Versatile Dual-Channel Loop-mediated Isothermal Amplification Assay Featuring Smartphone Imaging Enables Determination of Fecal Indicator Bacteria in Environmental Waters by Thin Film Microextraction

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Abstract

Rapid diagnostic assays are often a critical tool for monitoring water quality in developing and developed countries. Conventional testing requires 24 to 48 hours for incubation, resulting in delayed remediation and increasing the likelihood of negative outcomes. In this study, we report a workflow for detection of *E. coli,* a common indicator of fecal contamination. Following large volume filtration, *E. coli* is then solubilized enabling the facile isolation and recovery of genetic material by a thin film microextraction (TFME) device featuring a polymeric ionic liquid (PIL) sorbent. Rapid recovery of pure nucleic acids is achieved using a PIL sorbent with high affinity for DNA to significantly increase mass transfer and facilitate adsorption and desorption of DNA. Downstream detection is performed by a versatile, dual channel loop mediated isothermal amplification (LAMP) assay featuring a colorimetric dye and a sequence-specific molecular beacon. A portable LAMP companion box enables consistent isothermal heating and endpoint smartphone imaging while being powered by a single 12-volt battery. Programmable LEDs are switched from white or blue light to facilitate the independent imaging of the colorimetric dye or fluorometric probe following amplification. The methodology positively identified *E. coli* in environmental samples spiked to concentrations of 6,600 colony forming units (CFU) per milliliter and 660 CFU/mL with 100 % and 22% positivity, respectively.

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1. Introduction

Rising global temperatures are driving concern for the security and quality of water sources in both developed and developing countries. Environmental perturbations of clean waters, such as droughts and floods, concentrate underlying or introduce new contaminates.[1] The contamination of feces in water is a major health concern as it is a well-known vector for disease transmission of enteric pathogens,[2] which contributes to nearly two million deaths each year world-wide.[3] Comprehensive monitoring for each enteric pathogen is impractical as negative results for several pathogens does not indicate that zero pathogens are present. Individualized pathogen testing can be circumvented by monitoring concomitant bacteria originating from the digestive tracts of warm-blooded animals and the presence of such fecal indicator bacteria (FIB) in water.[4] Nonpathogenic *Escherichia coli* is a FIB recommended by the World Health Organization and others as it is present in higher concentrations than other FIB and is indicative of both human and animal excrement.[3, 5, 6]

The gold standard methodology for the determination of fecal contamination is performed by filtration of large (100 mL) volumes of water followed by cultivation of the captured FIB on differential or dyed media.[4] Alternatively, tube-based liquid cultures can determine the most probable number (MPN) of FIB through serial dilution and a comparison of replicate samples.[7] While these methodologies are simple to deploy, 1-2 days are often required for incubation of the FIB prior to enumeration, which delays results and subsequent action. Furthermore, it is important to note that the underlying assumption that all captured FIB is culturable and detectable is not factual in all cases. Following exposure to stressors like temperature change or lack of nutrients, bacteria can adopt a state where they are viable but nonculturable (VBNC).[8, 9] Bacteria that are VBNC can be “resuscitated” in proper conditions and maintain virulence, which has been

demonstrated in pathogenic *E. coli, V. cholerae,* and *salmonella* as well as routinely analyzed nonpathogenic FIB like *E. coli* and *E. faecalis*.[9] Recent work performed by Guo and co-workers monitored the distribution of culturable and VBNC cells in water through processing in a full-scale drinking water treatment plant.[10] They utilized molecular detection of VBNC nucleic acids and were able to detect up to 10^2 cells per 100 mL of various FIB in final chlorinated water, whereas culture-based methods were unable to detect any.[10]

Molecular detection of genetic material is a powerful alternative to determine fecal contamination as all possible cell types (culturable, VNBC, or dead) are equally detected. Additionally, these assays enable rapid determination in approximately two hours. The principal method employs polymerase chain reaction (PCR) which involves enzymatic amplification of a target region enabled by complementary, single-stranded DNA seqeunces and precise temperature modulation. Bernhard and co-workers leveraged PCR to detect members of the *Bacteroides-Prevotella* group, as the organisms are anaerobic and difficult to culture.[11] Additionally, quantitative polymerase chain reaction (qPCR) can be used to correlate FIB genetic material with the amount of fecal contamination. One study by Kapoor and co-workers used qPCR to monitor and localize fecal contamination within a river following dramatic flooding caused by a hurricane.[12] Furthermore, a panel of qPCR assays have been shown to classify serotypes of nonpathogenic *E. coli* and shiga toxin-producing *E. coli* based on genotyping.[13]

Isothermal assays have been developed to amplify target DNA and have advantages of not requiring precise temperature modulation as only 30-60 minutes is required for completion, while offering similar sensitivity to qPCR.[14] Loop-mediated isothermal amplification (LAMP) is among the most popular isothermal amplification methods and uses 4-6 primers along with a polymerase with strand displacement activity.[14] The low-resource requirements of LAMP have

enabled sensitive molecular diagnostic assays to be more easily deployed, which have been exemplified through commercially-available in-home diagnostic LAMP assays during the SARS-CoV-2 pandemic. Furthermore, LAMP has been employed in assays for the detection of FIB in coastal waters of Singapore by Lee and co-workers.[15]

An important consideration when using molecular detection techniques revolves around the difficulty in obtaining pure nucleic acid samples. In the detection of FIB, cells are collected through filtration and subsequently released through bead-beating, [16, 17] freezing and grinding, [18] or reversing the direction of flow.[15, 19] Consumable solid phase extraction (SPE) kits are used to release nucleic acids and separate them from co-filtered matrix components. These kits generally include a series of steps for cellular lysis, adsorption of nucleic acids on silica, washing, and elution of pure nucleic acids.[20] They also typically require extensive user intervention, utilize toxic organic solvents, and have a high cost per use. However, if these methods are not employed samples may contain residual matrix components which can inhibit the enzymatic reaction resulting in inaccurate results or false negatives.[21] Methodologies involving less user intervention while maintaining the ability to preconcentrate and purify nucleic acids are required. Sorbents comprised of polymeric ionic liquids (PILs) are a promising alternative to silica as they adsorb large amounts of DNA which can be selectively eluted with a high concentration of NaCl.[22] Various PIL compositions have been studied and sorbents which possessed an exchangeable anion enabled more DNA to be isolated and recovered due to a facile anion exchange with the negatively charged phosphodiester backbone of DNA.[23] Several works have employed this PIL sorbent for the extraction of spiked nucleic acids in human plasma,[24] artificial oral fluid, [25] and artificial sputum.[26] However, targeted nucleic acids are often sequestered behind one or more membranes of bacteria or viruses, but PIL-compatible lysis methodologies have yet to be

studied. Lysis solutions must effectively release more DNA and not inhibit the PIL-DNA interaction which has been demonstrated in the presence of anionic species.[24] Recently, application of these sorbents on a high surface area support has been demonstrated to provide significant improvements in the mass transfer kinetics that govern the extraction and release of nucleic acids allowing for increased downstream sensitivity of qPCR and LAMP assays for target nucleic acids.[25] These new thin film microextraction devices (TFME) drastically improved sorbent efficiency and provided consistent results over the course of more than 50 extraction/desorption steps demonstrating its reusability.

Herein, we report a rapid and easily deployable workflow for the generalized determination of *E. coli* in water using a thin film microextraction (TFME) device featuring a PIL sorbent. Prior to DNA recovery by TFME, the *E. coli* cells are collected through large volume filtration on a syringe filter and subsequently recovered by backflushing with a lysis solution. The time required for isolation and recovery of DNA from the devices was subsequently optimized for maximal sensitivity and decreased analysis time. A dual-channel LAMP assay featuring a colorimetric dye, phenol red, and a fluorescently-labeled molecular beacon (MB) was employed for the first time in the detection of the *uidA* gene present in *E. coli.* Facile LAMP assay implementation was achieved through the construction of a LAMP reactor platform which produces consistent isothermal heating while requiring only 12-volt batteries for operation. In addition, a single set of LEDs are programmed to produce colorimetric or fluorometric lighting conditions for smartphone visualization of completed LAMP reactions.

2. Experimental

2.1 LAMP and qPCR Assay Conditions

All reagents and methods for cell culturing and DNA stock preparation can be found in the supporting information. Sequences of oligonucleotide primers and the molecular beacon probe targeting the *uidA* gene of *E. coli* can be found in Table S1 of the Supporting Information. qPCR reactions were 20.0 µL in total volume and contained the following components: 1.0 µL of 20 µM forward and backward primers, 10.0 µL of SsoAdvanced Universal SYBR green supermix, 8.0 µL water, and 1.0 µL of 200 mM NaCl and DNA or only 200 mM NaCl for no-template control (NTC) reactions.

LAMP reactions were carried out at 65 °C for 60 minutes. The 20.0 µL reactions contained 1.0 µL of 100 mM ammonium sulfate and 1.0% v/v Tween 20, which was pH adjusted with KOH. Additionally, 2.0 µL 1.00 mM phenol red, 8.6 µL of water, 2.8 µL of 10 mM dNTPs, 0.8 µL of 8 U/µL *Bst* 2.0 WarmStart® polymerase, 1.2 µL of 100 mM magnesium sulfate, and 0.6 µL of 10 µM molecular beacon probe, and a 2.0 µL portion of a primer mix containing 1.6 µM FIP and BIP, 0.4 µM LB, and 0.2 µM F3 and B3 was added. For positive reactions, 1.0 µL of DNA and 1.00 M KCl was added and for NTC control samples, only 1.00 M KCl was used. Primer concentrations and reaction temperatures for assays featuring BRAF and ompW were performed as previously described [27], while all other components follow the procedure described herein.

To determine the status of the completed LAMP reactions, images were collected using a Samsung S20 smartphone (Suwon-si, South Korea). Each experiment featured four reactions including a triplicate of samples and a NTC, which was used as the blank. Image analysis yielded average G values for each reaction corresponding to the fluorometric or colorimetric channel. Corrected sample G values (G') were obtained using Equation 1, where Gs is the raw G value obtained from the sample and Gb is the G value from the blank or NTC sample.

$$G' = Gs - Gb. \quad \text{Eq. 1}$$

Positive reactions required both the colorimetric G' and fluorometric G' values to be greater than 25 and 28, respectively. The 3σ value of negative control samples was used to set thresholds (Figure S1).

2.2 General TFME Procedure and Filter Backflushing

TFME devices featuring a polymeric ionic liquid (PIL) sorbent with a 2:1 ratio (w/w) of the $[C_9COOHVim^+][Br^-]$ IL monomer and $[C_{12}(Vim^+)_2][2Br^-]$ IL crosslinker were prepared according to a previously published protocol with no modifications (Figure 1).[25] Prior to use, the devices were conditioned to remove unreacted components and immersed overnight in 6.14 M NaCl to replace $[Br^-]$ anions with $[Cl^-]$ ions. The devices were removed from the solution of NaCl, washed in DI water, and then added to the sample to facilitate DNA isolation. To decrease analysis times, the sample and TFME device were agitated in-hand or through the use of a vortex mixer. In-hand agitation was carried out using a moderate horizontal back-and-forth motion. After a pre-determined time was reached, devices were removed and washed again in fresh DI water before being placed in 90.0 µL of 1 M NaCl for DNA recovery. It is important to note that conditions including the sample, extraction time, and desorption time are specific for each experiment and were varied to determine optimal conditions. For experiments featuring the dual channel LAMP assay for downstream detection, a 1 M NaCl solution used for DNA recovery was replaced with 1 M KCl to facilitate better assay compatibility.

Samples were drawn into 50 mL syringes and filtered through a 0.22 µm PES syringe filter to completely sterilize the solution. To recover the bacteria and genetic material, a smaller volume of backflushing solution was passed through in the reverse direction of flow to recover and preconcentrate the genetic material prior to recovery by TFME. To perform this, 3.0 mL of backflushing solution was drawn along with 3.0 mL of air and passed through in the reverse

direction where it was collected in a 5.0 mL centrifuge tube. In several experiments, this backflushed solution was used as a sample for TFME and analyzed by serial plating. Optimized conditions for the workflow included the following: 15 minutes of in-hand agitation for DNA isolation, 10 minutes for DNA recovery, and use of a 750 µg/mL lysozyme and PBS backflushing solution.

3. Results and Discussion

3.1 Bacterial Filtration and Backflushing Coupled to TFME

Extraction devices featuring PILs are attractive for the purification of free nucleic acids as they can be easily manipulated, reused, and are compatible with complex matrices, such as oral fluid and plasma.[24, 25] Additionally, Nacham and co-workers demonstrated the extraction of both plasmid DNA and intact *E. coli* cells using PIL sorbents, which was confirmed by successful PCR amplification and scanning electron micrographs.[28] More recent work with PIL sorbents have discovered compositions with higher affinity for nucleic acids [23] and a more advantageous thin film geometry, which increases mass transfer and leads to higher sensitivities for downstream analysis.[25] For these reasons, TFME PIL devices were selected for this work and constructed as illustrated in Figure 1, where PILs are covalently bonded to a NiTi alloy support through free radical polymerization of the IL monomers.[25]

For determination of FIB in environmental water, the workflow illustrated in Figure 2 was employed. Initially, samples were passed through a syringe filter to collect all bacteria present in the initial solution. The sample volumes featured in Figure 2 are large (50 mL), as they are easy to obtain and contamination may be as little as one CFU per 100 mL.[4] The syringe filter was then reversed, and a standard 3.00 mL volume of backflushing solution is used to recover the cells to provide a preconcentrated sample prior to DNA isolation by the TFME device. It is important to

note that direct analysis of the initial solution by TFME would lead to higher detection limits compared to when the filtration and backflushing steps are used (Figure 2). Moreover, the composition of the backflushing solution can be varied to induce lysis and enable more sensitive downstream detection.

Reagents such as surfactants [29, 30] and enzymes [31, 32] have literature precedence for the lysis of *E. coli* cells and were selected for examination as backflushing solutions. The anionic surfactant SDS is known to disrupt the membranes of bacteria and solubilize DNA.[29] The enzyme lysozyme has been shown to cleave peptidoglycan, the major constituent of cell walls for gram-positive bacteria, resulting in cell lysis.[33] Additionally, the cationic surfactant CTAB has also been used in protocols for the purification of DNA from *E. coli.*[34] Individual lysis components were prepared in PBS solutions for comparison of cellular lysis and compatibility with the TFME devices (Figure 3). Large 50 mL solutions of PBS were prepared and spiked with a known amount of *E. coli* and filtered prior to backflushing, where an aliquot was removed and cultured on plates to determine the concentration of viable cells. TFME devices were then added to the backflushed solution to recover genetic material prior to analysis by qPCR. It can be observed in Figure 3 that CTAB and SDS effectively lysed the most bacteria, as few if any viable cells remained in the backflushing solution. However, SDS was found to not be compatible with the PIL sorbent, as two of the three tested devices exhibited stripping of all PIL coating. Interactions of SDS and an IL have been reported by McCutchen and co-workers where they showed increased aggregation and decreased diffusion coefficients for both components indicating a high degree of interaction and the formation of micelles.[35] Therefore, stripping of the PIL sorbent is likely driven by inclusion of SDS which disrupts the existing polymeric structure resulting in degradation during the agitation step. On the other hand, CTAB led to a high recovery

of DNA (581±199 pg) but produced a dense, thick foam during agitation which made removal of the TFME device challenging and required more thorough washing steps compared to the other solutions. Lysozyme was selected as it resulted in the detection of a high amount of DNA (731±326 pg) and did not offer the aforementioned drawbacks associated with CTAB. For lysozyme, the concentration of viable cells was similar to that of a PBS control solution; however, the increased DNA recovery could be a result of lysed cells which remained on the filter. Additionally, tris buffer containing no salts was examined as bacteria can exhibit hypotonic swelling resulting in induced stress on the cellular membrane of *E. coli*.[36] Furthermore, tris buffers have been extensively used in the isolation and recovery of DNA by PIL devices.[24, 25] However, the tris backflushing solution produced the largest concentration of viable cells (6,630 CFU/mL) indicating that tris buffer is an ineffective solution for cellular lysis. As a result of decreased cellular lysis, the quantification of DNA (236±186 pg) was found to be highly variable.

3.2 Kinetics of TFME Isolation and Recovery

Following selection of the optimal backflushing solution in Figure 3, it was important to identify necessary conditions for isolation and recovery. These processes are dependent upon the mass transfer of DNA at the PIL sorbent-solution interface, and thus the amount of DNA adsorbed or desorbed can be variable with time. Initially, the sorption or recovery of DNA should increase linearly with time until a steady state is achieved, corresponding to equilibration of DNA within the sorbent and the solution. It is crucial in microextraction workflows to identify the onset of equilibration to select the appropriate time-course to reduce analysis time while maximizing the amount of isolated DNA. To reduce experimental complexity, DNA was isolated from *E. coli* cultures and spiked into a 3 mL volume of the lysozyme-containing backflushing solution and subjected to TFME. TFME devices have previously utilized agitation by vortex to expedite mass

transfer of DNA.[25] However, decreasing the requirements of equipment and instrumentation is crucial to enable deployment of workflows in-field or in any resource deficient area. In this study, in-hand agitation was used as no additional equipment is required to facilitate agitation.

The duration of agitation was varied from 1 to 30 minutes, as shown in Figure 4A, while maintaining a 30 minute recovery time for all trials. The results clearly show the characteristic linear region in the extraction-time profile followed by a plateau region at around 15 minutes, representing the optimal time for maximal isolation of DNA. In the same manner, the time required to recover DNA was determined after loading the device with DNA and monitoring elution with increasing desorption time (Figure 4B). The obtained results indicate that the amount of DNA recovered approaches equilibrium at approximately 5 minutes, followed by a clear plateau at 10 minutes. While similar amounts of DNA were observed at both time points, 10 minutes was selected due to significantly lower variation in mass of DNA recovered.

3.3 Development of Dual-Channel LAMP Assay and Companion Box

The LAMP assay featured in this work simultaneously employs phenol red, a pH-sensitive dye, and a FAM-labeled MB specific for the *uidA* gene of *E. coli*. To perform these reactions, a LAMP reactor and dual-channel imaging system, shown in Figure 5, was designed for the purpose of providing highly reproducible smartphone imaging capability for both colorimetric and fluorometric channels. A photograph of the constructed companion system is shown in Figure S2 and utilizes a heating block capable of carrying out LAMP reactions by maintaining reaction temperatures of up to 65 °C for 1 hour, as shown by the green trace in Figure S3. For endpoint analysis, the imaging system employs a single set of four LEDs composed of individual red, green, and blue LEDS that can produce white light for colorimetry or blue light for the fluorometric channel (Figure 5B). To perform smartphone imaging in the fluorometric channel, a 460 nm long

pass filter is slid into place by the user, demonstrated in Figure 5A, to remove the blue light provided by the LEDs. For unbiased determination of results, smartphone images were obtained from both channels following amplification (Figure 6A) and analyzed by determining the average green, blue, and red values. The green value in both channels was found to increase following amplification and classification of reactions, as either positive or negative, was performed by comparing the sample reaction's green value to the green value of the NTC.

Positive and negative samples containing either the MB, phenol red, or both were imaged and plotted on the scatter plot shown in Figure 6B. For all reaction sets, positive reactions exhibited a large separation from the negative samples due to the drastic color change and quality of the imaging environment provided by the companion box. Additionally, the imaging channels are distinct as samples containing only phenol red do not show any substantial increase in the fluorometric channel; likewise, the MB produces no increase in the colorimetric channel (Figure 6B). The advantages of the sequence-specific MB probe are demonstrated in Figure 6C, where it was employed with LAMP primers for different DNA targets. Reactions containing DNA corresponding to the LAMP primers produced G' values above the colorimetric threshold. While providing facile detection by the naked eye, the phenol red dye only responds to changes in pH resulting from dNTP hydrolysis and cannot distinguish the products of the *uidA,* ompW, or BRAF LAMP assays. It is important to note that any amplification, specific or non-specific, would also be indistinguishable from true positive *uidA* reactions if only colorimetry was used. On the other hand, reactions containing *uidA* primers and DNA template exhibited an increase in the fluorometric G' value as the *uidA* is attributed to the successful hybridization of the *uidA* MB probe and the amplified *uidA* LAMP product. Reactions containing the *uidA* primers and ompW or BRAF DNA templates did not produce any amplification products (Figure 6C). The two distinct

modalities can be imaged separately and reinforce the reliability of obtained results. Additionally, the development of more versatile dual-channel LAMP assays enables wider adoption and utilization according to the availability of resources.

The LAMP assay buffer was adapted from Tanner and co-workers [38] with novel LAMP primers and MB targeting the *uidA* gene of *E. coli.* The MB was designed using the sequence of a loop primer and modified with self-complementary guanine and cytosine bases to form the stem region of the hairpin structure before functionalization with a fluorophore and quencher pair. Assay conditions, such as reaction temperature, Mg^{2+} ion concentration, polymerase concentration, and loop primer concentration, were determined using data obtained from real-time fluorescence studies shown in Figure S4. To determine assay sensitivity, purified *E. coli* DNA was serially diluted, and reactions were performed in triplicate from 700 pg to 70 fg of DNA per reaction. Since reactions containing 70 fg of DNA initially exhibited intermittent amplification, six additional reactions (n=9) were performed at 70 fg and 700 fg. When 70 fg of DNA was added to the reaction, 33.3% (n=9) of the reactions were positive while at 700 fg, 100 % (n=9) were positive (Figure S5). Reactions were determined to be positive only if the G' value in Equation 1 was observed to increase for both channels.

3.4 Application of Filtration, Backflushing, and TFME Workflow for the Detection of *E. coli*

Following development of the LAMP assay, the workflow (Figure 2) was employed to determine the limit of detection of *E. coli* in solutions of PBS. Solutions containing 66,000 to 660 CFU/mL were prepared in 50 mL solutions and filtered, backflushed with the lysozyme-containing solution, and subjected to TFME isolation and recovery. For these experiments, recoveries were performed in 1 M KCl instead of NaCl to enable direct addition of the recovery solution to the LAMP assay. At a concentration of 6,600 CFU/mL, 100% (n=9) of the reactions were positive,

while 11.1% (n=9) were positive at 660 CFU/mL as shown in Table S3. Environmental water samples were collected in Ames, IA from two ponds and a stream to determine fecal contamination by detecting FIB *E. coli.* Additionally, a portion of the samples were processed by filtration and cultivated for 72 hours on M-endo total coliform differential culture media. The media causes lactose fermenting bacteria like *E. coli* to produce metallic colonies while non-lactose fermenting bacteria will appear clear.[39] In all samples, no non-lactose fermenting bacteria cultures were observed, but two cultures were found to exhibit colonies with the characteristic metallic sheen associated with bacteria like *E. coli*. The samples were enumerated and data are provided in Table 1. Environmental samples were then processed via the workflow in Figure 2; however, no positive reactions were observed. The number of bacteria present in the environmental samples (0, 4, or 10 CFU/50 mL) was significantly lower than 6,600 to 660 CFU/mL, which were previously determined to be required for detection. To determine if the method could be applied to heavily contaminated water samples, *E. coli* was spiked into the negative environmental sample at concentrations of 6,600 and 660 CFU/mL. At a higher concentration of 6,600 CFU/mL, 100% of the reactions (n=9) were deemed positive and 22.22 % (n=9) were positive at 660 CFU/mL.

A comparison of the workflow procedure, sample volume, and sensitivity of this work and similar methodologies can be found in Table 2. It is clear that Khan and co-workers [5] have developed a more sensitive scheme which enables detection of as few as 10 cells/mL. However, the method also requires the non-trivial centrifugation of 1 L sample volumes and deploys two separate kits for DNA purification and concentration, which is costly and requires substantial user intervention. Lee and co-workers employed syringe filtration and backflushing to preconcentrate cells prior to DNA adsorption to magnetic beads, washing, and elution.[15] The method enabled detection of 10-100 CFU/mL in environmental water, but requires organic solvents and

consumption of magnetic beads. A chip-based LAMP detection platform was developed by Fu and co-workers to calculate the most probable number (MPN) of FIB in contaminated samples. Purified DNA was serially diluted and ran in quintuplicate LAMP reactions to determine the MPN by considering the amount dilution and number of successful reactions. Using this method, sensitivities of 102-5,870 MPN/mL were obtained for water samples taken from beaches, reservoirs, or rivers.[40] Additionally, the samples volumes deployed in this study (30 L) are very large and pose additional challenges for collection and transportation. Furthermore, dialysis and the commercial purification kit requires significantly more time compared to the method proposed in this work.

4. Conclusions

Results from this study demonstrate the successful detection of *E. coli* in environmental samples using a method that requires less than two hours for total completion. Additionally, the approach requires no toxic organic solvents or single-use consumables for the isolation and recovery of nucleic acids. Furthermore, the LAMP reactor and smartphone imaging system was constructed with low-cost materials enabling the entire workflow to be deployed in any setting with minimal equipment requirements. The dual channel LAMP assay developed in this study leverages two independent detection methodologies, which increases reliability of the results permitting users to take immediate action when analyzing analytes such as FIB. We envision that improvements in the methodology, such as miniaturizing the DNA recovery device or creating sandwich layers of the sorbent, may enable more sensitive detection of FIB while maintaining all other benefits. Versatile LAMP assays featuring several detection methods can be more easily adopted, following appropriate validation, for any setting employing the reactions.

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Table 1. Determination of *E. coli* in Environmental Samples by Filtration, Backflushing, TFME Isolation and Recovery, Followed by Downstream Dual Channel LAMP Detection.

Sample	Initial (CFU/50 mL)[a]	Spike (CFU/mL)[b]	Positive/Total[c]
Pond 1	0	n/a	0/3
Pond 2	4	n/a	0/3
Stream 1	10	n/a	0/3
Pond 1	0	6,600	9/9
Pond 1	0	660	2/9

[a]Enumeration was carried out following cultivation of filters following the processing of 50 mL of sample. [b]Spike concentrations were determined by dilution plating. [c]Number of positive LAMP reactions represented as a fraction of the total number of reactions.

Table 2. Experimental Details and Figures of Merit for Methodologies Reported in the Literature for the Detection of *E. coli* in Environmental Water using Nucleic Acid Amplification Assays

Assay	Sample treatment and DNA purification	Sample volume	Sensitivity	Reference
Dual-channel LAMP	Syringe filtration, backflushing, PIL TFME DNA purification	50 mL	660-6,600 CFU/mL for environmental water	This work
qPCR	Centrifugation, Mini Stool DNA purification kit, Pellet Paint kit for DNA concentration	1 L	10 cells/mL environmental water	Khan and co-workers [5]
MPN-LAMP	Dialysis for purification and concentration, filtration, DNeasy PowerSoil Kit for DNA purification.	30 L	102-5,870 MPN/mL for various environmental water	Fu and co-workers [40]
LAMP	Syringe filtration, backflushing, magnetic bead DNA purification.	100 mL	10-100 CFU/mL for environmental water	Lee and co-workers [15]

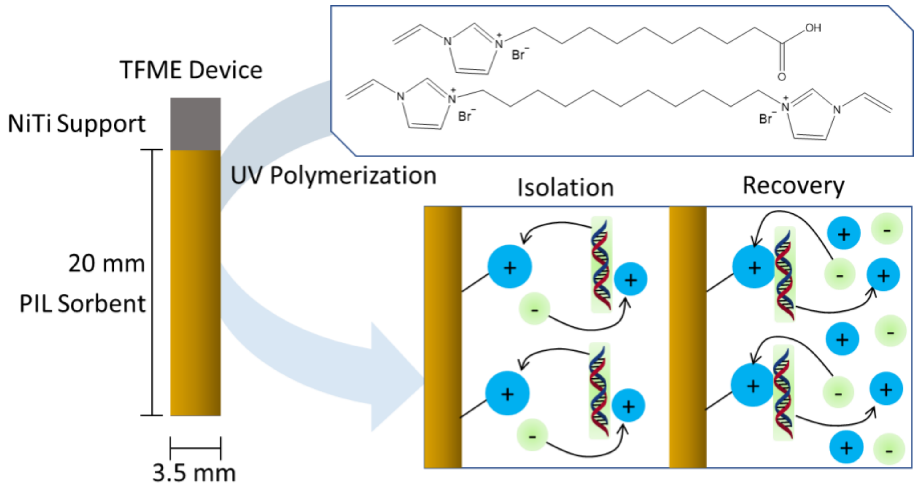


Figure 1. Pictorial representation of the TFME device employed in this work for the isolation and recovery of DNA from *E. coli* cells. A polymeric ionic liquid (PIL) sorbent composed of the $[VImC_9COOH^+][Br^-]$ IL monomer and $[(VIm^+)_2C_{12}][2Br^-]$ IL crosslinker were polymerized following UV irradiation on a nickel-titanium alloy support. The thin film of PIL sorbent possesses a high affinity for nucleic acids permitting the halide anion to be exchanged for the anionic phosphodiester backbone of nucleic acids. Likewise, nucleic acid can be recovered by immersing the device in an electrolyte solution with excess anions.

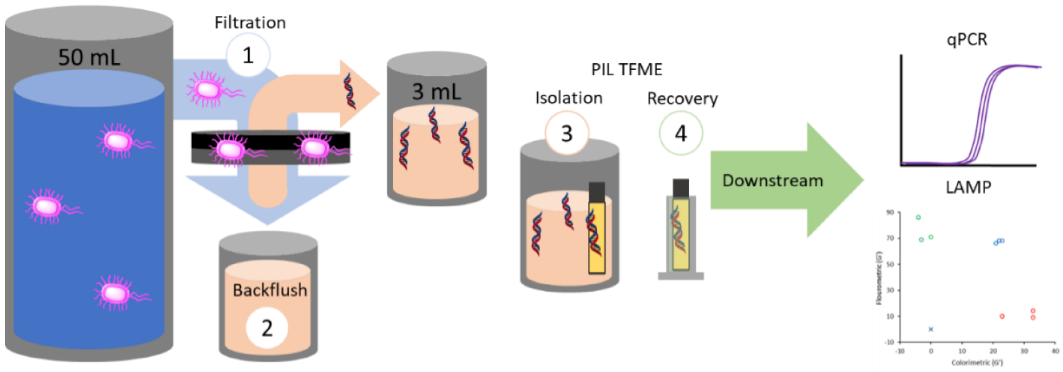
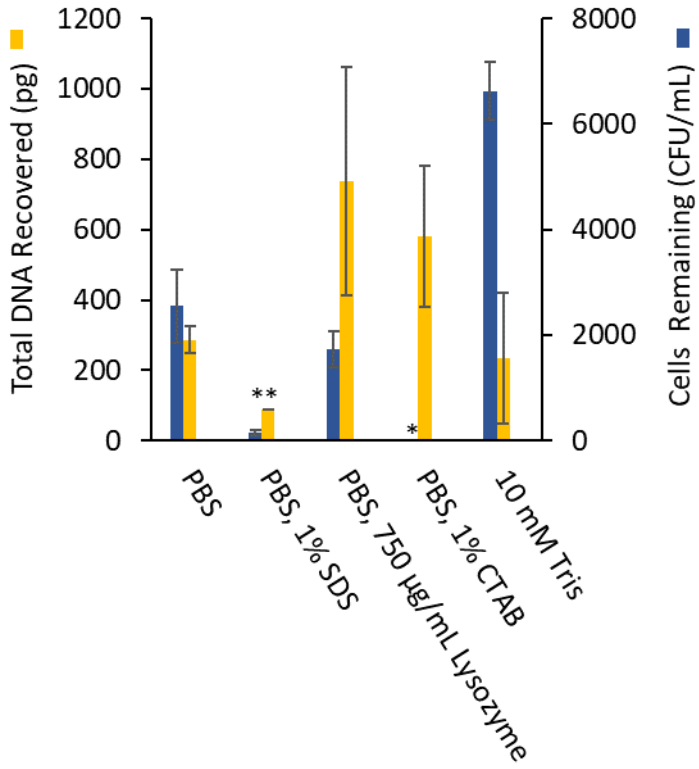


Figure 2. Schematic of the workflow used to determine FIB *E. coli* in water samples. (1) Large sample volumes are filtered through a syringe filter to extract all bacterial cells, which are recovered by backflushing (2) the filter providing a preconcentrated solution for isolation (3) and recovery (4) by the TFME device.



523

524 **Figure 3.** Comparison of backflushing solutions for DNA recovery using the TFME device from
525 filtered samples containing *E. coli.* The total amount of DNA recovered by TFME was
526 determined using qPCR and the cells remaining were enumerated following dilution plating
527 (n=3). Initial volumes (50 mL) of PBS were spiked with 33,000 CFU/mL *E. coli*. TFME
528 isolation was carried out for 15 minutes using in-hand agitation, followed by recovery for 30
529 minutes by immersion in 1 M NaCl (n=3). * Indicates that no cells were observed (n=3). **
530 Indicates only a single measurement (n=1) for DNA quantification was performed due to loss of
531 PIL coating from two other devices. Errors bars illustrate the standard deviation of the average
532 values.

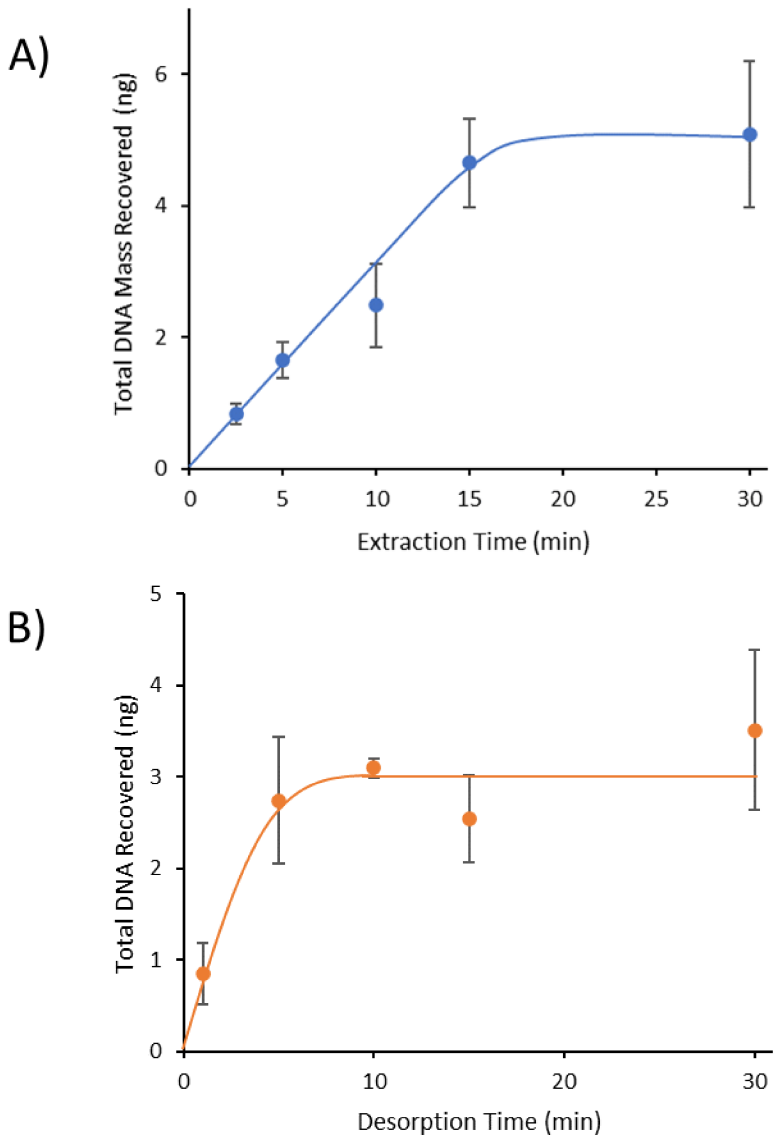


Figure 4. Time-course experiments for the optimization of DNA isolation from the backflushing solution and subsequent recovery using TFME devices. (A) DNA adsorption-time profiles featuring in-hand agitation of a 3.00 mL PBS solution containing 7.5 mg/mL lysozyme and spiked to 76 ng/mL of purified *E. coli* DNA. Recoveries were carried out in 1 M NaCl for 30 minutes prior to qPCR analysis. (B) Desorption-time profiles obtained with a 1.00 mL solution of 7.5 mg/mL lysozyme in PBS and vortex agitation for four minutes.

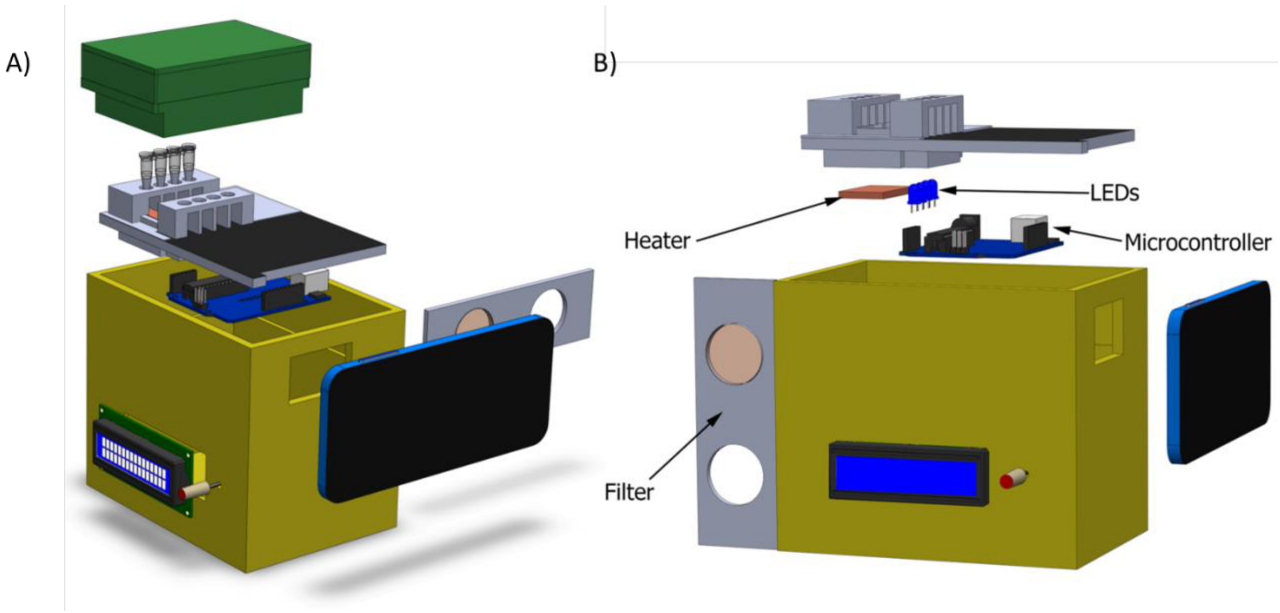
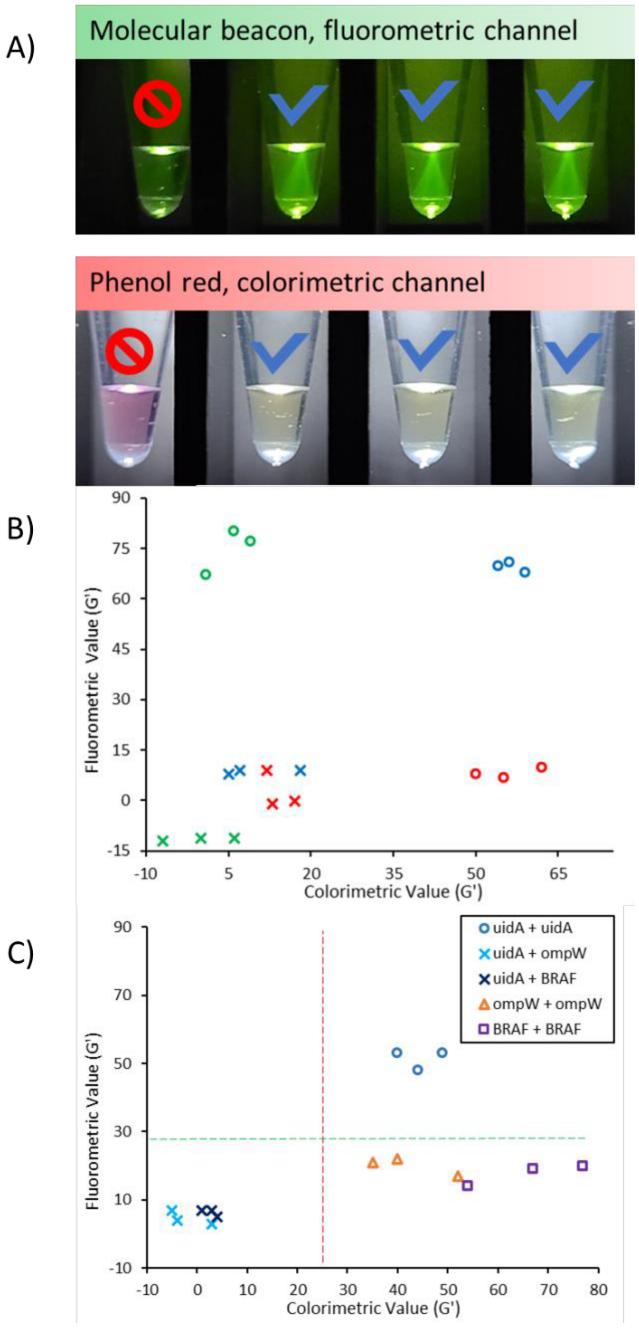


Figure 5. (A) Companion box developed for use with the dual-channel LAMP assay employed in this work. (B) Side-view of the box highlighting all essential components/devices that enable isothermal heating of the sample as well as colorimetric and fluorometric smartphone imaging. The housing, enclosure, and sample holders were constructed using FDM 3D printing.



548

549 **Figure 6.** (A) Smartphone images obtained following amplification from the fluorometric
550 channel of LAMP assays containing only the MB and colorimetric channel of reactions
551 containing only phenol red. Reactions which successfully amplified the spiked target DNA are
552 denoted with checkmarks while NTC samples are represented by a circle with a diagonal line.
553 (B) Scatter plot obtained from a set of six LAMP reactions. Open circles indicate positive

reactions obtained from reactions featuring only the molecular beacon (green), phenol red (red), and both the molecular beacon and phenol red (blue), while crosses indicate negative reactions. (C) Endpoint dual-channel analysis of the *E. coli* MB probe in the presence of various LAMP primer sets and input DNA. The first three sets of reactions were prepared with the *E. coli* LAMP primers and input DNA from *E. coli*, ompW gene, or the BRAF gene. The other two sets featured LAMP primers designed for ompW or BRAF and were paired with its corresponding DNA target. The red and green dashed lines enable visualization of the threshold G' value for the colorimetric or fluorometric channel.