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NAIL: **N**ucleic **A**cid detection using **I**sotachophoresis and **L**oop-mediated isothermal amplification

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ABSTRACT

Nucleic acid amplification tests are the gold standard for many infectious disease diagnoses due to high sensitivity and specificity, rapid operation, and low limits of detection. Despite the advantages of nucleic acid amplification tests, they currently offer limited point-of-care (POC) utility due to the need for complex instruments and laborious sample preparation. We report the development of the Nucleic Acid Isotachophoresis LAMP (NAIL) diagnostic device. NAIL uses isotachophoresis (ITP) and loop-mediated isothermal amplification (LAMP) to extract and amplify nucleic acids from complex matrices in less than one hour inside of an integrated chip. ITP is an electrokinetic separation technique that uses an electric field and two buffers to extract and purify nucleic acids in a single step. LAMP amplifies nucleic acids at constant temperature and produces large amounts of DNA that can be easily detected. A mobile phone images the amplification results to eliminate the need for laser fluorescent detection. The device requires minimal user intervention because capillary valves and heated air chambers act as passive valves and pumps for automated fluid actuation. In this paper, we describe NAIL device design and operation, and demonstrate the extraction and detection of pathogenic *E. coli* O157:H7 cells from whole milk samples. We use the Clinical and Laboratory Standards Institute (CLSI) limit of detection (LoD) definitions that take into account the variance from both positive and negative samples to determine the diagnostic LoD. According to the CLSI definition, the NAIL device has a limit of detection (LoD) of 1,000 CFU/mL for *E. coli* cells artificially inoculated into whole milk, which is two orders of magnitude improvement to standard tube-LAMP reactions with diluted milk samples and comparable to lab-based methods. The NAIL device potentially offers significant reductions in the complexity and cost of traditional nucleic acid diagnostics for POC applications.

INTRODUCTION

Diagnostics impact healthcare and food safety decisions by providing professionals the information needed to improve care, safety, and decision making. Point-of-care (POC) diagnostics can provide faster results with minimal instrumentation and user interaction compared to lab-based diagnostics.[1] The rapid results and simplified testing provided by POC tests can lead to improved clinical outcomes, while reducing the cost of testing and care. POC diagnostic platforms offer benefits for quickly diagnosing and monitoring hospital infections, viral and bacterial diseases in developing nations, and the presence of pathogenic bacteria in the field or at food production facilities.

Nucleic acid amplification tests have become the gold standard for many infectious disease diagnoses due to their high sensitivity and specificity, rapid operation, and low limits of detection.[2–4] As of June 2014, the Food and Drug Administration (FDA) has approved over 150 different nucleic acid diagnostic tests for detecting pathogens including influenza, *Mycobacterium tuberculosis*, and *Staphylococcus*, with many more under commercial development for applications including global health and food safety testing.[5] Some nucleic acid tests also have benefits including quantitative detection, such as real-time quantitative polymerase chain reaction (qPCR), and the versatility to detect multiple targets using multiplexing strategies.[6]

Despite the advantages of nucleic acid tests, the requirements of complex sample preparation and lab-scale instrumentation limits PCR's use to centralized laboratories with skilled personnel. All current clinical diagnostic nucleic acid tests are considered high complexity by the United States Clinical Laboratory Improvement Amendments (CLIA), with the exception of a few systems, such as the Cepheid GeneXpert, BD Diagnostics BD Max System, and Biofire Diagnostics FilmArray RP system which are characterized as medium complexity, and the Alere i influenza A&B test, which was recently the first nucleic acid test to be granted waived status (Jan 2015).[7–9] High complexity tests are limited to central laboratories, while medium complexity tests can be performed near patients at a hospital, but are not CLIA waived tests necessary for POC applications. Extraction and purification for PCR tests typically involves procedures that include multiple binding, washing, centrifugation, and elution steps that are labor intensive and time consuming. Commercial PCR instruments have been developed to automate sample preparation, amplification, and detection, but these systems still require thermocyclers, complex fluidic systems, and/or fluorescent laser detection that increase the cost and infrastructure requirements for testing.

Isothermal amplification methods (e.g. NASBA, HDA, RPA, NEAR, LAMP) have been developed to replace thermocycling equipment with heating blocks, water baths, or other simple heating methods.[10–

[13] Some isothermal amplification methods, including loop-mediated isothermal amplification (LAMP), also do not require laser fluorescent detection.[14,15] These characteristics make isothermal amplification techniques advantageous for nucleic acid diagnostics—the Alere i uses isothermal nicking enzyme amplification reactions (NEAR)—but integration with sample preparation and detection remains challenging.[7,10] Work has been done to improve sample preparation and overall device integration for isothermal amplification diagnostics, but further improvements with regards to necessary user steps, required instrumentation, or overall complexity are still necessary for point-of-care application.[16–19]

We have developed the NAIL (**N**ucleic **A**cid **I**sotachophoresis **L**AMP) nucleic acid detection device that extracts nucleic acids from complex samples in a single step, uses automated, user-free valves and pumps, and provides sensitive and specific results in less than an hour, making it well-suited for potential POC use. NAIL uses isotachophoresis (ITP) and loop-mediated isothermal amplification (LAMP) to extract and amplify nucleic acids from complex samples in an integrated chip, with mobile phone detection of results.

ITP is an electrokinetic technique that separates and concentrates ionic molecules, including DNA and RNA, from complex samples with little or no sample preparation.[20] ITP only requires leading electrolyte (LE) buffer, trailing electrolyte (TE) buffer, and an electric field to extract, purify, and transport nucleic acids from complex samples. The need for laborious centrifuging, binding, washing, and eluting protocols are avoided using single-step ITP purification. ITP compatibility with nucleic acids in whole blood, urine, and soil samples has been demonstrated.[20–27] Rogacs, Marshall, and Santiago offer a detailed review of nucleic acid purification using ITP.[20]

LAMP is a sequence specific, isothermal nucleic acid amplification protocol developed by Notomi and co-workers.[28] LAMP nucleic acid amplification is differentiated from PCR in that it requires a single constant temperature to amplify so that a thermocycler is not required. LAMP also uses four or six primers to detect six distinct sequences for specificity. Finally, it produces large amounts of DNA that can be more easily detected using fluorescent calcein dye, eliminating the need for post-amplification confirmation.[15]

The flow in the NAIL device does not require user intervention. We integrate capillary valves and heated air chambers that act as passive valves and pumps, which reduce fabrication complexity and operation compared to devices with moving parts. Reagents are also stored dried on-chip. The device requires minimal user interaction and infrastructure to achieve sample-to-answer results. Device operation consists of adding buffer and sample to the inlets of the device, applying an electric field for ITP, and heating the device before reading out the answer using a mobile phone camera.

Using our NAIL device, we demonstrate detection of *Escherichia coli* (*E. coli)* O157:H7 from whole milk samples. *E. coli* O157:H7 is a strain of bacteria that produces shiga-like toxins that can cause infec-

tions in humans and is a main source of food-borne illness.[29,30] Infection by *E. coli* O157:H7 often results in severe hemorrhagic diarrhea and in a small percentage of cases, hemolytic uremic sydrome, which can cause kidney failure.[31] Routine screening of food products for *E. coli* O157:H7 and related pathogens is necessary to prevent food-borne illness outbreaks. Scharff recently estimated the overall negative economic impact of foodborne illness in the United States to be approximately $77 billion per year.[32]

Food samples are complex matrices with low levels of target pathogens and high levels of background flora that makes detecting targets challenging. Pre-enriched cell cultures followed by confirmatory tests are commonly used for food screening and patient diagnosis, but require specialized laboratories and time-to-results can take multiple days to complete.[33] POC food screening diagnostics that can rapidly test in the field or at food production facilities may reduce the negative health and economic impact of food-borne illness.

In this paper, we detail NAIL device design and demonstrate extraction, amplification, and detection of *E. coli* O157:H7 cells inoculated in whole milk using the device. We use whole milk because *E. coli* O157:H7 contaminated raw milk has previously caused infection outbreaks, whole milk is a complex sample that poses challenges for nucleic acid analysis, and it is widely available.[34] We describe geometry considerations for valves, pumps, and separation channels, as well as LE and TE electrolyte chemistry. The limit of detection (LoD) of the NAIL device is reported according to the Clinical and Laboratory Standards Institute's definitions and compared directly to standard tube-LAMP amplification reactions with unprocessed, diluted samples. Our results show that using the NAIL device with ITP purification can reliably detect 10^3 CFU/mL, which is two orders of magnitude lower than LAMP in unprocessed, diluted milk. This LoD compares favorably to PCR with manual purification, as well as other reported detection methods. The NAIL device potentially offers significant reductions in the complexity and cost of traditional nucleic acid diagnostics for POC applications.

EXPERIMENTAL METHODOLGY

NAIL Device Overview

The NAIL device consists of six main components, shown in Figure 1A: LE and TE reservoirs, LE channel and ITP separation channel, extraction chamber, capillary valve, enclosed air chamber, and reaction chamber. The LE and TE inlets allow filling of the device with buffer and sample, and hold electrodes for applying electric fields. The ITP separation channel is where extraction and purification of DNA from impurities in the sample occurs, and the LE channel connects the LE inlet to the extraction chamber. Purified DNA arrives in the extraction chamber before being pumped into the reaction chamber where amplification and detection occurs. The capillary valve between the extraction and reaction chambers prevents buffer from wetting the reaction chamber with dried LAMP reagents before purified DNA has reached the extraction chamber. The enclosed air chamber generates pressure when heated to

65°C that forces the fluid in the extraction chamber to break the capillary valve, pump purified DNA from the extraction chamber to the reaction chamber, and isolate the reaction chamber from the rest of the device. The solution containing target DNA dissolves the LAMP and indicator reagents and undergoes isothermal amplification at 65°C. The amplified DNA is imaged in the reaction chamber using a mobile phone camera.

NAIL testing requires one hour (15 minutes for lysis, 4 minutes for ITP extraction, 45 minutes for LAMP amplification, and 1 minute for imaging) and five basic steps to operate. First, the sample containing target cells is added to TE buffer with lysing agents in a microtube that effectively dilutes the sample by two, and allowed to incubate for 15 minutes. Second, the NAIL device is filled with LE and the TE-sample mixture is added to the TE inlet. Third, applying an electric field extracts the DNA from whole milk and transports it to the extraction chamber. Fourth, the chip is placed onto a hot plate at 65°C to activate the air channel pump and the LAMP reaction occurs for 45 minutes. Finally, the chip is placed into the mobile imaging unit for fluorescent detection using a mobile phone.

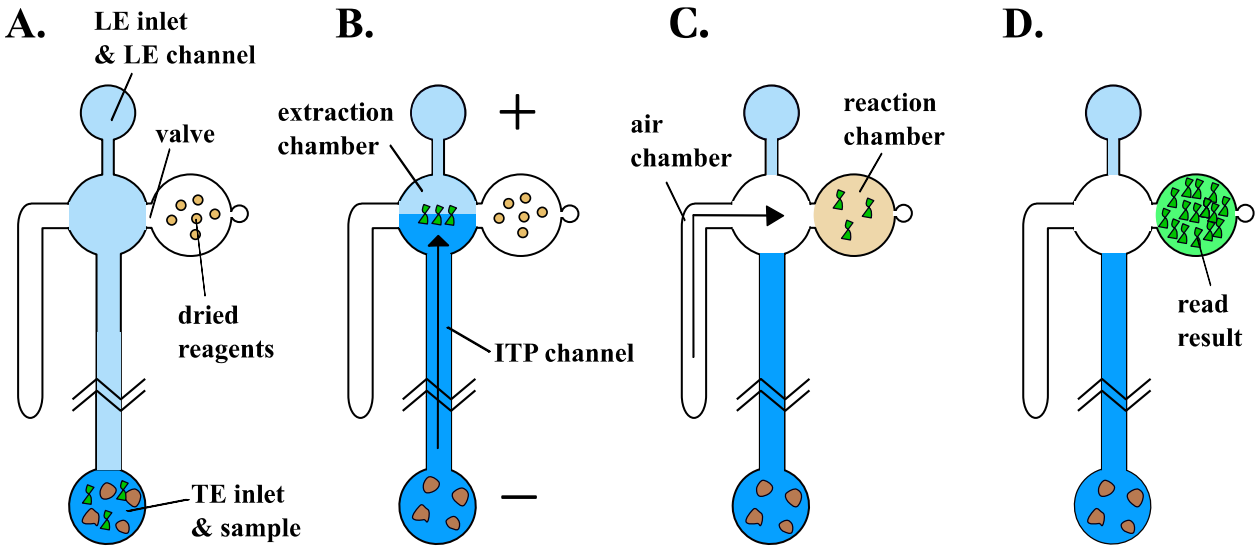

Figure 1. Schematic of device components and operation. Panel A shows the LE inlet and chip filled with LE buffer (light blue), and TE inlet filled with sample mixed with TE buffer (dark blue). (B) Applying an electric field isolates the DNA by ITP in the extraction chamber, purifying it from inhibitors in the sample. (C) Heating the chip to 65°C increases the pressure in the air chamber that breaks the capillary valve and drives flow of purified DNA solution in the extraction chamber to the reaction chamber. The purified DNA solution rehydrates the dry stored LAMP reagents and the air expansion isolates the reaction chamber from the rest of the chip. (D) LAMP specifically amplifies the target DNA sequence to produce a fluorescent signal that can be imaged with a mobile phone to detect the presence (or absence) of target nucleic acid.

NAIL Device Design and Operation

The geometry and surface properties of the microfluidic channels control the majority of device operation. Native styrene-ethylene/butylene-styrene (SEBS), our substrate material, is a moderately hydrophobic polymer, but offers a relatively stable hydrophilic surface following plasma treatment.[35] We plasma treat the flat and molded substrates prior to assembling the device to create hydrophilic surfaces for capillary flow. Following plasma treatment, adding LE buffer to the LE inlet causes the entire channel to fill almost instantaneously as shown in Figure 2A and supplementary video S1. Liquid does not flow into the air channel because it is sealed, resulting in a dead-end channel. The capillary valve is designed to prevent liquid flow into the reaction chamber during filling and ITP DNA extraction, and then burst when the air pressure is increased by heating the chip to 65°C. The capillary valve, shown in Figure 2B and Supplementary Video S2, works by forming a liquid-gas interface at a rapidly diverging channel wall that effectively increases the contact angle of the flowing solution and creates an energetic barrier to flow into the reaction chamber.[36] An external pressure greater than the pressure drop across the bulging interface must be applied to force liquid to wet the diverging wall and allow fluid to continue advancing into the reaction chamber. A detailed description of the capillary valve is given in the Supplementary Information (SI).

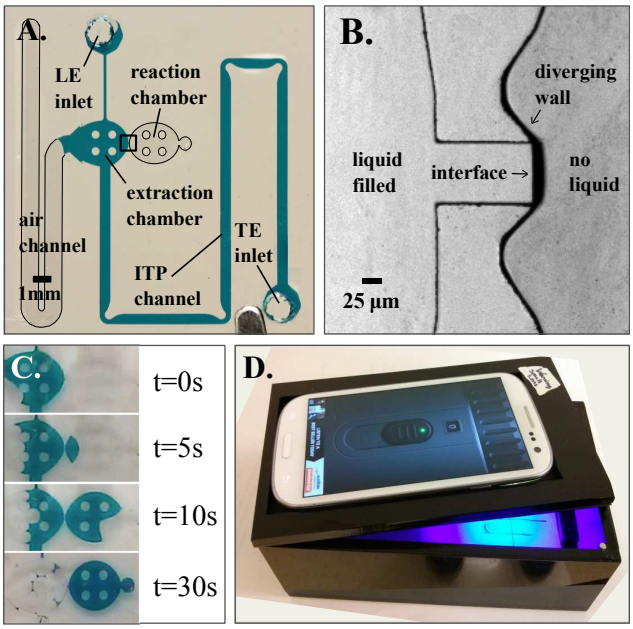

Figure 2. Image A shows a fabricated device filled with colored LE via capillary flow. The black square is the location of the capillary valve between the extraction and reaction chambers. (B) A 4x micrograph of the capillary valve that prevents LE from wetting the reaction chamber. The liquid has fully filled the extraction chamber on the left and the valve up to the diverging wall, with no liquid present in the reaction chamber on the right. (C) Sequential images of fluid flow from the extraction chamber to the reaction

chamber after the NAIL chip is placed onto a hotplate at 65°C. The liquid in the reaction chamber is completely isolated after 30 seconds of heating. (D) The mobile imaging unit consisting of a dark box with components that snap into place with magnets, an excitation and emission filter, and mobile phone.

Heating the microchannel to the 65°C required for LAMP activates the air channel pump. The pressure generated by the heated air channel achieves three goals: (1) it overcomes the energetic barrier created by the capillary valve; (2) assists in driving flow towards the reaction chamber along with capillary forces; and (3) displaces liquid from the extraction chamber so that the reaction chamber is fluidically isolated during amplification. The pressure generated by the air channel is dependent on the temperature after heating, T_2, and the temperature during device filling, T_1, and is given by the ideal gas or Gay-Lussac's law, $P_2 / P_1 = T_2 / T_1$. This pressure breaks the capillary valve and, along with capillary flow, drives liquid to fill the reaction chamber. The volume change caused by the enclosed air chamber is given by,

$$\Delta V = V_1\left[\left(\frac{T_2}{T_1}\right)-1\right] \quad (1)$$

where ΔV is the change in air volume, V_1 is the initial air channel volume, T_1 is the initial temperature before heating, and T_2 is the final temperature after heating. We designed the enclosed air chamber to have a volume change that is 90% of the extraction chamber volume when heated from room temperature, 25°C, to 65°C. This volume change causes liquid to evacuate the extraction chamber and isolate the filled reaction chamber during amplification of the target DNA, as seen after 30 seconds in Figure 2C. Isolation is necessary to prevent dilution and diffusion of LAMP reagents and purified nucleic acids during amplification.

The balance between the hydraulic resistances of the LE channel, ITP channel, as well as the valve and reaction chamber control the thermopneumatic flow driven by the air channel. First, the hydraulic resistances in the LE and ITP channels should be large relative to the valve burst pressure such that the pressure generated by heated air causes the valve to burst with only marginal flow through the LE and ITP channels. Second, the hydraulic resistance of the valve channel should be less than the hydraulic resistance of the ITP channel and LE channel to promote pressure driven flow, along with capillary flow, towards the reaction chamber after the valve bursts. Finally, the hydraulic resistances of the ITP channel and LE channel should be approximately equal to avoid preferential flow through either channel. After filling the reaction chamber, the remaining liquid will begin to evacuate the extraction chamber, thereby isolating the reaction chamber, by flowing through the LE and/or ITP channels towards the LE and/or TE inlets. Preferential flow through the LE or ITP channel may result in the reaction chamber retaining a fluid connection to one of the channels and prevent isolation of the reaction chamber. For example, Figure

2C at t=10s would have the possibility to retain fluid connection if there was preferential flow through either the ITP or LE channel. We performed hydraulic circuit calculations to model the resistances and flows into each channel during heating, using $\Delta P = QR_H$, where ΔP is the pressure differential, Q is the volumetric flow through the channel, and R_H is the hydraulic resistance of the channel.[37] The hydraulic circuit calculations are shown in the SI.

The ITP channel must contain sufficient ITP separation capacity in order to fully separate and purify DNA from contaminants.[38] Separation capacity is the amount of charge that passes through an ITP system before the target analyte reaches its final destination, in this case DNA reaching the extraction chamber, and is approximately dependent on channel volume and leading ion concentration.[20,38] The separation capacity should be greater than the separation parameter, the amount of charge required to separate two groups of ions with different mobility, to isolate one group of ions from the other during ITP.[38,39] The SI details the separation capacity of the NAIL device and separation parameter for DNA and fatty acids in whole milk. We also use optimized turns to reduce dispersion of the plug during ITP.[40]

We use qualitative fluorescent calcein detection to determine positive and negative samples.[15] The fluorescence from calcein is imaged following amplification using a mobile phone camera, excitation and emission filters, and a dark box.[41] Figure 2D shows the mobile imaging unit. Magnets hold the components in place so that the mobile phone and filter alignments are consistent for each image.

Device fabrication

We molded our NAIL devices by solvent casting styrene-ethylene/butylene-styrene (SEBS, 42 wt% PS, A1536H, Kraton) polymer onto a silicon wafer patterned with SU-8 photoresist as previously described with minor modifications.[35,42] The full fabrication procedure is detailed in the SI.

After fabrication of the molded substrate, ITP inlet ports and an air valve at the edge of the reaction chamber were punched using a hand punch (Fiskars 1/16" Circle Hand Punch) and 20-gauge needle respectively. Molded and flat substrates were air plasma treated at 200 mTorr and 30 W for 15 s (Harrick Plasma Cleaner, Ithaca, NY). After air plasma treatment, LAMP reagent mixture was pipetted onto the reaction chamber surface and the substrate was placed under vacuum with desiccant for 1-2 hours to dry the reagents on the surface before being manually pressed together with the flat substrate to seal. To ensure good room temperature bonding strength, the substrates were handled inside of a decontaminated biosafety cabinet with laminar flow as much as possible to keep the substrate surfaces clean. After bonding, we attached 5 mm high wells cut from polyvinyl chloride (PVC) tubing (3.175 mm ID, 6.35 mm OD) with UV curable epoxy and a UV transilluminator to hold LE and TE solutions during ITP. Aluminum foil strips were placed under the reaction chamber during UV exposure to protect the dried LAMP reagents.

ITP chemistry and operation

The LE consisted of 600 mM Tris and 200 mM HCl buffer with 0.001% (w/v) Tween-20, 0.075% (w/v) polyvinylpyrridine (PVP), and 1x SYBR green (Life Technologies, Grand Island, NY). The TE was 90 mM Tris buffer and 37.5 mM HEPES buffer with 0.5 mg/mL proteinase K (EO0491, Thermo Scientific, Pittsburgh, PA), 0.1% (w/v) Tween-20, 0.1% (w/v) PVP, 1x SYBR green, 5000 U Ready-Lyse™ Lysozyme (lysis buffer, Epibio, Madison, WI), 50% (v/v) whole milk (local grocery store), and varying amounts of *E. coli* O157:H7 genomic DNA (0801622DNA-10UG, Zeptometrix, Buffalo, NY) or inactivated *E. coli* O157:H7 cells (NATECO(933)-ERCM, Zeptometrix, Buffalo, NY). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

To initiate ITP, 45 μL of LE buffer was pipetted into the LE inlet and allowed to fill the device, followed by pipetting 30 μL of the TE buffer mixed with sample into the TE inlet. The increased LE inlet volume applies slight pressure against the migration of the ITP plug, resulting in increased separation and extraction of DNA.[21,25,43] We used platinum electrodes placed into the LE and TE inlets to apply 450 μA of constant current using a sourcemeter (Keithley 2410, Cleveland, OH). ITP was monitored using DNA-intercalating SYBR green dye and an inverted epifluorescence microscope (Nikon TE2000, Melville, NY), a fluorescent optical filter set (XF108-2, Omega, Brattleboro, VT), 2x or 4x objective, and cooled digital camera (Cascade IIb, Photometrics, Tucson, AZ).

After viewing the DNA plug enter the extraction chamber, the current was stopped and electrodes were removed before setting the chip onto a hotplate. The current or voltage can also be monitored to track the location of the plug without optics or labeling.[44] We quantified the ITP extraction efficiency by mixing known amounts of genomic DNA with the TE and imaging the fluorescence of DNA 3 mm away from the extraction chamber during ITP. This fluorescent intensity was compared to a calibration curve of fluorescent intensity versus DNA concentration to determine the extraction efficiency. Between each test, the platinum electrodes were soaked in DNA Erase solution for 2 min, followed by ethanol for 5 min, and DI water for 5 min to reduce contamination.

LAMP reactions

We conduct LAMP amplification experiments with three different methods. First, we conduct LAMP in the NAIL device with a range of target *E. coli* concentrations in whole milk diluted 2x and purified by ITP. Second, we perform LAMP experiments in centrifuge tubes with a fixed concentration of pure *E. coli* genomic DNA in a range of dilutions (10-10000x) of whole milk, as well as with no milk. Third, we complete LAMP experiments in centrifuge tubes with a range of *E. coli* target concentrations (10^2-10^6 CFU/mL) in a fixed milk dilution of 1000x.

For the experiments conducted in the NAIL devices, we prepared 10 μL of LAMP reagents in a 1-mL microtube for drying on the reaction chamber surface. The six LAMP primers (F3, B3, FIP, BIP, LF, LB) are provided in supplementary table S1 and were developed by Wang et al. to target the *eae* gene of *E.*

coli O157:H7.[45] The primers used in this study were shown to be specific for seven shiga toxin producing E. coli strains, including O157:H7, among 90 different bacteria tested to evaluate assay specificity.[45] A 10 µL stock mixture consisted of 8mM $MgSO_4$, 0.5 mM $MnCl_2$, 25 µM calcein disodium salt, 1.6 mM FIP and BIP, 0.8 mM LF and LB, 0.2 mM F3 and B3, 1.4 mM of each nucleotide, 2% (w/v) sucrose, and 0.32 U/µL Bst 2.0 Warmstart polymerase (M0538M, New England Biolabs, Ipswich, MA). The sucrose helps to stabilize the dried reagents and we observed quality amplification for up to two weeks after drying, the longest period tested in this study.[46] The LE and TE serve as the reaction buffer, so the 1x Thermopol buffer (New England Biolabs) is omitted in the drying mixture. 500 nL of this reaction mixture (the reaction chamber has a 500 nL volume) was pipetted and dried under vacuum on the reaction chamber surface before sealing the device. We placed the NAIL device onto a hotplate set at 65°C for 45 mins after DNA entered the extraction chamber from ITP. Silicone oil was pipetted over the air valve and into the inlets to prevent evaporation and equalize hydrostatic pressures.

For the tube-LAMP milk dilution tests, 10 µL reactions were prepared consisting of 1x Thermopol buffer (New England Biolabs), 6 mM $MgSO_4$, 0.5 mM $MnCl_2$, 25 µM calcein, 1.6 mM FIP and BIP, 0.8 mM LF and LB, 0.2 mM F3 and B3, 1.4 mM of each nucleotide, 0.32 U/µL Bst 2.0 Warmstart polymerase, 100 pg of pure *E. coli* O157:H7 genomic DNA, and 10-fold dilutions of whole milk. Positive and negative (*E. coli* DNA omitted) controls consisting of the same reaction mixture without the milk were run with each test. The reaction tubes were placed into a water bath heated to 65°C for 45 minutes. We determined the results by placing the tubes flat inside our mobile imaging unit and imaging the calcein fluorescence.

For the tube-LAMP reactions at 1000x milk dilution with varying cell concentrations, a 10 µL reaction consisted of the same reagents as the milk dilution tests except with 0.001 (v/v) whole milk, and 10-fold dilutions of *E. coli* O157:H7 cells (CFU/mL). The cells were lysed with 5000 U of Ready-Lyse Lysozyme, the same as the NAIL experiments. Negative and positive controls with no milk and no *E. coli* (negative) or 100 pg of *E. coli* genomic DNA (positive) were tested with each experiment. The reaction tubes were placed into a water bath heated to 65°C for 45 minutes. We determined the results by placing the tubes flat inside our mobile imaging unit and imaging the calcein fluorescence.

Mobile phone imaging

The mobile imaging unit was made with 0.6 cm thick (0.23 inch) black acrylic and consisted of a box with detachable lid and embedded optical filters, as well as a mobile phone aligner. The box had a length of 16.5 cm (6.5 inches), width of 9.5 cm (3.75 inches), and height of 6.9 cm (2.72 inches). Acrylic cement was used to bond the walls of the box together. We allowed the lid and bottom of the box to be detachable from the walls by imbedding magnets (1/8 x 1/8 inch cylinder neodymium) into the plastic. A mobile phone alignment piece was also held in place with magnets on top of the lid to ensure proper alignment of

the mobile phone. Two holes were cut in the lid for the excitation filter (Omega XF1073, Stamford, CT) and the emission filter (Omega XF3084, Stamford. CT). The holes were cut specifically to align the excitation and emission filters with a Samsung Galaxy III (Samsung, South Korea) camera and flash. The filters were held in place with a plastic piece attached to the bottom side of the lid with magnets to create a lip for the filters to rest on. A 0.5 mm (0.02 inch) thick silicon rubber sheet, cut with holes specifically aligned to the camera and flash, was placed over the lid to seal out contaminating light.

We imaged the reactions by placing the chip or tube inside of the mobile imaging unit and taking a picture using a Samsung Galaxy III mobile phone set on top of the box. The free Android application "Camera FV-5 Lite" was used for imaging so that camera settings could be controlled and better transferred between Android phones. We used the close-up focus mode, auto white balance, 1600 ISO, and +1 exposure for imaging. The images were analyzed in ImageJ. Each image was split into RGB images, the blue image was subtracted from the green image for background correction, and the reaction chamber or reaction volume in-tube was manually selected to calculate the average intensity. Images were also taken using an epifluorescence microscope to ensure quality of mobile phone results.

Limit of detection

We determined the LoD of the NAIL device and tube-LAMP for detecting *E. coli* O157:H7 cells in whole milk. To control for differences between the tests, we based our LoD results on the initial concentration (CFU/mL) of the raw sample before any dilution or extraction. Initial concentration is commonly used for defining detection limit requirements because the goal in many testing environments is to detect a certain pathogen load present in a sample of interest. For example, the FDA typically sets detection requirements in terms of CFU/mL or CFU/g in the original sample. For the NAIL experiments, we diluted the initial sample 2x in TE buffer before adding it to the device. For the tube-LAMP experiments, we diluted the initial sample 1000x in the final reaction because it is the least amount of milk dilution that gave quality results. We tested 10-fold dilutions of cell samples ranging from 1-10^5 CFU/mL in the original milk samples for NAIL. We also ran identical NAIL tests without cells for negative controls. Devices were disposed into 10% bleach after each experiment to reduce the risk for contamination. For tube-LAMP, we diluted the original milk sample with lysed cells 100x and then added 1 µL of this 100x dilution to 9 µL of LAMP reagents for a 1000x dilution. The initial cell concentrations for these tests were 10^2-10^6 CFU/mL.

The cutoff and LoD for these qualitative tests was determined according to the Clinical and Laboratory Standards Institute's EP12 "User protocol for Evaluation of Qualitative Test Performance; Approved Guideline—Second Edition" and EP17 "Protocols for Determination of Limits of Detection and Limits of Quantification; Approved Guideline" documents.[47,48] The limit of blank (LoB) was used as the cutoff and calculated by the parametric procedure, $LoB = \mu_B + 1.645\sigma_B$, where μ_B is the mean of the negative con-

trol samples and σ_B is the standard deviation of the negative control samples.[47] The LoD was defined as the upper endpoint of the 95% interval, C_{95}. The C_{95} is the lowest analyte concentration where at least 95% of the tested samples are determined to be positive according to the established cutoff. The LoD was determined to be the sample concentration that has a mean response that is at least 1.645 sample standard deviations above the LoB, $LoD = \mu_S = \mu_B + 1.645\sigma_B + 1.645\sigma_S$, where μ_S is the sample mean and σ_S is the sample standard deviation.[47] Using these definitions, we have a 5% probability of committing either type I (false positive) or type II (false negative) error at the LoD. The confidence that our LoD is at or above the C_{95} concentration is based on the number of correct tests run at the LoD and calculated according to Table A2 in the EP12 document.[48]

RESULTS AND DISCUSSION

Isotachophoresis

We have demonstrated the extraction and purification of nucleic acids from *E. coli* O157:H7 cells inoculated in whole milk samples using ITP. Whole milk samples contain an abundance of calcium ions, proteins, and fatty acids that can inhibit amplification reactions. ITP extracts and purifies nucleic acid for downstream amplification with minimal sample preparation and reduces manual steps compared to standard bead or column based purification techniques. ITP requires 2 pipetting steps (adding sample to TE and adding TE/sample mixture to chip) and pushing a button to apply an electric field, while standard purification techniques require over 10 pipetting steps and vacuum or centrifuging procedures. To prepare the samples, we add whole milk inoculated with target cells or genomic DNA to TE buffer in a microtube at a 1:1 ratio, resulting in two times dilution of the original milk sample. The TE buffer contains enzymatic lysing agent to lyse the cells at room temperature and release the DNA, as well as proteinase K to digest proteins that may bind DNA and alter its mobility.[20] The Ready-Lyse Lysozyme is added in excess to efficiently lyse the cells at room temperature and release the DNA into solution, while the tween-20 assists in lysis and solubilizing proteins following lysis.[20] After 15 minutes of incubation, the TE and sample mixture can be added directly to a device previously filled with LE buffer in order to extract and purify the DNA for amplification using ITP.

After the NAIL device is filled with LE and sample has been added, applying an electric field extracts the DNA from amplification inhibitors present in the milk. Cations, such as calcium present in milk, electromigrate in the opposite direction of the anionic DNA and do not exit the TE well. Anions, such as proteins and fatty acids electromigrate in the same direction as DNA, but have slower electrophoretic mobilities and are not able to overcome the TE buffer to enter the ITP plug. We adjust the pH of the TE buffer to 7.9, which results in a HEPES mobility of approximately $1.4\text{x}10^{-8}$ $Vm^{-2}s^{-1}$ after conductivity corrections.[49–52] The measured mobility of the proteins and fatty acids from milk in TE buffer are approximately $0.6\text{x}10^{-8}$ $Vm^{-2}s^{-1}$, while the DNA should have an electrophoretic mobility of approximately

$2.7x10^{-8}$ V/m^2s in the TE buffer.[53] Fully separating the DNA from the fatty acids and proteins requires 51.7 mC of separation capacity, as shown in the SI, which is less than the 108 mC separation capacity of the system, meaning DNA should separate from the fatty acids and proteins in the milk sample.[38] The ITP separation process only requires four minutes after applying the electric field.

Figure 3A shows a fluorescent image of *E. coli* O157:H7 DNA extracted and purified from cells inoculated in whole milk using ITP. The image is taken approximately 3 mm below the extraction chamber. We extract up to 10% of the DNA from an initial TE/sample volume of 30 uL using a semi-infinite injection scheme. Marshall has shown up to 80% extraction of nucleic acid from a 25 uL whole blood sample using finite injection ITP with large separation channel volume and high buffering capacity,[26] which may be incorporate into future designs to improve extraction efficiency. Figure 3B shows an image of the chip after DNA has entered the extraction chamber. The fatty acids and proteins, shown in Figure 3C, only migrate about 60% of the channel length before the current is stopped, indicating that the DNA has been purified from the milk.

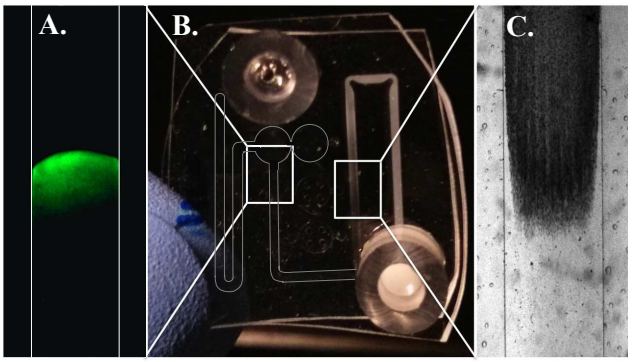

Figure 3. (A) Fluorescent image of an ITP plug containing purified DNA from *E. coli* O157:H7 cells bound to 1x SYBR green dye 3 mm below the extraction chamber. (B) An image of the NAIL device after DNA has reached the extraction chamber, showing milk in the TE reservoir and filling approximately 60% of the channel. Approximate channel dimensions are drawn to show the location of the plug. (C) Transmission micrograph of milk in the channel (dark) at the termination of ITP. ITP sufficiently purifies the DNA from inhibitors present in the milk sample and allows for amplification downstream from only a 2x diluted sample.

LAMP is known to be robust to a number of perturbations and more tolerant to inhibitors than PCR reactions.[54] However, we found that significant dilution of unprocessed whole milk samples is required for the tube-LAMP experiments before quality amplification occurs. Figure 4 shows the normalized fluorescent intensity of LAMP experiments conducted in centrifuge tubes with 100 pg of pure genomic DNA in milk with various dilutions ranging from 10 to 10,000x. This data is normalized using a positive

control with 100 pg of genomic DNA and a negative control with no DNA in water, denoted by the infinite (inf) data point. The data shows that the LAMP is unable to properly amplify the DNA in the presence of high concentrations of milk, *i.e.* at or below ~500x dilution. 1000x dilution was the smallest dilution amount that gave consistent, expected results according to the established LoB cutoff. At the dilutions above 1000x, the fluorescence intensity is relatively stable, indicating proper amplification. Using samples purified by ITP, we are able to consistently amplify nucleic acids from milk using only two times dilution of the original milk sample, indicating that ITP is sufficiently purifying the DNA for amplification. These results are supported in Figure 3 and Figure 5, by the separation capacity calculations in the SI, and by previous work that has shown ITP-purified DNA is compatible with PCR, which is known to be more sensitive to inhibitors than LAMP.[20,24,26,54–56]

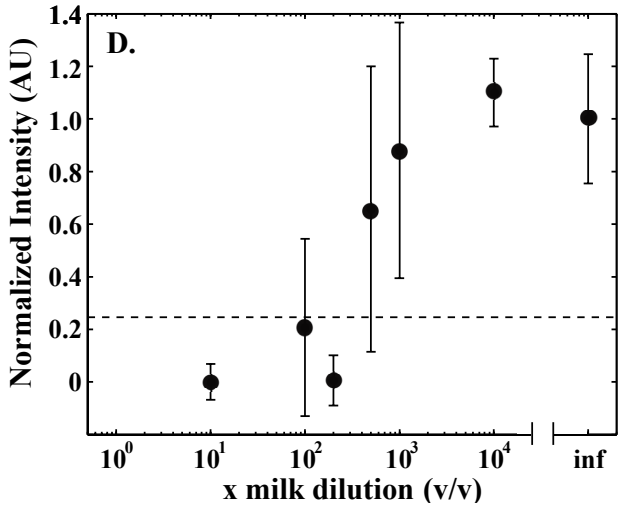

Figure 4. Average normalized fluorescent intensity of tube-LAMP reactions containing various dilutions of milk. The data is normalized by $(I_P - I_N)/(I_{P,\mathrm{inf}} - I_{N,\mathrm{inf}})$, where I is the fluorescent intensity; subscript P is the positive reaction; N is the negative reaction; P,inf is a positive control with no milk (infinite dilution); and N,inf is the negative control with no milk. The dashed horizontal line shows the cutoff used to determine positive versus negative reactions and is given by the LoB using the standard deviation of negative control reactions. Error bars represent $1.645\sigma_s$ where σ_s is the standard deviation of the samples. 1000x milk dilution was the lowest dilution amount that gave reproducible LAMP results above the cutoff according to the LoD definition.

Limit of Detection

To determine the limit of detection of the NAIL device, we tested 10-fold dilutions of cells (CFU/mL) inoculated in whole milk with the device. We compare these results to tube-LAMP amplification using 10-fold dilutions of cells (CFU/mL) inoculated in whole milk and then diluted 1000x, and report results

from previous studies using immunological or molecular methods to detect bacteria from milk. The purpose of these experiments is to show that ITP sufficiently purifies DNA for amplification from a minimally diluted sample, that the limit of detection for an initial sample concentration may be improved by using NAIL with ITP purification as opposed to simply diluting the sample, and that the NAIL LoD is comparable to other molecular methods such as lab-based PCR that require user intensive sample extraction preparation protocols. We use the Clinical and Laboratory Standards Institute (CLSI) definitions of limit of blank (LoB) and limit of detection (LoD) for determining our LoD.[47,48] Many publications currently report LoDs based only on measurements from blank samples, if any statistical measure is used at all. The rigorous CLSI statistical definitions take into account the variance in both positive and negative (blank) results when determining the LoD, and ensure that test concentrations at or above the LoD (up to the upper measuring limit) are truly positive at least 95% of the time according to the cutoff established by the LoB.

Figure 5A and 5B show unprocessed negative and positive mobile phone images of the reaction chamber following 45 min of LAMP amplification of *E. coli* O157:H7 DNA purified from cells inoculated in whole milk. The positive reaction chamber is clearly visible in the mobile phone image, while the negative reaction chamber is only faintly visible. Figure 5C shows the average normalized fluorescent intensities of at least 5 measurements at each cell concentration for the NAIL device (black circles) and at least 3 measurements for tube-LAMP with 1000x diluted milk (blue crosses). Supplementary Table S5 shows a summary of all experimental results. Both the NAIL device and tube-LAMP exhibit roughly sigmoidal responses due to the qualitative response of the calcein dye.

The solid horizontal line represents the cutoff for determining positive and negative samples for the NAIL device and the dashed horizontal line represents the cutoff for the tube-LAMP tests. The cutoffs are given by the LoB, or 1.645 times the standard deviation above the mean blank (negative reactions) response. Repeated tests at a concentration directly at the cutoff should yield positive results 50% of the time and negative results 50% of the time, meaning there is equal probability of committing type I and type II error.[48] The errors bars in Figure 5 may appear to show large variance, but note that we have plotted 1.645 times the standard deviation in order to provide clarity for the CLSI LoD definitions because the error bars, along with the cutoff, are used to determine the LoD. The lower bound of $1.645\sigma_S$ should be equal to or greater than the cutoff value to be considered at or above the LoD, which is visually shown in Figure 5. Figure S4 shows the same data with the 95% confidence intervals that have more narrow intervals for the fluorescence signal at each tested concentration. The 95% confidence intervals show our confidence in creating an interval that contains the true mean and that the mean fluorescence for the tests conducted above the LoD are unambiguously larger than those conducted below the LoD.

We determined the LoD of our NAIL device to be 10^3 CFU/mL because it is the lowest concentration for the given data that has a mean that is $1.645\sigma_S$ above the cutoff. Due to the qualitative response of the dye, we only considered actual test concentrations as possible LoD concentrations, as opposed to directly calculating the LoD concentration using the pooled sample standard deviation protocol from section 4.3 of the EP17-A document, which gives a lower LoD concentration. At this concentration, 10/10 samples were determined to be positive, giving us 76.6% confidence that 10^3 CFU/mL is at or above the C_{95} concentration.[48] Testing more samples would likely increase this confidence according to Table A2 in the EP17 document. For the 1000x dilution tube-LAMP experiments, the LoD was determined to be 10^5 CFU/mL, or two orders of magnitude higher than the NAIL device using ITP purification. The differences in initial dilution and ITP extraction efficiency approximately account for the LoD difference between NAIL and tube-LAMP. The tube-LAMP requires 1000x dilution, or 500x more dilution than NAIL, but the NAIL device has 10% extraction efficiency, which means that NAIL should be 50x better by a first approximation. The LoD of the NAIL device also has potential to be further improved with larger sample volumes and higher extraction efficiency, which are not feasible with diluted samples.

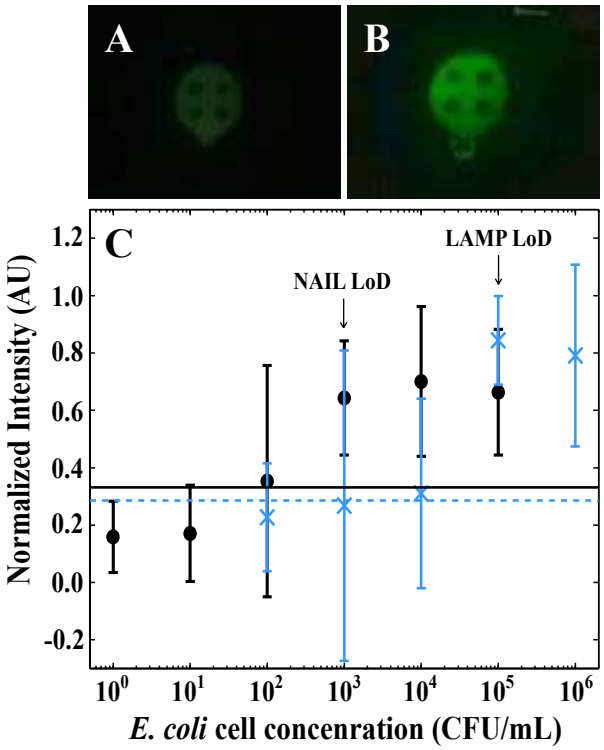

Figure 5. (A) Unprocessed image of the NAIL reaction chamber following a negative reaction. (B) Unprocessed image of the NAIL reaction chamber following a positive reaction. The fluorescent intensity of the positive reaction is noticeably greater than the intensity of the negative reaction. (C) Average normalized intensity of the NAIL device reaction chamber (black circles) and tube-LAMP reactions with 1000x

diluted milk (blue cross) as a function of cell concentration (CFU/mL) in the original milk sample. Error bars represent 1.645 times the sample standard deviation, $1.645\sigma_S$, for each data point according to the CLSI definition of LoD. The two separate data sets were normalized between 0 and 1 using $(I-I_{min})/(I_{max}-I_{min})$, where I is fluorescent intensity, I_{min} is the lowest intensity from negative responses, and I_{max} is the highest intensity from positive responses for each data set. The black solid line represents the NAIL device cutoff, while the blue dashed line represents the tube-LAMP cutoff as determined by the means and 1.645 times the standard deviations of negative control experiments. The data shows that the NAIL device can reliably detect 10^3 CFU/mL in the original milk sample, while the 1000x diluted milk samples can detect 10^5 CFU/mL. Using the NAIL device with ITP purification improves upon the limit of detection by two orders of magnitude.

Culture based methods are generally considered the gold standard for pathogen detection in food, but typically consist of multiple incubation steps (pre-enrichment, selective enrichment, selective and differential plating) before a biological, serological, or molecular confirmation test. More rapid tests including immunological and molecular tests have been developed to speed the detection process. Our LoD compares favorably to LoDs reported for other immunological and molecular based tests. Nogva et al. and Ibekwe et al. reported qPCR detection limits on the order of 10^3 (between 2E2-2E3 and 6.4E3 CFU/mL respectively) for detecting *L. monocytogenes* from milk and *E. coli* from milk wastewater, respectively.[57,58] Both of these studies used manual purification techniques such as bead based assays or other extraction kits. Hoehl et al. reported an LoD of 1E3 inserted *E. coli* cells from milk for qPCR and LAMP in their semi-automated LabSystem that corresponds to 1E4 CFU/mL for their 100 µL sample input. Daly et al. combined PCR amplification with ELISA detection to achieve an LOD of 1E2 *E. coli* CFU/mL from milk.[59] They state that manual purification and PCR without ELISA results in an LoD of 1E3 CFU/mL. Immunological based tests with surface plasmon resonance or electrochemical detection have reported detection limits of 1E2-1E3 and 1E4 *E. coli* CFU/mL from milk and lettuce respectively.[60,61] NAIL shows a comparable LoD, but has the advantage of being a rapid, integrated device that does not rely on considerable user interaction and significant ancillary, lab equipment.

SUMMARY

NAIL offers a potential POC solution to nucleic acid diagnostics. Our NAIL device has an LoD of 1000 CFU/mL, which is two orders of magnitude improvement compared to tube-LAMP with diluted samples. The LoD was determined using CLSI definitions that take into account the variance from both positive and negative samples when determining the LoD. The LoD is also comparable to many reported molecular and immunological based methods, but requires one hour total, has integrated sample extraction and amplification, few rapid manual steps, and is amenable to POC applications. Increasing extrac-

tion efficiency and processing larger sample volumes will serve to improve this LoD for future applications. Altogether, the lysis, extraction, and amplification process requires approximately one hour (15 minutes for lysis, 4 minutes for ITP, 45 minutes for LAMP, and 1 minute for detection) and five steps: adding sample to TE buffer, adding LE and TE-sample mixture to NAIL, applying an electric field for ITP, heating the chip, and taking an image of the chip with a mobile phone.

We expect this device to be applicable to a wide range of complex samples and target organisms. ITP has been shown to be compatible with a host of complex matrices including whole blood, urine, and soil, and we have demonstrated its compatibility with whole milk. We have also previously shown that ITP can be run using a watch battery and a low-cost electrical circuit for POC applications.[62] Detecting other organisms will only require slight modifications to the presented procedure, such as changing primers in the reaction mixture. We anticipate that the entire device including ITP, heating, imaging, and analysis can be powered and controlled using a mobile phone with low-cost electronics. The mobile phone will also allow for geo-tagging of field-based results and data transmission to storage databases for tracking disease transfer and record keeping. Future work will focus on integrating a low-cost heating solution, on-chip lysis, increased extraction efficiency, larger volume processing, and further automation of the device, with examples of many of these goals previously demonstrated.[11,12,23,26,63]

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