisherbrand digital vortex mixer (Fisher Scientific, Hampton, NH, U.S.A.) for 2 min. Immediately following the extraction, the PIL-SPME fiber was desorbed in 10 μL of 1 M NaCl for 30 min. To avoid qPCR inhibition caused by 1 M NaCl, the desorption solution was diluted 5-fold to achieve a final concentration of 10 mM NaCl in the qPCR mix. After each extraction, the fibers were washed with 2 M NaCl for 30 min to desorb any remaining DNA from the fiber. Figure S4 shows representative qPCR plots of the diluted desorption solution as well as the subsequent wash step, revealing that carryover is negligible (Cq > 38) after this step.

RESULTS AND DISCUSSION

Optimization of Extraction and Desorption Conditions. The chemical structure of the PIL sorbent coating used in this study is shown in Figure S5 and was prepared using a previously reported method.[30] This PIL was chosen for method optimization as it had previously been successfully applied for the extraction of DNA from aqueous solutions and *Escherichia coli* cell lysate.

In order to develop a method that could be easily interfaced with HNB-IMSA for potential POC applications, the total analysis time should be as short as possible. The step in the PIL-SPME workflow that can be most readily expedited is the extraction time. To determine the effects of different parameters on the extraction of DNA, qPCR was used to quantify the DNA extracted by the PIL sorbent coating.

In an attempt to decrease the extraction time, vortex agitation was employed as an alternative to traditional magnetic stirring for the extraction of a 280 bp fragment of DNA. As shown in Figure 2C, vortex agitation for 2 min at 2500 rpm yielded comparable extraction efficiency to stirring at 650 rpm for 30 min. This corresponds to a drastic 15-fold decrease in extraction time while maintaining similar extraction performance. When a 2 min extraction at 650 rpm with magnetic stirring was performed, qPCR analysis of the desorption solution indicated that insufficient DNA was extracted for quantification. The vigorous mixing afforded by vortex agitation results in faster mass transfer of the nucleic acid to the PIL fiber. Short extraction times (i.e., 2 min) are achieved due to the rapid ion-exchange process,[31] facilitated by the exchangeable halide anions of the PIL sorbent.[22] Furthermore, the good reproducibility [relative standard deviation (RSD) of 0.8%, n = 3 for Cq values] indicates that there is no significant loss of fiber performance due to loss of coating when applying vortex mixing.

Faster vortex speeds should be expected to provide faster mass transfer of the DNA from the sample solution to the PIL and therefore allow for higher extraction of DNA at shorter times. To investigate this, several vortex speeds ranging from 500 to 2500 rpm were investigated. As shown in Figure S6, the amount of DNA extracted increased from 500 to 1000 rpm, remaining constant at approximately 0.030 pg of DNA. However, a sharp increase in the mass extracted was observed from 1000 to 2000 rpm, with masses of 0.029 ± 0.010 to 0.135 ± 0.028 pg, respectively, being achieved. A less pronounced increase was seen at 2500 rpm where the mass extracted was 0.161 ± 0.005 pg. Therefore, a vortex speed of 2500 rpm was chosen for subsequent extractions.

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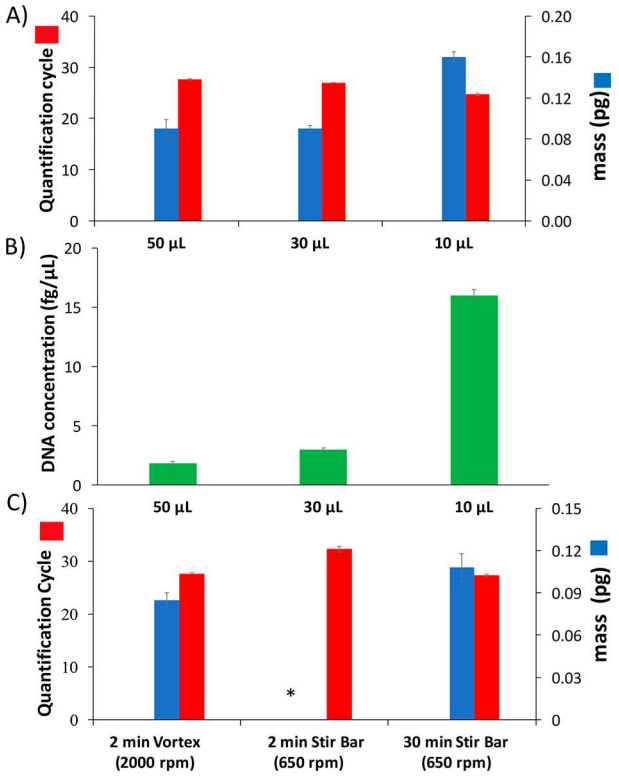

Figure 2. (A) Effect of desorption volume on the recovery of DNA following extractions. (B) Concentration of the desorption solution when employing different desorption volumes. (C) Effect of the agitation method on the extraction of DNA. All extractions were performed in triplicate from a 10 pg mL^{-1} solution of DNA: total volume, 1.5 mL, pH 8 TE buffer; extraction time, 2 min with vortex (2500 rpm); desorption time, 30 min; desorption solvent, 1 M NaCl; desorption solvent volume, 10, 30, or 50 μL. Cq values are indicated in red, while mass is indicated in blue. * Indicates insufficient DNA was extracted for quantification.

The effect of extraction time on the extraction of DNA was also investigated. This was carried out by performing extractions from a 10 pg mL^{-1} solution of DNA in TE buffer at pH 8 and varying the extraction time from 0.5 to 15 min. Figure S7 shows an increase in the mass of DNA extracted in the range from 0.5 to 6 min. Beyond 6 min, no significant increase in the amount of DNA extracted was observed. An extraction time of 2 min was chosen for subsequent experiments as it allowed for very rapid and reproducible extractions (RSD = 3.1% based on mass extracted) and precludes exposing the fiber, sample, and analyst to long vortex times.

Dilution of the desorption solution is necessary to relieve inhibition caused by the high concentration of NaCl in qPCR. Therefore, the use of smaller desorption volumes should increase the concentration of DNA within the final desorption solution and ultimately produce better detection limits. To examine this, three different desorption volumes were investigated. As shown in Figure 2A, the amount of DNA recovered after a 2 min extraction was greater when a desorption volume of 10 μL was used. The difference in the mass recovered when using 10 μL instead of 30 or 50 μL could be attributed to the significantly smaller surface area of the desorption container that makes contact with the 10 μL solution (0.67 cm^2) versus using 50 or 30 μL solutions (2.45 and 1.63 cm^2, respectively). Since DNA is known to adsorb onto polypropylene under high ionic strength conditions,[32] decreasing the surface area of the desorption container that is exposed to the 1 M NaCl desorption solution likely minimizes this effect and allows for higher DNA recoveries. Figure 2B further highlights the benefits of using 10 μL as the desorption solvent volume by showing the concentration of DNA in the desorption solution plotted as a function of the desorption volume. This results in nearly a 1 order of magnitude increase in the concentration of DNA when 10 μL is used as the desorption volume instead of 50 μL (16 and 1.8 fg μL^{-1}, respectively). The intraday fiber-to-fiber reproducibility was tested with three fibers using a 2 min extraction at 2500 rpm, and a 5% RSD was found for all quantification cycle (Cq) values.

Optimization of HNB-IMSA Buffer for Direct Analysis of SPME Desorption Solution. IMSA is an INAA technology developed by Ding et al. that is similar to LAMP. However, a particular advantage of IMSA is the creation of multiple self-matching structures, allowing for faster reaction times and better detection limits than traditional LAMP reactions.[12] IMSA reactions are commonly performed in the isothermal amplification buffer supplied by NEB. However, inhibition of the reaction was observed when 1 μL of the desorption solution (1 M NaCl) was directly transferred into the IMSA reaction mix (Figure S8). The minimum inhibitory concentration of NaCl was determined by testing a range of NaCl concentration in the HNB-IMSA mix and monitoring the fluorescence change in real time. This allowed for reaction effectiveness to be gaged by using the threshold times obtained to determine the inhibitory effects of NaCl. As shown in Figure S8, the minimum inhibitory concentration of NaCl in the reaction mixture was 14 mM, which would require a 10-fold dilution of the 1 M NaCl desorption solution. This dilution step would severely hinder the analysis and detection of low concentrations of bacteria (e.g., 10^5 cfu/mL) of cells. Ideally, a buffer compatible with the 1 M NaCl desorption solution would be used in the HNB-IMSA reaction to avoid unnecessary dilution steps and achieve optimal sensitivity.

According to NEB, the Bst 2.0 WarmStart polymerase used for IMSA is compatible with a variety of buffers including NEBuffer 3 (100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris−HCl pH 7.9). When the reaction was attempted under the same reaction conditions as the previously optimized reaction (63 °C, 60 min), no amplification was detected. However, upon increasing the reaction temperature to 70 °C, the amplification of target DNA was recovered as indicated by an increase in real-time fluorescence. The recovery in amplification can potentially be attributed to the increased melting temperature of the primers to the target due to the high concentration of NaCl. Unfortunately, visual detection was not achieved due to the difficulty in discerning between positive and negative samples. Since the color of the solution is dependent on a combination of pH and HNB/dNTPs chelating Mg^{2+},[33] these components were investigated to determine the source of the lacking color change. The concentration of Mg^{2+} was varied in a mixture of 1× NEBuffer 3, dNTPs, and HNB to determine the optimal Mg^{2+} concentration to produce a solution of purple color corresponding to negative amplification. Figure S9 illustrates that the negative color is not achieved until the total concentration of Mg^{2+} is 24 mM. However, when this concentration of Mg^{2+} is used for a standard reaction, the target was not amplified due to the excessive amounts of magnesium present in the reaction system, possibly leading to

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inhibition of the polymerase or a change in melting temperature of the primers.

One significant difference between the isothermal amplification buffer (pH 8.8) and the NEBuffer 3 (pH 7.9) is their vast difference in pH. Although previous reports have suggested that the solution color results from the chelation of Mg^{2+} by the dNTPs and is independent of small changes in pH,[33] it is conceivable that the large difference in pH (nearly 1 unit) influences the color of the IMSA reaction system. This hypothesis was tested and confirmed when the pH of NEBuffer 3 was raised to 8.8 and the color of the negative reaction became purple. Since 10 mM Mg^{2+} was already present in the reaction, a new buffer was prepared with 100 mM NaCl, 1 mM dithiothreitol (DTT), and 50 mM Tris−HCl at pH 8.8. Figure S10A shows a series of reactions in which the Mg^{2+} concentration was varied and the color change examined prior to amplification. The reactions following amplification are illustrated in Figure S10B. A Mg^{2+} concentration of 8 mM was chosen as it produced the most easily identified color change. The sensitivity of this reaction system was tested across a wide range of mycobacterial DNA concentrations (from 1.39×10^4 to 1.39 copies) and is shown in Figure 3. The data shown in Figure 3 demonstrate the capability of the method for detecting down to 139 copies of mycobacterial DNA, making it suitable for the detection of low quantities of template.

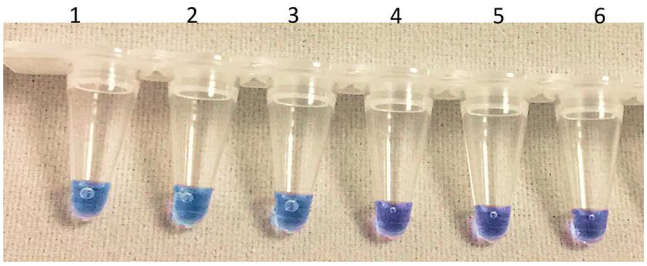

Figure 3. Visual detection sensitivity of HNB-loaded IMSA. HNB: 240 μM. Tubes 1−6: the reactions with templates of (1) 1.39×10^4, (2) 1.39×10^3, (3) 1.39×10^2, (4) 1.39×10^1, and (5) 1.39×10^0 copies, and (6) no-target control (NTC) with isovolumetric nuclease-free water replacing the template. Positive reactions turn light blue (1−3), while negative ones remain purple (4−6). Template: *M. smegmatis* genomic DNA. Reaction time: 90 min. Reaction temperature: 70 °C.

Extraction of Mycobacterial DNA from Cells in Artificial Sputum and Its Visual Detection Using HNB-IMSA. Extracting DNA from mycobacteria in sufficient quantity and purity for downstream molecular biology techniques represents a formidable challenge. Besides being difficult to lyse due to their notoriously thick cell walls, pathogenic mycobacteria such as *M. tuberculosis* are commonly found in the sputum of the infected individual, increasing the difficulty of analysis due to the complex nature of this matrix.[34] PIL-based SPME holds promise as a sample preparation method for the extraction of DNA directly from mycobacterial cells in sputum due to its ease of use and lack of multiple centrifugation steps that are required in typical methods. Furthermore, by coupling PIL-SPME with the visual detection afforded by HNB-IMSA, this method is ideally suited for a POC-oriented application in resource-limited settings. *M. smegmatis* was chosen as the target organism due to its morphological similarity to *M. tuberculosis*.

To demonstrate the compatibility of PIL-based SPME with HNB-IMSA for the extraction of DNA from *M. smegmatis*, extractions were initially conducted from an aqueous cell lysate. The crude lysate was diluted to 1.5 mL with TE buffer (pH 8) and subjected to the optimized PIL-based SPME method for the extraction of DNA. The extraction performance was analyzed with HNB-IMSA using the previously optimized buffer system (50 mM Tris−HCl at pH 8.8 and 1 mM DTT) with each extraction analyzed by HNB-IMSA in triplicate. The exhaustion of Mg^{2+} ions during amplification resulted in the color of the reaction turning from purple (negative) to sky blue (positive), as shown in Figure 4B, allowing for easy identification of DNA positive and negative samples. Furthermore, the reactions can be viewed under blue light (~470 nm) to exploit the fluorescent properties of both SYBR green and HNB for detection (Figure 4C).[35] As illustrated in Table 1, all the reactions showed positive for target DNA amplification, indicating that extraction and detection of DNA from *M. smegmatis* was possible by coupling PIL-based SPME with HNB-IMSA. Primer specificity was also tested in triplicate each time to ensure that false positives did not interfere with the analysis. It was observed that all no-target controls (NTCs)

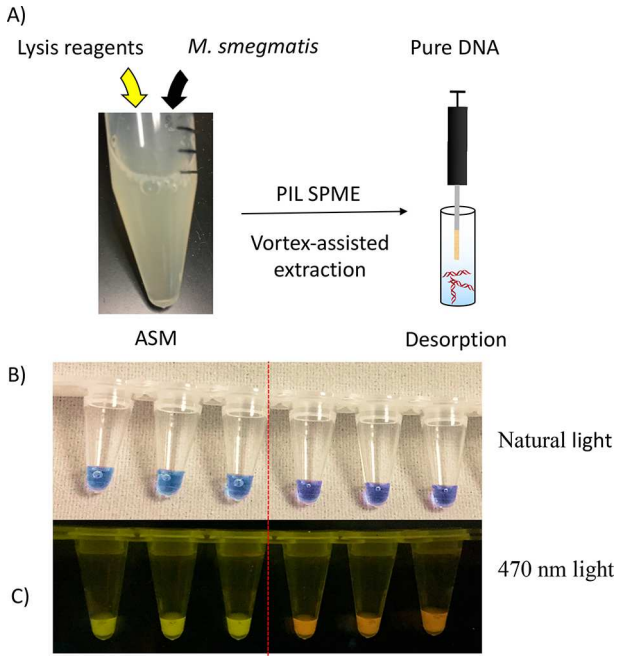

Figure 4. Visual representation of the artificial sputum media (ASM) used as well as the PIL-SPME workflow for DNA extractions (A). A representative example of all HNB-IMSA reactions performed and their visual detection under natural light (B) and 470 nm irradiation (C). Reactions are performed in triplicate, and three NTCs are also included to assess reaction specificity.

Table 1. Summary of Visual Results Following the Extraction of Mycobacterial DNA from Various Cell Suspensions

concn (cfu/mL)[a]	no. of positive samples/no. of samples tested	NTC
2.375×10^8	(9/9)	0/9
1.9×10^7	(9/9)	0/9
1.9×10^6	(9/9)	0/9
1.9×10^5	(9/9)	0/9

[a]Triplicate extractions were performed from each of the indicated concentrations. For each extraction, three HNB-IMSA reactions were performed along with three NTCs. NTC: no-target control with isovolumetric nuclease-free water replacing the template.

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were negative and did not change in color, indicating good primer specificity.

The capability of the method for extracting mycobacterial genomic DNA from a range of cellular suspensions was further explored. The cell suspensions were prepared by serially diluting an initial suspension of OD_{600} = 1 to three different concentrations (from 2.99×10^7 to 2.99×10^5 cfu/mL). A 200 μL aliquot from each suspension was subjected to chemical lysis followed by PIL-SPME and visual detection with HNB-IMSA. Triplicate extractions were performed for each concentration of cells. For each extraction, triplicate HNB-IMSA reactions were performed. As shown in Table 1, the results demonstrated that all HNB-IMSA reactions performed after extractions from each of the tested concentrations gave a positive result, as indicated by the color change of the reaction solution. This shows that sufficient mycobacterial DNA was recovered from *M. smegmatis* for detection by HNB-IMSA at cell suspension concentrations as low as 2.99×10^5 cfu/mL.

To test the potential applicability of the method for extracting mycobacterial DNA from sputum, ASM spiked with *M. smegmatis* cells was used to closely simulate a clinical sample. ASM was prepared following an example from the literature (details can be found in the Supporting Information).[36] Briefly, 200 μL of ASM was spiked with *M. smegmatis* at concentrations typical for infected individuals (2.99×10^7 cfu/mL).[28] Figure 4A shows a representative example of ASM with spiked mycobacterial cells. The cells were chemically lysed and their DNA extracted using PIL-based SPME. Following HNB-IMSA, results showed that all (9/9) reactions tested positive, indicating that genomic DNA of sufficient quantity and quality was extracted from the mycobacteria to yield positive visual identification with HNB-IMSA.

The developed PIL-SPME method was then compared with TE boiling and SPE methods for the extraction of mycobacterial DNA from artificial sputum samples.[37] A comparison of the methodologies is shown in Table S2. All three methods were tested at the same concentration (2.99×10^7 cfu/mL) of *M. smegmatis* cells in ASM. The TE boiling method is simpler and requires fewer steps than the SPE kit. However, multiple centrifugation steps are required in both cases, rendering these methods incompatible at the POC or in resource-limited settings. The PIL-SPME method is rapid and does not require sophisticated equipment. For all of the extractions performed with the SPME device, all (9/9) reactions indicated a positive result. It is also important to note that all three methods are compatible with HNB-IMSA demonstrating the compatibility of this INAA technology with a variety of sample preparation methods. The DNA extracted by the SPE kit could also be detected using a traditional qPCR-based method which amplified a region in the 16S rRNA gene giving Cq values of 17.63 ± 0.79. While detection was easily achieved using qPCR, the technique requires expensive and sophisticated equipment that is not compatible with POC applications.

CONCLUSIONS

In summary, a rapid method based on PIL-SPME capable of extracting genomic DNA from mycobacteria in ASM was developed. This method was seamlessly interfaced with HNB-IMSA to afford visual detection of the extracted nucleic acid by developing a compatible reaction buffer. The method was capable of extracting and detecting mycobacterial genomic DNA from the crude lysate of mycobacteria at concentrations as low as 2.99×10^5 cfu/mL. Moreover, extraction of DNA was also possible from clinically relevant concentrations of mycobacteria in ASM, demonstrating its incredible promise for use in POC applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b01160.

Calibration curve, IMSA primer design, representative cycling amplification, qPCR plots, chemical structure of the PIL sorbent coating, effect of vortex speed, sorption−time profile, effects of NaCl and Mg^{2+}, sequences of IMSA primers, comparison of different sample preparation methods, and additional experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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