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Facile Coupling of Droplet Magnetofluidic-Enabled Automated Sample Preparation for Digital Nucleic Acid Amplification Testing and Analysis

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.0c02454>.

Detailed description of the automated magnetofluidic sample purification and extraction routine; evaluation of the Amplitaq Gold polymerase with homemade buffers of various pH values; calculation of the extraction efficiency; digital high-resolution melt analysis in the Nanoarray; and single-plex digital melt curves for *S. Aureus* and *E. coli* (PDF)

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Abstract

Digital nucleic acid amplification testing (dNAAT) and analysis techniques, such as digital polymerase chain reaction (PCR), have become useful clinical diagnostic tools. However, nucleic acid (NA) sample preparation preceding dNAAT is generally laborious and performed manually, thus creating the need for a simple sample preparation technique and a facile coupling strategy for dNAAT. Therefore, we demonstrate a simple workflow which automates magnetic bead-based extraction of NAs with a one-step transfer to dNAAT. Specifically, we leverage droplet magnetofluidics (DM) to automate the movement of magnetic beads between small volumes of reagents commonly employed for NA extraction and purification. Importantly, the buffer typically used to elute the NAs off the magnetic beads is replaced by a carefully selected PCR solution, enabling direct transfer from sample preparation to dNAAT. Moreover, we demonstrate the potential for multiplexing using a digital high-resolution melt (dHRM) after the digital PCR (dPCR). The utility of this workflow is demonstrated with duplexed detection of bacteria in a sample imitating a coinfection. We first purify the bacterial DNA into a PCR solution using our DM-based sample preparation. We then transfer the purified bacterial DNA to our microfluidic nanoarray to amplify 16S rRNA using dPCR and then perform dHRM to identify the two bacterial species.

Graphical Abstract

INTRODUCTION

Digital nucleic acid amplification testing (dNAAT) techniques such as digital polymerase chain reaction (dPCR) have become indispensable tools in molecular diagnostics. By digitizing nucleic acids (NAs) with single molecule resolution, dNAAT can outpace conventional bulk-based NAAT in sensitivity, quantification capability, and—when coupled to pos-amplification analysis such as digital high-resolution melt (dHRM) analysis—the capacity for multiplexed detection.^{1–5} These advantages are especially attractive for clinical

diagnostic applications, which have led to the development of a range of dNAAT-based assays for detecting and analyzing copy number variation,^{6–8} biomarkers,^{5,9–11} gene expression,^{12–14} and pathogenic microorganisms.^{15–17} Despite these advantages, robust performance of dNAAT requires that the input NA be extracted from biological samples and purified from dNAAT-inhibiting impurities via sample preparation procedures, which frequently involve cumbersome hands-on steps and can consequently bottleneck dNAAT in clinical diagnostic settings. Given that simplification or automation of sample preparation can shorten analysis time, lessen sample handling, reduce contamination, and minimize human error,^{18,19} coupling dNAAT to a simple sample preparation method offers a promising approach for further expanding the utility of dNAAT for clinical diagnostic applications. Specifically, liquid biopsies which frequently utilize droplet dPCR^{20,21} and emerging technologies for detecting pathogens of infectious diseases²² will benefit from a simple and efficient coupling between sample preparation and analytical technique.

Droplet magnetofluidics (DM)^{23–26} offers a particularly attractive option for simplifying and even automating sample preparation. In DM-enabled sample preparation, surface-modified magnetic beads that allow NA binding are transported between discrete droplets to achieve NA extraction and purification in a short time with simpler and more cost-effective instrumentation²⁷ than other sample preparation methods such as microfluidics based on integrated microvalves and micropumps^{28–30} and digital microfluidics based on electrowetting-on-dielectrics.^{31,32} In more recent DM devices, permanent magnets are actuated by servo motors to transport the magnetic beads between static droplets of reagents to purify and then elute NAs in a polymerase chain reaction (PCR) solution. Thus, DM-enabled sample preparation can be simplified to a single injection of the sample into a plastic cartridge for purification. We have previously implemented DM-enabled sample preparation within portable devices and coupled it to bulk-based loop-mediated isothermal amplification for detecting *Chlamydia trachomatis*,³³ real-time quantitative PCR for quantifying the viral load of Hepatitis-C virus,³⁴ and detection of DNA methylation cancer markers.³⁵ While plastic cartridges have previously been designed for DM-enabled sample preparation capable of performing isothermal amplification and real-time qPCR after purification, DM-enabled sample preparation suitable for dNAAT has not been established.

In this work, we demonstrate facile coupling of a portable and automated DM-enabled sample preparation instrument to a microfluidic device that supports various dNAAT techniques. The magnetofluidic-based sample preparation purifies NAs into a PCR solution comprising the reagents required to perform dNAAT. This dPCR solution is then transferred from the automated magnetofluidic instrument into dNAAT platforms by a single transferring step. Specifically, we demonstrate the purification of bacterial DNA using the magnetofluidic system coupled to the microfluidic device for dPCR. Moreover, we demonstrate the potential for multiplexing of dPCR through postamplification analysis using dHRM. To that end, a polymicrobial sample containing *Escherichia coli* and *Staphylococcus aureus* is purified with the magnetofluidic device and then transferred to the nanoarray for dPCR targeting 16S rRNA of both species. The dHRM of the isolated 16S rRNA amplicons produces a unique melt signature of both species' amplicon. The magnetofluidic sample preparation is completed in 5 min and the dPCR–HRM analysis is completed within 3 h. Matching parameters of an unknown melt curve such as shape and melt temperature can

be used to identify bacterial species from a known database. Here, we demonstrate the discrimination of *E. coli* and *S. aureus* through a dHRM of the 16S amplicons generated during dPCR.

EXPERIMENTAL SECTION

Preparation of dPCR Solution for Universal Amplification of Bacterial 16S Region.

A dPCR solution of 65 μL was created with the following composition: 1 $\times$ Gold Buffer II (Thermo Fisher Scientific, Waltham, MA), 3.5 mM MgCl_2 (Thermo Fisher Scientific, Waltham, MA), 1 $\times$ EvaGreen (Biotium, Freemont, CA), 0.10 U/ μL of Amplitaq Gold LD (Thermo Fisher Scientific, Waltham, MA), 1 $\times$ ROX (Thermo Fisher Scientific, Waltham, MA), 200 μM of each deoxynucleotide triphosphate (Thermo Fisher Scientific, Waltham, MA), 1 mg/mL BSA (New England Biolabs, Ipswich, MA), 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO), 0.3 μM forward primer (5'-GYGGCGNACGGGTGAGTAA-3'; Integrated DNA Technologies, Coralville, IA), 0.3 μM reverse primer (5'-AGCTGACGACANCCATGCA-3'; Integrated DNA Technologies, Coralville, IA), and Ultra-Pure PCR water (Quality Biological Inc., Gaithersburg, MD).

Sample Preparation Device and Thermoformed Cartridge.

The structural components comprising the magnetofluidic sample preparation system were created on a Formlabs Form 2 SLA 3D printer. A rotational and linear servo motor, Hitec HS-422 and Actuatorix PQ12-P, was utilized to enable magnetofluidic transport. A 3D printed arm controlled by servo motors containing two permanent magnets when placed in close proximity to the outside of the cartridge attracts the magnetic beads encased within a thermoformed cartridge. An Arduino microcontroller was programmed to actuate the servo motors to perform a predetermined sample preparation sequence in the Arduino IDE.

The sample preparation cartridge consisted of four layers: a thermoformed layer which defines the wells, a spacer to set the volume of the cartridge, a cap to provide sealing as well as an inlet and outlet, and a thin silicone sheet sealing the inlet and outlet while allowing insertion of a needle for the extraction of components. A 0.25 mm thick polyethylene terephthalate glycol (PETG) film was used to construct the thermoformed wells with a vacuum forming process. A commercial dental vacuum forming instrument (Meta Dental Corp, Glendale, NY) was used to soften the film via heating after which vacuum is applied, which pulls the softened plastic around a 3D printed master mold. The spacer was formed using a 1.50 mm polymethylmethacrylate (PMMA) sheet, which was laminated on both sides with a pressure-sensitive adhesive material (468MP, 3 M, Maplewood, MN) and then cut using a CO₂ laser cutter. The cap consisted of a PMMA sheet laser cut with a Teflon film laminated onto one side laser cut to fit atop the spacer, enclosing the cartridge except for the inlet and outlet cut into the cap. A 0.5 mm silicone sheet was laminated onto the outward section of the cap, sealing the inlet and outlet. The silicone sheet was thin enough to permit puncturing with a syringe allowing for the extraction of the PCR solution. The cartridge was assembled by compressing the spacer with pressure-sensitive adhesive against the PETG mold.

The sample preparation cartridge, comprising three wells, contained solutions to bind DNA to ChargeSwitch magnetic beads (Thermo Fisher Scientific, Waltham, MA). The three wells in the thermoformed cartridge were loaded with (1) a mixture of 101 μL of 99.8% ChargeSwitch binding solution AP051 and 0.2% Tween-20, 4 μL of the ChargeSwitch magnetic beads, and 5 μL of a lysed bacterial cells or unlysed bacterial cells, (2) 75 μL of a solution containing 99.8% Thermo ChargeSwitch wash solution AP001 and 0.2% Tween-20, and (3) 65 μL of the universal dPCR solution. Following loading of these reagents, 750 μL of silicone oil 100 cSt was injected through the inlet to displace the air in the cartridge and provide a fluidic bridge between the three wells. In this work, *E. coli*, ATCC 25922, and/or *S. aureus*, ATCC 29213, were used. A detailed description of the automated sample purification and extraction routine is included in the Supporting Information.

Fabrication of Nanoarray Devices.

Microfluidic devices comprising polydimethylsiloxane (PDMS) and glass were fabricated to enable dPCR via a unique technique to isolate a PCR solution containing DNA into 5040 1 nL droplets. First, a 15:1 mixture of PDMS (SYLGARD 184 Silicone Elastomer Kits Dow Corning, Midland, MI) was spin-coated onto a master mold at 500 rpm for 1 min, producing a thin, $\sim 80\text{ }\mu\text{m}$, layer of PDMS patterned with the nanoarrays and microfluidic channels and then partially cured for 6 min at 80 °C. A sacrificial layer is created to remove the fragile, 80 μm thin, PDMS from the wafer after baking. This sacrificial layer was created using a 6:1 mixture of PDMS spin-coated onto a blank 4" silicon wafer at 100 rpm for 2 min and then partially cured at 80 °C for 6 min. After cooling, the thick 6:1 PDMS layer was removed from the silicon wafer and placed on top of the 80 μm thin PDMS layer still attached to the wafer. This stack of sacrificial 6:1 PDMS on the thin 15:1 PDMS was again baked at 80 °C for 6 min to weakly bond these two layers. The stack was then carefully peeled off the wafer and then cut into a 50 mm $\times$ 75 mm piece. The inlets and outlets were formed with a 2 mm biopsy punch. Next, a glass slide (1 mm thick, 75 mm $\times$ 50 mm; Ted Pella, Redding, CA) was coated with an ultrathin layer of 15:1 PDMS by spin coating the glass slide at 2100 rpm for 1 min. The slide was then baked at 80 °C for 6 min. The PDMS-coated glass slide and PDMS stack were bonded by first treating them with oxygen plasma at 40 W for 45 s and then quickly placing these two pieces into contact with one another, ensuring that all air was removed. The mated coated glass slide and PDMS stack were baked at 80 °C for 6 min. After cooling, the 6:1 PDMS sacrificial layer was carefully peeled from the thin, patterned 15:1 PDMS layer. Finally, the 15:1 patterned PDMS was bonded to a thin glass coverslip (0.13–0.16 mm thick, 24 mm $\times$ 60 mm; Ted Pella, Redding, CA) and two 4 mm thick 10:1 PDMS tubing adapters using the same oxygen plasma and heat treatment as described above. Finally, a thin layer of 10:1 PDMS was coated around the adapters to ensure vacuum-tight sealing and the nanoarray device was baked at 80 °C overnight. The devices were prepared for use by sealing the inlets and outlets of the device with adhesive tape prior to desiccation of the PDMS device in a vacuum chamber for at least 12 h.

Digitization and dPCR–HRM Assay.

The nanoarray was loaded with the PCR solution through a vacuum-assisted loading and digitized via oil-driven flow from a syringe. A 4 ft long Tygon tubing loaded with silicone oil and the PCR solution was connected to a blunt-end needle. The nanoarray was removed

from the vacuum chamber upon removal of the blunt-end needle connected to the tubing punctures through the adhesive tape covering the inlets of nanoarray. This vacuum-assisted loading pulled the PCR solution into the microfluidic channels and nanowells. Moreover, the vacuum within the vacuum-primed PDMS ensured that no air bubbles were trapped within the nanowells. After displacement, the PCR solution was entrapped in each of the 1 nL wells by the silicone oil/PDMS mixture completing digitization.

After digitization, a three-step PCR using universal primers targeting the conserved 16S region amplifies the DNA trapped within the nanowells. This amplification procedure comprised an initial denature at 95.0 °C for 10 min prior to thermocycling, 60 cycles of three-step thermocycling, and a final extension at 72.0 °C for 7 min. The three-step PCR on the nanoarray comprised the following steps: 95.0 °C for 15 s, annealing at 67.0 °C for 30 s, and extension at 72.0 °C for 60 s. The dPCR step took about 2.5 h after which the HRM was performed.

The HRM process may be used to identify the species in the sample of interest from the 16S amplicon produced in the dPCR step. The HRM step-up comprised a GeneTouch flatbed heater (Bulldog Bio Inc., Portsmouth, NH) to perform the melt process, a Sony Alpha 7s Camera with Nikon 60 mm Macro Lens for imaging the nanoarray during melt, a filter matching the emission wavelength of the EvaGreen dye, and a set of blue LEDs for excitation of the EvaGreen dye. The nanoarray was first placed on a silicon wafer on a GeneTouch flatbed heater (Bulldog Bio Inc., Portsmouth, NH). This silicon wafer was used to improve the temperature uniformity of the flatbed heater. Afterward, the nanoarray was secured using electrical tape to prevent movement of the nanoarray. Then, a microcontroller was activated to turn on the blue LEDs to excite the fluorescent EvaGreen dye for the course of the melt. A GeneTouch program was run to precisely ramp the temperature of the nanoarray from 75 to 99.9 °C at a rate of 0.1 °C every 2 s after an initial 20 s holding period at 75 °C. After starting the GeneTouch thermocycler, the Nikon camera was activated to image the nanoarray at a rate of 1 image per second over the course of the temperature ramping. A thermocouple was attached to the silicon wafer to monitor the temperature of the nanoarray.

RESULTS AND DISCUSSION

Overview of Workflow.

For coupling the DM-enabled sample preparation and dNAAT, we have designed a simple workflow that entails loading the sample into a sample preparation cartridge, inserting the sample preparation cartridge into a DM instrument, transferring the processed sample from the cartridge to a dNAAT device with a single-step process, and finally performing dNAAT and analysis. We begin by adding the sample containing cells with a lysis/binding solution containing charge-functionalized magnetic beads, loading them into the sample well of the sample preparation cartridge (Figure 1, left), and then inserting the cartridge into the magnetofluidic instrument. Within the sample well, the acidic lysis/binding solution (pH 5) lyses the cells and renders the charge-functionalized magnetic beads capable of electrostatically binding to NAs (Figure 1, green). Inside the magnetofluidic instrument, permanent magnets attached to automated actuators first transport the NAs bound to charge-

functionalized magnetic beads to the wash well of the cartridge, which contains a pH 7 solution that can remove nonspecifically bound components such as proteins (Figure 1, red). The permanent magnets then transport the NAs bound to charge-functionalized magnetic beads to the elution well of the cartridge that contains a basic PCR mix (pH 8.3), which allows the charge-functionalized magnetic beads to release NAs for extraction (Figure 1, blue). Finally, the magnetic beads are transported back to the wash well, leaving behind only purified NAs in the elution well. Utilizing the PCR mix for direct elution of NAs enables a one-step transfer process in which the PCR mix containing NAs is extracted from the cartridge and loaded into dNAAT platforms. We note that although sample preparation based on charge-functionalized magnetic beads is commonly performed manually on a benchtop, we have employed magnetofluidics to automate the process in a simple cartridge to produce purified NAs within 5 min upon cartridge insertion into the instrument.

Purified NA prepared by DM is compatible with various dNAAT platforms. In this work, we focus on coupling DM-enabled sample preparation to our previously reported “Nanoarray”³⁶ as an example (Figure 1, right). While this DM-enabled sample preparation was presented previously, notably by Shin et al. 2018,³⁴ only isothermal or qPCR-based detection mechanisms have been utilized for detection. In this work, the DM sample preparation system was adapted to function with a generic dPCR-based method by modifying the cartridge to allow for extraction of the PCR solution and injection into the dPCR system. This work represents the first instance of coupling this DM-enabled sample preparation to any dPCR method. Importantly, the nanoarray facilitates not only dPCR but also dHRM and therefore offers broader analytical capabilities such as identification of multiple bacteria species in a polymicrobial sample. Transferring purified NA in the PCR mix from the sample preparation cartridge to the Nanoarray device is achieved conveniently in a single step. The PCR mix is simply drawn from the cartridge into a tubing preloaded with silicone oil by a syringe and subsequently injected into the Nanoarray device, where single copies of NAs and the PCR mix first fill the thousands of 1 nL “nanowells” within the device and then become isolated after the silicone oil is injected through the device and partitions individual nanowells. Subsequently, during dPCR, each single NA molecule entrapped in a nanowell is amplified to produce amplicons within the nanowell. During dHRM that immediately follows dPCR, thermally induced melting of the amplicons within each nanowell results in a melt curve, whose specific melting temperature and unique shape can be used to differentiate NA sequences—such as those from multiple bacteria species—in the sample.

Sample Preparation Cartridge and Magnetofluidic Instrument.

We have designed a simple sample preparation cartridge and employed rapid prototyping techniques to produce the 33 mm-long cartridges, in which the sample preparation steps are reliably executed. Each cartridge comprises four components that are easily assembled: (1) a PETG thermoformed base with a sample well, a wash well, and an elution well reserved for the aqueous reagents required for sample preparation, (2) a laser-cut acrylic spacer with pressure adhesives on both the top and bottom faces, (3) a laser-cut cap with a sample inlet opening and an extraction outlet opening, and (4) a silicone septum that seals both the inlet and the outlet during sample preparation (Figure 2A). The silicone septum, unique to the cartridge in this work, enables extraction of the purified DNA in the PCR solution while

maintaining a seal. Each assembled cartridge holds the lysis/binding solution in the sample well, the wash solution in the wash well, and PCR solution in the elution well (Figure 2B, green food dye, pink food dye, and blue food dye, respectively). Silicone oil is loaded in the cartridge to form an immiscible layer above the three wells to provide a medium for transporting the magnetic beads in the cartridge and to prevent mixing of aqueous solutions between the wells. Compared to the cartridges in our previous magnetofluidic platforms, this cartridge accomplishes effective washing in only a single wash well. Moreover, the additions of the extraction outlet opening in the acrylic cap and the silicone septum that can be easily pierced facilitate ready extraction of purified DNA from the sample preparation cartridge.

We have also constructed a 110 mm (length) $\times$ 90 mm (width) $\times$ 90 mm (height) instrument to automate sample preparation, thus maximizing both portability and user-friendliness of our workflow. At the core of this instrument is a motorized magnet holder arm that is actuated by both a linear servo motor and a rotational servo motor (Figure 2C). The sample preparation cartridge and the magnet holder arm are aligned such that when the cartridge is inserted into the faceplate of the instrument (Figure 2D) and the faceplate is subsequently magnetically clipped onto the frame of the instrument, the sample well of the cartridge posits between the pair of permanent magnets of the magnet holder arm (Figure 2Ei). An Arduino microcontroller controls actuation of linear and rotational servo motors to manipulate the magnet holder arm to transfer the magnetic beads between the lysis/binding solution, the wash solution, and the PCR solution, thereby accomplishing sample preparation without user intervention. Briefly, the linear servo first moves the magnet holder arm orthogonally with respect to the sample preparation cartridge, pulling the magnetic beads in the sample well out of the lysis/binding solution and into the silicone oil above the well (Figure 2Eii). The rotational servo then rotates the magnet holder arm along the sample preparation cartridge, dragging the magnetic beads through the oil from above the sample well to above the wash well (Figure 2Eiii). The linear servo finally moves the magnet holder arm orthogonally with respect to the sample preparation cartridge, pulling the magnetic beads into the wash solution to commence washing (Figure 2Eiv). Washed magnetic beads are then similarly transported into the PCR solution for DNA elution and subsequently back into the wash solution, leaving only purified DNA in the PCR solution ready for transferring into the Nanoarray device. Finally, we note that our emphasis on sample preparation for this DM instrument allows us to strip away thermocycling and fluorescence detection hardware used in our earlier DM instruments, thus reducing the footprint.

One-Step Transfer from Cartridge to a Nanoarray and Streamlined Digitization within a Nanoarray.

To demonstrate one-step transfer from the sample preparation cartridge to the Nanoarray, we performed this facile process using a food dye as mock PCR solution containing purified DNA (Figure 3, blue food dye). Here, we employ a previously reported PDMS-based Nanoarray, which houses 5040 1 nL 6 in a 90 $\times$ 56 grid. The mock PCR solution is extracted using a blunt-end needle connected to a tubing that is preloaded with silicone oil for the subsequent Nanoarray digitization step and a 1 mL syringe. The blunt-end needle pierces through the PDMS septum, extends through the extraction outlet opening and into the elution well, and immerses in the mock PCR solution (Figure 3i). Using the syringe, the

mock PCR solution is then pulled into the tubing such that the PCR solution sits underneath the silicone oil (Figure 3ii). The extracted mock PCR solution in the tubing is then directly injected into a Nanoarray device through our previously established vacuum-assisted sample loading. Briefly, prior to sample loading, the PDMS-based Nanoarray device is held under vacuum while the inlets and outlets are sealed with an adhesive tape. This vacuum treatment evacuates the air from both the microfluidic channels and the gas-permeable PDMS. Vacuum-assisted sample loading into the Nanoarray is initiated by simply piercing the tape covering the inlet of the device with the blunt-end needle transferred from the sample prep cartridge (Figure 3iii). The vacuum-primed nanoarray pulls the mock PCR solution from the needle and tubing into the channels and nanowells, completely filling the device (Figure 3iv). Importantly, the nanowells do not contain air because of the desiccation step and therefore no air is trapped in the nanowells during loading. Finally, the mock PCR solution is digitized into the nanowells through a pressure-driven injection of the preloaded silicone oil, which displaces the PCR solution in the microfluidic channels while retaining the PCR solution in the nanowells. We note that, compared to our previous vacuum-assisted sample loading and pressure-driven digitization processes that entail first inserting the sample tubing and then switching to the silicone oil tubing, our current process obviates switching between multiple tubings. This simplified and streamlined vacuum-assisted sample loading and pressure-driven digitization process thus offers a convenient complement to the one-step transfer between the sample preparation cartridge and the subsequent dNAAT in the Nanoarray.

Optimization of Dual-Function PCR Solution.

The pH of our PCR solution was carefully tuned to endow the dual functions of eluting DNA from magnetic beads and digital amplifying DNA, thereby bridging magnetofluidic sample preparation and dNAAT in the Nanoarray. Specifically, the optimal buffer for low DNA (LD) Amplitaq Gold polymerase—the most reliable dNAAT polymerase with the least residual bacterial DNA based on our experiences—has a pH of 8.0. On the other hand, the magnetofluidic-based extraction requires a solution with pH ~8.5 to neutralize the positive charge of the polymer coating the charge-functionalized magnetic beads used for purification. When the Gold buffer was tested in our magnetofluidic purification system, we observed low extraction efficiency. However, polymerase activity is very sensitive to the pH of the buffer; therefore, careful tuning of the pH was required to maintain activity while enabling purification. Therefore, testing of the enzymatic activity, as well as the extraction efficiency, was performed using buffers created in-house at pH 8.0, 8.3, 8.5, and 9.0. The efficiency of the PCR solution monotonically decreases with increasing pH. The efficiency of the buffer at pH 8.0 is the highest, while pH 8.3 worked similarly to the Gold buffer with reduced PCR efficiencies. The buffer adjusted to pH 8.5 and 9.0 shows no amplification (Figure S1).

After observation of the effects of pH on the assay, we purchased a less commonly employed buffer II, pH 8.3, with the LD Amplitaq Gold to aid in the removal of the NAs from the charge-functionalized magnetic beads. Assuming a magnetic particle coating pK_a of ~6.5 based on previously demonstrated charge-based NA purification,³⁷ the theoretical percentage of positively charged functional groups on the bead in elution solutions of pH

8.0, 8.3, and 8.5 were 3.1, 1.5, 1.0%, respectively. Although there appear to be minor differences in charge, the quantity of DNA purified into buffer II, pH 8.3, was 17.6%, while DNA purified into the Gold buffer, pH 8.0, was calculated to be ~7.6%, Table S1. The fine-tuning and selection of this PCR solution enabled the integration of the magnetofluidic system and the dPCR-HRM.

Bacteria Detection via DM Sample Preparation and dPCR–HRM in a Nanoarray.

We evaluate our DM sample preparation by comparing it with manual benchtop sample preparation in extracting and purifying DNA for dPCR–HRM (Figure 4A). To do so, we divide a sample containing *E. coli* for either automated DM sample preparation or manually performed benchtop sample preparation procedures and subsequently detect both extracted *E. coli* DNA via dPCR-HRM in separate Nanoarray devices. We perform dPCR-HRM by employing 16S V1–V6 PCR primers and EvaGreen dye to broadly amplify DNA from many bacterial species including *E. coli* while also yielding melt curves with species-specific melting temperatures and shapes, which allows us to identify *E. coli*. dPCR–HRM is conducted by placing the Nanoarray device in a custom set-up comprising a flatbed thermocycler (for dPCR) and DLSR camera for fluorescence imaging with concurrent temperature ramping (for dHRM), detailed description in methods section. Upon completion of dPCR, strongly fluorescent (i.e., positive) nanowells that indicate successful dPCR are observed from both the DM prepared sample and benchtop prepared sample. Subsequently, during dHRM, as the temperature increases, dissociation of the amplicon occurs within the nanowell, resulting in the disassociation of the EvaGreen dye from the double-stranded DNA, thereby decreasing the fluorescence intensity of the nanowell (Figure S2). Upon completion of dHRM, digital melt curves with comparable melting temperatures and shapes are observed from both the DM prepared sample and benchtop prepared sample. These results thus support that our automated, 5 min DM sample preparation can extract and purify *E. coli* DNA from a sample with comparable performance as manual benchtop sample preparation procedures.

We finally demonstrate our workflow with duplexed detection of bacteria (akin to polymicrobial infection) by first purifying bacterial DNA from the sample using our magnetofluidic sample preparation, then transferring purified bacterial DNA to our Nanoarray, and finally performing dPCR–HRM to identify the two bacterial species. For duplexed detection, the sample comprises spiked *S. aureus* and *E. coli* in equal concentration. After DM sample preparation and dPCR–HRM, we acquire two distinct groups of digital melt curves, which are consistent with the two species in our sample. Using these previously determined melting temperatures, 200 melt curves from both bacterial species were color-coded to indicate those produced from the *E. coli* 16S amplicon (Figure 4B, red), and the *S. aureus* 16S amplicon (Figure 4B, blue). The shape and melting temperature of the *E. coli* 16S amplicon in the polymicrobial sample is identical to the *E. coli* 16S amplicon when injected into the nanoarray alone (Figure 4A); the same is true of the *S. aureus* amplicon (Figure S3, blue). The differentiation of these two bacterial species through melt temperature is visualized through a histogram of the melting temperatures of the 16S amplicons of these two bacteria (Figure 4C). We again note that our duplexed detection results are comparable to those that we reported in our previous work, but our

current results are obtained after automated magnetofluidic sample preparation in 5 min instead of benchtop-based, laborious manual sample preparation as in previous work. These results therefore clearly illustrate the potential of our workflow.

CONCLUSIONS

In this work, we demonstrated the coupling of an automated magnetofluidics-based sample preparation system with a dNAAT platform. We developed a sample preparation platform which automates magnetic bead extraction using magnetofluidic transport between droplets of purification reagents. Specifically, we demonstrated facile coupling between this automated sample preparation system and a microfluidic device on which we preformed dPCR. Through this facile coupling, we first purified the NAs from a polymicrobial sample containing both *S. aureus* and *E. coli* and then transferred the purified NAs in a PCR solution to the microfluidic chip in one step. dPCR was performed on the purified NAs, isolated in droplets on the microfluidic chip, from both species. Moreover, we demonstrated the potential for multiplexed detection by employing dHRM following dPCR to distinguish these two bacterial species by the melting temperatures of their 16S amplicons. By coupling sample preparation to dNAAT, we have drastically reduced the number of manual steps required to process a sample for digital analysis. The one-step transfer process is easy for operation and reduced dependence on laboratory equipment makes this platform more suitable toward use in a clinical setting. In future work, enlarging the sample well and optimizing the number of beads to accommodate a larger sample volume will be of interest to address applications with various sample concentration-volume products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Athamanolap P; Hsieh K; O'Keefe CM; Zhang Y; Yang S; Wang T-H Anal. Chem 2019, 91, 12784–12792. [PubMed: 31525952]
- (2). Velez DO; Mack H; Jupe J; Hawker S; Kulkarni N; Hedayatnia B; Zhang Y; Lawrence S; Fraley SI Sci. Rep 2017, 7, 42326. [PubMed: 28176860]
- (3). O'Keefe CM; Pisanic TR; Zec H; Overman MJ; Herman JG; Wang T-H Sci. Adv 2018, 4, No. eaat6459.
- (4). O'Keefe CM; Giammanco D; Li S; Pisanic TR; Wang T-HJ Lab Chip 2019, 19, 444–451. [PubMed: 30623957]
- (5). O'Keefe CM; Kaushik AM; Wang T-H Anal. Chem 2019, 91, 11275–11282. [PubMed: 31356737]
- (6). Huggett JF; Cowen S; Foy CA Clin. Chem 2015, 61, 79–88. [PubMed: 25338683]
- (7). Salipante SJ; Sengupta DJ; Rosenthal C; Costa G; Spangler J; Sims EH; Jacobs MA; Miller SI; Hoogestraat DR; Cookson BT; McCoy C; Matsen FA; Shendure J; Lee CC; Harkins TT; Hoffman NG PLoS One 2013, 8, No. e65226.

- (8). Didelot X; Bowden R; Wilson DJ; Peto TEA; Crook DW Nat. Rev. Genet 2012, 13, 601–612. [PubMed: 22868263]
- (9). Day E; Dear PH; McCaughan F Methods 2013, 59, 101–107. [PubMed: 22926236]
- (10). Brychta N; Krahn T; von Ahlsen O Clin. Chem 2016, 62, 1482–1491. [PubMed: 27591291]
- (11). Hudecova I Clin. Biochem 2015, 48, 948–956. [PubMed: 25828047]
- (12). Heredia NJ; Belgrader P; Wang S; Koehler R; Regan J; Cosman AM; Saxonov S; Hindson B; Tanner SC; Brown AS; Karlin-Neumann G Methods 2013, 59, S20–S23. [PubMed: 23036330]
- (13). Taylor SC; Laperriere G; Germain H Sci. Rep 2017, 7, 2409. [PubMed: 28546538]
- (14). Sanders R; Mason DJ; Foy CA; Huggett JF PLoS One 2013, 8, No. e75296.
- (15). Bian X; Jing F; Li G; Fan X; Jia C; Zhou H; Jin Q; Zhao J Biosens. Bioelectron 2015, 74, 770–777. [PubMed: 26226346]
- (16). Abram TJ; Cherukury H; Ou C-Y; Vu T; Toledano M; Li Y; Grunwald JT; Toosky MN; Tifrea DF; Slepkenkin A Lab Chip 2020, 20, 477. [PubMed: 31872202]
- (17). Yu F; Yan L; Wang N; Yang S; Wang L; Tang Y; Gao G; Wang S; Ma C; Xie R Clin. Infect. Dis 2020, 71, 793. [PubMed: 32221523]
- (18). Buchan BW; Ledebor NA Clin. Microbiol. Rev 2014, 27, 783–822. [PubMed: 25278575]
- (19). Thatcher SA Clin. Chem 2015, 61, 89–99. [PubMed: 25451869]
- (20). Olmedillas-López S; García-Arranz M; García-Olmo D Mol. Diagn. Ther 2017, 21, 493–510. [PubMed: 28477149]
- (21). Lim M; Kim C-J; Sunkara V; Kim M-H; Cho Y-K Micromachines 2018, 9, 100.
- (22). Li H; Bai R; Zhao Z; Tao L; Ma M; Ji Z; Jian M; Ding Z; Dai X; Bao F Biosci. Rep 2018, 38, BSR20181170.
- (23). Shin DJ; Zhang Y; Wang T-H Microfluid. Nanofluid 2014, 17, 425–430. [PubMed: 25386112]
- (24). Zhang Y; Park S; Liu K; Tsuan J; Yang S; Wang T-H Lab Chip 2011, 11, 398–406. [PubMed: 21046055]
- (25). Zhang Y; Wang T-H Adv. Mater 2013, 25, 2903–2908. [PubMed: 23529938]
- (26). Chiou C-H; Jin Shin D; Zhang Y; Wang T-H Biosens. Bioelectron 2013, 50, 91–99. [PubMed: 23835223]
- (27). Shin DJ; Wang T-H Ann. Biomed. Eng 2014, 42, 2289–2302. [PubMed: 25008142]
- (28). Feng D; Liu T-F; Su J; Bosch M; Wei Z; Wan W; Yuan D; Chen Y-P; Wang X; Wang K; Lian X; Gu Z-Y; Park J; Zou X; Zhou H-C Nat. Commun 2015, 6, 5979. [PubMed: 25598311]
- (29). Du K; Cai H; Park M; Wall TA; Stott MA; Alfson KJ; Griffiths A; Carrion R; Patterson JL; Hawkins AR; Schmidt H; Mathies RA Biosens. Bioelectron 2017, 91, 489–496. [PubMed: 28073029]
- (30). Park BH; Oh SJ; Jung JH; Choi G; Seo JH; Kim DH; Lee EY; Seo TS Biosens. Bioelectron 2017, 91, 334–340. [PubMed: 28043075]
- (31). Shi X; Chen C-H; Gao W; Chao S.-h.; Meldrum DR Lab Chip 2015, 15, 1059–1065. [PubMed: 25519439]
- (32). Prakash R; Pabbaraju K; Wong S; Tellier R; Kaler K Biomed. Microdevices 2016, 18, 44. [PubMed: 27165104]
- (33). Shin DJ; Athamanolap P; Chen L; Hardick J; Lewis M; Hsieh Y-H; Rothman RE; Gaydos CA; Wang TH Sci. Rep 2017, 7, 4495. [PubMed: 28674410]
- (34). Shin DJ; Trick AY; Hsieh Y-H; Thomas DL; Wang T-H Sci. Rep 2018, 8, 9793. [PubMed: 29955160]
- (35). Stark A; Shin DJ; Wang T-H Biomed. Microdevices 2018, 20, 31. [PubMed: 29594810]
- (36). Athamanolap P; Hsieh K; Chen L; Yang S; Wang T-H Anal. Chem 2017, 89, 11529–11536. [PubMed: 29027789]
- (37). Cao W; Easley CJ; Ferrance JP; Landers JP Anal. Chem 2006, 78, 7222–7228. [PubMed: 17037925]

Figure 1.

Facile coupling of automated DM NA sample preparation to digital NAAT and analysis. A sample containing cells is mixed with an acidic lysis/binding buffer and charge-functionalized magnetic beads and then loaded into a sample preparation cartridge. Upon inserting the cartridge in the DM instrument (not shown), a four-step sample preparation procedure is enabled by DM and automated in the cartridge in 5 min: negatively charged bacterial DNA in the acidic binding solution (green) binds to the positively charged magnetic beads, cellular materials nonspecifically bound to the magnetic beads are removed in neutral wash solution (red), a basic PCR solution (blue) neutralizes the charge on the magnetic beads and releases the NAs, and the PCR solution is withdrawn into a tubing preloaded with silicone oil. The tubing containing the PCR solution is transferred to a nanoarray device where each processed NA molecule is digitized within a 1 nL-sized “nanowell” and subsequently analyzed via dPCR and dHRM analysis. dPCR and HRM take place in each of the 5040 nanowells within the array, thereby physically isolating single copies of DNA from different bacterial species and enabling individual profiling within each nanowell.

Figure 2.

Design and construction of sample preparation cartridge and magnetofluidic instrument. (A) Sample preparation cartridge comprises a PETG thermoformed base which holds aqueous reagents required for purification, a laser-cut acrylic spacer which defines the volume of the cartridge, a laser-cut top providing a seal, inlet and outlet, as well as a silicone septum to seal the inlet and outlet. (B) Assembled cartridge containing colored solutions representing the binding solution, wash solution, and PCR solution in the three thermoformed wells. These three aqueous solutions are separated by silicone oil which fills the remainder of the cartridge. (C) Components forming the automated magnetofluidic system. The 3D printed frame holds the rotational servo motor, which is attached to a linear servo motor, enabling the rotational and linear movements of the magnet holder arm. The 3D printed magnet holder arm contains two permanent magnets on either side of the thermoformed cartridge. An Arduino microcontroller manipulates the servos to perform the NA purification. (D) Cartridge is loaded into the magnetofluidic system by placing it into a 3D printed faceplate, which contains a small slot where the cartridge is held. The face plate is placed onto the body of the magnetofluidic device to complete loading. (E) Beads transfer between wells is accomplished by moving the permanent magnets using the linear and rotational actuators. (i) Initially, the cartridge is positioned such that the sample well aligns with the magnetic holder arm. (ii) Magnet holder arm is “lowered”, pulling the magnetic beads toward the “upper” permanent magnet into the silicone oil. (iii) Magnet holder arm is then rotated over the wash well. (iv) Magnet holder is then “raised” such that the magnetic beads are pulled toward the “lower” permanent magnet and into the wash well. The same sequences repeat to transfer magnetic beads (and NA) to the PCR solution.

Figure 3.

One-step transfer from the cartridge to the Nanoarray and streamlined digitization within the Nanoarray. A blunt-end needle connected to a 0.02" tubing and syringe containing the PCR solution and silicone oil punctures the hermetically sealed Nanoarray. Upon puncturing the seal, the vacuum within the vacuum-primed nanoarray draws the PCR solution from the tubing into the channels and nanowells loading the device. Positive pressure on the syringe displaces the sample residing in the microfluidic channels with silicone oil, thereby partitioning the nanowells.

Figure 4.

Bacteria detection via magnetofluidic sample preparation and dPCR–HRM in the nanoarray.

(A) Extraction and purification of *E. coli* DNA from a sample via either DM sample preparation or manual benchtop sample preparation is compared by detecting the extracted and purified DNA with dPCR–HRM in the Nanoarray devices. Comparable dPCR results—indicated by similar strongly fluorescent (i.e., positive) nanowells—and comparable digital melt curves—indicated by similar melting temperatures and shapes—suggest comparable performance from both sample preparation methods, but DM sample preparation is automated in 5 min. (B) Simulated polymicrobial sample containing both *S. aureus* and *E. coli* is prepared by automated magnetofluidic sample preparation and analyzed via dPCR–HRM in a Nanoarray. The positive nanowells after dPCR become strongly fluorescent because of the EvaGreen dye; however, the identity of the 16S amplicon within each well is unknown prior to HRM. dHRM curves for *S. aureus* (blue) and *E. coli* (red) are identified using the melting temperature. (C) Bimodal histogram of the 16S amplicon melting temperatures within each positive nanowell demonstrates the capability of the dPCR–HRM to discriminate between two melt profiles.