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Technical Note

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Bacterial RNA Extraction and Purification from Whole Human Blood Using Isotachophoresis

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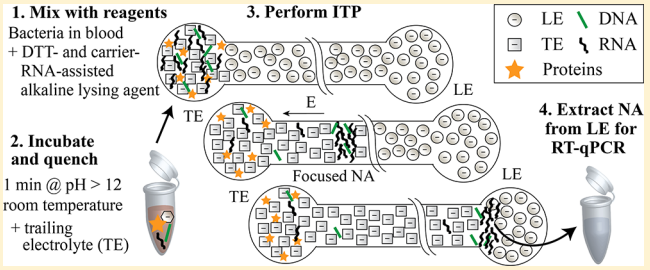
Supporting Information

ABSTRACT: We demonstrate a novel assay for physicochemical extraction and isotachophoresis-based purification of 16S rRNA from whole human blood infected with *Pseudomonas putida*. This on-chip assay is unique in that the extraction can be automated using isotachophoresis in a simple device with no moving parts, it protects RNA from degradation when isolating from ribonuclease-rich matrices (such as blood), and produces a purified total nucleic acid sample that is compatible with enzymatic amplification assays. We show that the purified RNA is compatible with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and demonstrate a clinically relevant sensitivity of 0.03 bacteria per nanoliter using RT-qPCR.



Nucleic acid amplification methods, including reverse transcription-quantitative polymerase chain reaction (RT-qPCR), targeting the 16S rRNA (16S rRNA) enable fast and specific detection of bacteria in complex samples such as whole blood.[1,2] 16S rRNA is a universal constituent of bacterial ribosomes present at high copy numbers (10^3–10^4 per actively growing cell).[3,4] Targeting these biomarkers can potentially increase assay sensitivity compared with the assays targeting the corresponding DNA.[5,6] Sample preparation is time-consuming and labor-intensive, involving one or more centrifugation steps, buffer exchange(s), lysing, and associated multistep processes of nucleic acid extraction and purification from PCR inhibitors. The latter can include endogenous species, such as heme proteins, lactoferrin, and immunoglobin, as well as species used in preparation, including heparin, denaturants, surfactants, chaotropic salts, and alcohols.[7,8] These sample preparation steps can be difficult to integrate, automate, or hasten, particularly in a miniaturized system.[9,10]

Fundamentally, RNA can be an exceedingly challenging blood biomarker due to its extreme lability. RNA is susceptible to the ubiquitous action of ribonucleases (RNases)[11] and degradation to physicochemical conditions including elevated temperature (>65 °C)[12] and high pH.[13−15] Careful decontamination together with assay standardization or automation can mitigate the effects of exogenous RNase, but only careful design of assay chemistry can provide sufficient protection against the abundant endogenous RNases in blood. As one salient example, without adequate RNase control, free RNA is nonamplifiable after 15 s of incubation in plasma or serum.[11]

To date, the majority of microfluidic-based sample preparation approaches have focused on DNA isolation, and mainly via the miniaturization of solid-phase extraction (SPE) methods.[16−19] SPE-type approaches include specialized structures such as packed beads, monolithic porous structures, and magnetic beads. These approaches are well-established in traditional settings but require multiple reagent wash steps and specialized fabrication or manipulation (e.g., pumping liquids for wash steps or moving magnets for beads). Further, SPE binding capacity can be low due to competitive protein absorptions (e.g., to silica) and the presence of PCR inhibiting chemistry such as guanidinium thiocyanate (GuCN), guanidinium chloride (GuHCl), isopropyl alcohol, or ethanol.

Of the few studies reporting RNA purification using a microfluidic platform,[20−27] to our knowledge, only two have reported using blood or blood product as a sample matrix.[20,22] Witek et al. employed an array of photoactivated polycarbonate micropillars as the solid phase to purify total RNA from bacterial cells suspended in whole blood.[22] Their protocol eliminated the PCR-inhibiting guanidine chemistry by adopting thermal and mechanical lysing approaches. They also report a high, postextraction RNA integrity. However, their assay did not address the need for adequate RNase control[4,22] (see Tsui et. al[11] for a discussion of RNA stability in blood products). We attribute their high RNA integrity to the very large number of bacteria they spiked into their blood sample. We estimate they spiked 18 000 ¢/nL-blood (where ¢ is the number of bacterial cells), which is on the order 10^6-fold higher bacteria concentration than the current work. Further, their protocol required ethanol, which can inhibit PCR if not sufficiently removed.

Instead of SPE, Root et al.[20] used an oligonucleotide polymer capture matrix to purify free RNA spiked into serum. This aqueous purification process reported an impressive 375 RNA

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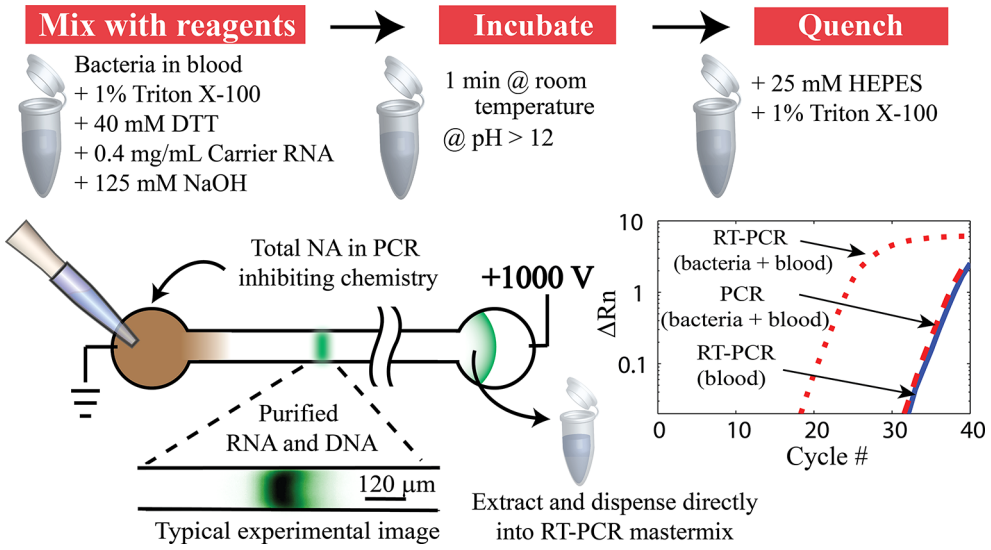

Figure 1. Schematic summarizing protocol with one mixing and two dispensing steps for otherwise automated on-chip RNA extraction from whole blood. TE mixed with lysate (brown) contains target nucleic acid (green), proteins, and potential PCR-inhibiting chemistries. Appropriate selection of trailing and leading ions enables selective focusing of target nucleic acid while leaving PCR inhibitors behind. The detail view shows on-chip extraction of RNA from blood stained with SYBR Green II, focused into a concentrated zone. The amplification plot shows the result of alkaline-based lysing (enhanced with Triton X-100, DTT, and carrier RNA) of total nucleic acid from whole blood spiked with *P. putida* at 30 ¢/nL, followed by purification of total nucleic acid (NA) from lysate using ITP. The NA collected from the output well was split to perform both RT-qPCR and qPCR to verify successful extraction of 16S rRNA (red dotted) and 16S rDNA (red bold dashed). For the negative control template (uninfected blood), RT-qPCR amplified 16S rRNA (blue solid line) above 30 cycles and qPCR did not amplify 16S rDNA within 40 cycles, as expected.

copies/μL of serum.[20] However, very importantly, the Root assay included no lysing strategy; instead, they demonstrated purification by spiking free RNA into a preprepared serum sample that already contained 0.5% w/v concentration of lithium dodecyl sulfate, a powerful RNase inhibitor.

We know of no reports of miniaturized systems that can extract RNA from blood or blood lysate at RNA copy numbers which are within a factor of about 10^5 orders of magnitude of clinically relevant RNA levels. For that matter, we know of no studies that have combined lysing and RNase control into a single chemistry. There is, therefore, still a significant need for a microfluidic assay that has adequate RNase control and can enable direct integration of lysis and nucleic acid purification (particularly RNA) and also provide nucleic acid samples compatible with amplification methods.

Isotachophoresis (ITP) offers an alternative approach to nucleic acid extraction and purification, and the input and output reagents used in ITP can be compatible with lysis, RNase control, and amplification. ITP does not require specific surfaces, specialized geometries, or pumping of reagents. It uses an electric field to extract and preconcentrate only target analytes whose electrophoretic mobility is bracketed between the anions of its trailing (TE) and leading electrolytes (LE). Anionic inhibitors with mobilites lower than of the TE do not focus, but do electrophorese into the microchannel. The separation distance between these potentially PCR-inhibiting contaminants and the focused nucleic acid in the ITP zone increases over time. For a channel length, L, the separation distance between the ITP and the inhibitor zone front, ΔL, can be expressed as $\Delta L = (1 - \mu_i/\mu_{TE})L$, where μ_i and μ_{TE} are the inhibitor and TE anion mobilities, respectively. For example, the zone front of an inhibitor with mobility $\mu_i = 0.9\ \mu_{TE}$ will lag 0.6 cm behind the ITP zone at the end of our 6 cm microchannel. ITP is a highly sensitive,[28,29] robust[30,31] sample preparation method that can, under ideal conditions, provide up to 1,000,000-fold preconcentration.[32] We have demonstrated successful ITP extraction of small RNA from cell culture lysate,[33] micro-RNA from total RNA,[34,35] genomic DNA (gDNA)[36] and pathogenic DNA (malaria)[37] from whole blood lysate, and rRNA from bacteria in urine lysate.[38] However, no previous nucleic acid extraction protocols using ITP have been designed for adequate RNase control and RNA integrity.

In this study, we offer new lysing and ITP chemistry that can isolate total nucleic acid from Gram-negative bacteria suspended in whole blood. We demonstrate integration of our method with qPCR, one of the most common transduction assays in molecular biology and an assay with well-characterized sensitivity to impurities and buffer conditions. Our protocol is unique in that it provides a combined lysis and TE chemistry that protects RNA from both exogenous and endogenous RNase degradation during extraction and ITP-based purification. This aspect is critical for RNA isolation from RNase-rich matrices, such as whole blood. Although the purified extract can be used to target a wide range of DNA and RNA from whole blood, we here target 16S rRNA to demonstrate assay compatibility with RNA and to highlight the enhanced sensitivity achievable by targeting highly transcribed genes.

MATERIALS AND METHODS

A schematic of our extraction process is shown in Figure 1. We started with human whole blood infected with known concentrations of *Pseudomonas putida* cells. We chemically lysed the blood at room temperature for 1 min in a mixture of sodium hydroxide (NaOH) (pH > ~12), dithiothreitol (DTT) reducing agent, Triton X-100 nonionic surfactant, and synthetic carrier RNA. We then "quenched" this high pH with ice-cold TE buffer and then directly pipetted this combined lysate and TE into the input well of a microfluidic chip with a single connecting channel (and single output well) prefilled with LE.

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We placed 600 μm diameter platinum wire electrodes into the wells, and applied +1000 V to the extraction well and grounded the TE well using a Keithley 2410 sourcemeter (see the Supporting Information for the experimental setup, Figure S-3) We recorded current versus time using the sourcemeter interfaced with a computer running a custom Matlab (Mathworks, Inc.) script. As shown in Figure S-4, with constant voltage applied, the current monotonically decreased as the ITP zone (containing the focused NA) advanced in the channel and as the lower-conductivity trailing electrolyte replaced the higher-conductivity leading electrolyte. The current signal plateaued near t = 220 s, coincident with the time at which the focused NA eluted into the extraction well. With a standard pipet, we gently mixed the content of the extraction well and collected 4 μL aliquots for each of the off-chip RT-qPCR and PCR assays.

In this study, we also performed SYBR Green-based NA visualizations to provide supporting evidence for the extracted and purified NA; however, the process can be performed by monitoring current alone (see the discussion regarding timing control and repeatability of the process on page 5 of the Supporting Information.)

***P. Putida* and Blood Samples.** Blood samples from healthy donors were collected in heparin tubes at the Stanford Blood Center. Aliquots of 100 μL of blood were prepared and stored at −80 °C. *P. putida* cells were purchased from ATCC (no. 12633), and cultured in Luria broth (Invitrogen) at 37 °C to a final concentration of 3×10^6 ¢/mL, and quantified by the plate count method. Bacteria suspensions were pelleted before diluting them in blood at 3×10^7, 3×10^6, 3×10^5, and 3×10^4 ¢/mL concentrations, and were stored at −80 °C.

Lysis. We lysed 20 μL of whole blood with suspended *P. putida* using a mixture of 1% Triton X-100, 125 mM NaOH, 40 mM DTT, and 0.4 mg/mL carrier polyA RNA. After 1 min of incubation at room temperature, we mixed 10 μL of lysate with 90 μL of ice-cold TE buffer (28 mM HEPES, 1% Triton X-100, pH ∼ 7.4).

ITP Extraction. The leading electrolyte used to fill the microchannel, LE1, contained 1 U/μL RNasin Plus, 0.1% Triton X-100, 1% 1.3 MDa poly(vinylpyrrolidone) (PVP), and 1X SYBR Green II in 100 mM Tris hydrochloride (Tris−HCl) at pH 7.5. The PCR-compatible leading electrolyte, LE2, contained 1 U/μL RNasin Plus and 1% 1.3 MDa PVP in 20 mM Tris−HCl at pH 7.5. We added PVP to LE to suppress electroosmotic flow and Triton X-100 to TE and LE to aid solubility of the denatured proteins.

Triton X-100, NaOH, Tris, HEPES, and HCl were obtained from Sigma-Aldrich (St. Louis, MO); DTT, carrier RNA, and SYBR Green II were obtained from Invitrogen (Carlsbad, CA); PVP (MW 1.3 MDa) was purchased from ACROS Organics (Thermo Fisher Scientific, NJ); and RNasin Plus was purchased from Promega (Madison, WI). All solutions were prepared in UltraPure DNase-/RNase-free deionized (DI) water (GIBCO Invitrogen, Carlsbad, CA).

Channel Preparation. We performed isotachophoretic purification of RNA on a 60.7 mm long, 120 um wide, 35 um deep Crown glass microchannel (NS12A) interfaced with 3.5 mm deep and 3.5 mm diameter wells from Caliper Science Life Sciences, Mountain View, CA. Before first use, we rinsed the channel with the following successive washes: methanol (2 min), DI (1 min), 1 M HCl (2 min), DI (1 min), 1 M NaOH (10 min), and DI (1 min). Between experiments, we rinsed the channel with 10% household bleach for 2 min to remove any residual nucleic acid contaminants and then with washes of 1 min DI, 10 min NaOH, and 1 min DI. After filling the microchannel with LE1 (2 min) (see filling instructions in the Supporting Information), we emptied and rinsed the extraction and sample wells and pipetted 10 μL of LE2 into the extraction well and 10 μL of TE into the sample well.

Imaging System. We performed on-chip visualizations using an inverted epifluorescence microscope (Nikon Eclipse TE300) (Nikon, Tokyo, Japan) equipped with a 10× objective (Plan, NA 0.30; Nikon, Tokyo, Japan). A blue LED (Thor Laboratories, Newton, NJ) was used for excitation of SYBR Green II (Invitrogen, Carlsbad, CA) nucleic acid dye. We used a filter cube optimized for detection of FITC (FITC-A-Basic, Semrock, Rochester, NY) and a 0.5× demagnification lens (Diagnostic Instruments, Sterling Heights, MI). We captured images using a 512 × 512, 16 bit, CCD camera (Cascade 512F, QImaging/Photometrics, Canada). We controlled the camera using Winview32 (Princeton Instruments, Trenton, NJ) and processed the images with MATLAB (R2007b, Mathworks, Natick, MA).

RT-qPCR and qPCR. We used off-chip RT-qPCR and qPCR to validate the purity of our sample and PCR compatibility of our ITP assay. We added 4 μL of total nucleic acid extract from the chip's output well to a PCR tube containing 10 μL Power SYBR Green RT-PCR Mix (2X) (Applied Biosystems), 0.16 μL RT Enzyme Mix (125X) (Applied Biosystems), 5.84 μL RNase free water, and 150 nM primers targeting 16SrRNA of *P. putida*. The forward (5′-CAAAACTGGCAAGCTAGAGTACG) and reverse (5′-TAAAATCTCAAGGATTCCAACGGCT) primer sequence reagents were purchased from IDT (Coraville, IA). To confirm that the RT-qPCR amplification is specific to the RNA of the 16S rRNA gene, we also performed parallel qPCR reactions without the RT enzyme for each ITP extraction. We performed off-chip RT-qPCR and qPCR using a real-time PCR thermocycler (7500 Fast, Applied Biosystems, Carlsbad, CA) with the following thermal profile: 30 min initial hold at 48 °C, followed by 10 min hold at 95 °C, and 40 cycles composed of 15 s denaturation at 95 °C and 1 min annealing and extension at 60 °C. We obtained post-PCR dissociation curves using the same instrument.

RESULTS AND DISCUSSION

Assay Design and Evaluations. The design of a sample preparation process requires careful evaluation of the integration and interrelation of each of its steps. For example, we observed that choices of specific initial lysing steps can have profound influence on the efficiency of the final analysis (e.g., compatibility with enzyme-based amplification). We designed the current sample preparation method for compatibility and use with PCR. Each of the following sections describes a major process step or chemistry, a brief reasoning for or background behind its inclusion, and a brief description of its integration with the rest of the assay.

Lysis. Guanidinium-based lysing followed by phenol−chloroform extraction and ethanol precipitation is the gold standard chemistry for RNA isolation from whole blood.[39] When combined with enzymes (e.g., lysozyme), strong detergents (e.g., sodium dodecyl sulfate, SDS), and chelating agents (e.g., ethylenediaminetetraacetic acid), the guanidinium-based approach can also be used to lyse bacterial cells suspended in blood.[40] However, the high ionic strengths (e.g., >500 mM) of these mixtures require careful purification,

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including wash and buffer exchanges because SDS, chaotropic agents, organic solvents, and alcohols are strong PCR inhibitors. We rejected these here because high ionic strength (especially when including high mobility anions) can be challenging to integrate with ITP. Instead, we opted for alkali-based lysis. The alkali approach is widely used for isolation of plasmid DNA from bacterial cells,[41] but it is not commonly applied for RNA isolation, likely because of the instability of RNA at high pH.[42] Although brief incubation in NaOH may contribute to some RNA degradation, we found it had negligible effect on RT-qPCR amplification of our target RNA sequences. For example, in a set of preliminary experiments (data not shown), we found negligible differences in RT-qPCR threshold cycles for samples of prepurified (using a standard SPE column, PureLink RNA Mini kit from Life Technologies Corp., Carlsbad, CA) 16S rRNA with and without treatment with 125 mM NaOH for 1 min. With respect to lysing, we found (again, through preliminary repeated trials and lysing quantitation studies) that this 1 min incubation (required to protect RNA) showed lysing performance on par with even 5 min of 130 mM NaOH or higher concentration (data not shown).

RNase Control. RNA is easily hydrolyzed at elevated temperature and at either alkali or acidic conditions. Further, its stability is significantly compromised by the abundance of ribonucleases (RNases) in blood. Tsui et al. showed that free RNA can no longer be amplified after incubation in blood plasma for 15 s![11] RNase activity requires its disulfide (S−S) bond be intact. Although NaOH can reduce these S−S bonds, the half-life of RNase in 0.2 M NaOH is ∼30 min.[43] Meanwhile, as we have mentioned, long incubation in alkali conditions degrades RNA. However, RNase degradation can be greatly accelerated during alkali lysing by adding detergents (e.g., SDS), or reducing agents (e.g., DTT).[44] Although ITP can be compatible with anionic detergents, we found that the addition of a reducing agent alone, 40 mM DTT to 125 mM NaOH in the presence of 1% nonionic detergent (Triton X-100) results in adequate mitigation of RNA degradation in our assay. We hypothesize that this mixture adequately destabilizes and covalently destroys disulfide bonds of the normally very stable RNases, even during 1 min of incubation.

After the lysate is quenched to normal pH, residual RNase may be present. To inhibit the activity of the remaining ribonucleases, we explored the use of formamide as the solvent for the lysate and TE. Although pure formamide stabilizes RNA in the presence of RNase,[45] we found (again through preliminary work) that a combination of 50% formamide and 20% blood unfortunately resulted in enhanced RNA degradation. Similar observations were reported by Strauss and Sinsheimer[46] in an evaluation of initial kinetics of RNA degradation by pancreatic ribonucleases.

Instead of formamide, we opted for an indirect approach: the addition of carrier RNA. The term "carrier RNA" refers to exogenous RNA spiked into a sample.[47] Addition of carrier RNA to lysate reportedly enhances the recovery of low traces of DNA and RNA in ethanol precipitation[48,49] and SPE-based extraction[21,50] procedures. In our application, we spiked large amounts of polyA synthetic carrier RNA sequences into the lysate to serve as a competitive substrate for RNase activity. That is, we hypothesize that the abundant carrier RNA reduces enzymatic activity on our trace-concentration, target RNA by acting as a high-abundance inhibitor to RNase. Figure S-5 of the Supporting Information summarizes example preliminary experiments we performed in part to confirm our hypotheses concerning the combined effect of both DTT and carrier RNA on target RNA stability and recovery.

Last, to further guard against exogenous RNase contamination and residual RNase from lysate, we also included PCR-compatible RNase inhibitor RNasin Plus (Promega) in the LE.

ITP Chemistry. In addition to requiring TE and LE anions with mobilities that bracket that of RNA, the ITP chemistry prompted two additional concerns: pH and ionic strength. Again, throughout the course of a series of preliminary experiments, we observed severe repeatability problems at lower pH, 5−7 (e.g., using Bis−Tris as the buffering weak base). We attribute these to the effects of (observed) protein aggregation and protein adsorption to channel walls.[51,52] Recovery efficiency stabilized at pH ∼ 7.5, so we chose Tris as the buffering counterion. We also quantified LE buffer compatibility with RT-PCR (via independent, ex situ runs with the PCR system) and found no change in the amplification efficiency when using 20 mM Tris−HCl (pH = 7.5) as the sample buffer added to the RT-PCR Mastermix. We filled the channel with 100 mM Tris−HCl (pH = 7.5) to improve the rate of nucleic acid accumulation[53] but used 20 mM Tris−HCl (pH = 7.5) as the LE buffer in the extraction well. The latter chemistry provided a good balance between PCR compatibility and buffering capacity in that well.

Demonstration of Extraction Purity and Compatibility with RT-qPCR. *Flourescence Imaging.* We monitored accumulation of RNA during ITP by visualizing the scalar fluorescence of RNA-specific intercalating dye, SYBR Green II. Figure S-6 illustrates the results of extractions from blood containing 0 and 30 ¢/nL *P. putida* cells. Our 1× concentration of SYBR Green II (in original LE1; equivalent to 0.003× concentration in the final PCR mix) showed no observable effect on the amplification signal.

RT-qPCR and qPCR. Figure 2 shows typical RT-qPCR threshold cycles for 16S rRNA extracted from *P. putida* suspended in whole human blood. We explored bacterial cell densities ranging 4 orders of magnitude from 0.03 to 30 ¢/nL. In Figure S-7, we show examples of raw data from RT-qPCR reactions containing ITP-processed, lysed blood with and without bacteria. We observed no amplification below 30

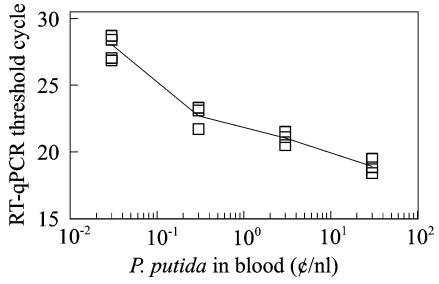

Figure 2. RT-qPCR threshold cycles for 16S rRNA extracted from whole human blood infected with *P. putida* using our DTT- and carrier-RNA-assisted, alkaline-based lysing and ITP purification protocol. Plot contains results for a total of 13 experiments, four at 0.03 ¢/nL and three each at 0.3, 3, and 30 ¢/nL bacterial cell concentrations. All negative controls for RT-qPCR (RNA extracted from uninfected blood) amplified above 30 cycles, as expected (not shown). Threshold amplification cycles for the (non RT) qPCR reactions targeting DNA were above 30 for all experiments, also demonstrating our sensitivity to RNA versus DNA.

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thermal cycles for the negative controls in either the RT-qPCR or qPCR reactions. (Low threshold cycles associated with negative controls are, of course, a sign of contamination of the RT-PCR master mix or ITP buffers.) Amplified sequences dissociated at ∼82 °C, a measured temperature that matches the calculation from theory for dissociation temperature (mFold, RNA Institute, University of Albany, Albany, NY).

Detection Limits. We detected *P. putida*-infected whole blood at bacterial cell concentrations of 0.03−30 ¢/nL-blood (4.5−8.5 $\log_{10}$¢/mL-blood). With the current channel design, our sensitivity is limited by contamination of our reagents with 16S rRNA. We hypothesize that we can improve sensitivity with a more carefully sterilized, dedicated lab space for RNA extraction work. In comparison, our sensitivity is still 6 orders of magnitude greater than the only other microfluidic RNA extraction from whole blood lysate, as reported by Witek et al.[22] On the other hand, Mahalanabis et al.[19] presented a method for on-chip DNA extraction from bacteria-infected whole blood using an SPE-based extraction method which processed about 100 μL of whole blood using a protocol with five reagent pumping steps and which achieved 10^2 ¢/mL sensitivity. This sensitivity is order 300-fold higher than our assay, but it used about 5 orders of magnitude higher processed sample volume than our assay's ∼1 nL processed volume. We hypothesize that we can exceed this sensitivity by scaling up the geometry of our channel to process order 1000-fold higher sample volume (order ∼ 1 μL) and hope to demonstrate this in future work.

In addition to the *P. putida* results we present here, we have also successfully performed RNA extraction from *Escherichia coli* cells suspended in whole blood (data not shown). Together, our more extensive *P. putida* work and our limited work with *E. coli* suggest that our protocol can be successfully adapted to RNA extraction and detection from many Gram-negative bacteria species by simply changing the primer sequence and thermal cycling conditions for RT-qPCR. One particular application that may benefit from our technique is diagnosis of *Menigococcemia*, an acute and potentially life-threatening infection of the bloodstream caused by bacteria *Neisseria meningitidis*. One large study including 1045 adult patients evaluated the full range of bacterial loads upon hospital admission, 2.87−6.3 $\log_{10}$¢/mL,[54] using qPCR analysis of whole blood samples for *N. meningitides*. Another study, including a smaller cohort of 51 pediatric patients reported a slightly higher *N. meningitides* load range of 4.3−8.2 $\log_{10}$¢/mL[55] using a similar qPCR-based assay.

CONCLUSIONS

We have demonstrated a novel assay for alkali-based extraction and ITP-based purification of 16S rRNA from *P. putida* bacteria suspended in whole human blood. The assay can be implemented with minimal manual steps and can automate the extraction, preconcentration, and purification of nucleic acid in less than 5 min. For example, we can envision a disposable plastic chip that can be discarded after each use, largely mitigating decontamination and obviating surface reconditioning procedures.

RNA is easily hydrolyzed at elevated temperature, and at either alkali or acidic conditions. Further, its stability is significantly compromised by the abundance of RNases in blood. We developed a novel ITP- and PCR-compatible chemistry that ensures RNA stability and recovery using the addition of DTT, a strong reducing agent, and large amounts of polyA carrier RNA. We showed that the extracted 16S rRNA was purified of PCR inhibitors and compatible with RT-qPCR. We demonstrated a sensitivity of 0.03 bacteria per nanoliter of blood, and hypothesize our sensitivity is currently limited by the presence of RNA contaminants in our reagents.

With the current channel design, we expect, at most, 2 orders of magnitude improvement in sensitivity by addressing the presence of contamination. To achieve higher sensitivity, our method will likely require redesign of chip geometry to achieve higher extraction efficiency and to accommodate processing of larger sample volumes. We hypothesize that, given sufficient sample capacity, our RNA extraction method can be made compatible with DNA or RNA chips, perhaps without the need for nucleic acid amplification. We hope to address these issues in future work.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Matsuda, K.; Tsuji, H.; Asahara, T.; Kado, Y.; Nomoto, K. *Appl. Environ. Microbiol.* **2007**, *73*, 32−39.
(2) Fujimori, M.; Hisata, K.; Nagata, S.; Matsunaga, N.; Komatsu, M.; Shoji, H.; Sato, H.; Yamashiro, Y.; Asahara, T.; Nomoto, K. *BMC Pediatr.* **2010**, *10*, 53.
(3) Arfvidsson, C.; Wahlund, K. G. *Anal. Biochem.* **2003**, *313*, 76−85.
(4) Neidhardt, F. C.; Umbarger, H. E. *Escherichia coli* and *Salmonella typhimurium*. In *Chemical Composition of Escherichia coli*, 2nd ed.; Neidhardt, F. C., Ed.; ASM Press: Washington, D.C., 1996; Vol. *1*, pp 13−16.
(5) Wellinghausen, N.; Kochem, A. J.; Disqué, C.; Mühl, H.; Gebert, S.; Winter, J.; Matten, J.; Sakka, S. G. *J. Clin. Microbiol.* **2009**, *47*, 2759−2765.
(6) Zhang, Y.; Isaacman, D. J.; Wadowsky, R. M.; Rydquist-White, J.; Post, J. C.; Ehrlich, G. D. *J. Clin. Microbiol.* **1995**, *33*, 596−601.
(7) Chiu, M. L.; Lawi, W.; Snyder, S. T.; Wong, P. K.; Liao, J. C.; Gau, V. *JALA* **2010**, *15*, 233−242.
(8) Al-Soud, W. A.; Rådström, P. *J. Clin. Microbiol.* **2001**, *39*, 485−493.
(9) Kim, J.; Johnson, M.; Hill, P.; Gale, B. K. *Integr. Biol.* **2009**, *1*, 574−586.
(10) Mariella, R. *Biomed. Microdevices* **2008**, *10*, 777−784.
(11) Tsui, N. B. Y.; Ng, E. K. O.; Lo, Y. M. D. *Clin. Chem.* **2002**, *48*, 1647−1653.
(12) Farrell, R. E. *RNA Methodologies*; Academic Press, Inc.,: San Diego, CA, 2005, p 45.
(13) Cox, R.; Gould, H. J.; Kanagalingam, K. *Biochem. J.* **1968**, *106*, 733.
(14) Lipkin, D.; Talbert, P. T.; Cohn, M. *J. Am. Chem. Soc.* **1954**, *76*, 2871−2872.
(15) Markham, R.; Smith, J. *Biochem. J.* **1952**, *52*, 552.

(16) Wen, J.; Legendre, L. A.; Bienvenue, J. M.; Landers, J. P. *Anal. Chem.* **2008**, *80*, 6472−6479.
(17) Breadmore, M. C.; Wolfe, K. A.; Arcibal, I. G.; Leung, W. K.; Dickson, D.; Giordano, B. C.; Power, M. E.; Ferrance, J. P.; Feldman, S. H.; Norris, P. M.; Landers, J. P. *Anal. Chem.* **2003**, *75*, 1880−1886.
(18) Legendre, L. A.; Bienvenue, J. M.; Roper, M. G.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2006**, *78*, 1444−1451.
(19) Mahalanabis, M.; Al-Muayad, H.; Kulinski, M. D.; Altman, D.; Klapperich, C. M. *Lab Chip* **2009**, *9*, 2811−2817.
(20) Root, B. E.; Agarwal, A. K.; Kelso, D. M.; Barron, A. E. *Anal. Chem.* **2011**, *83*, 982−988.
(21) Bhattacharyya, A.; Klapperich, C. M. *Sens. Actuators, B* **2008**, *129*, 693−698.
(22) Witek, M. A.; Hupert, M. L.; Park, D. S. W.; Fears, K.; Murphy, M. C.; Soper, S. A. *Anal. Chem.* **2008**, *80*, 3483−3491.
(23) Jiang, G.; Harrison, D. J. *Analyst* **2000**, *125*, 2176−2179.
(24) Satterfield, B. C.; Stern, S.; Caplan, M. R.; Hukari, K. W.; West, J. A. A. *Anal. Chem.* **2007**, *79*, 6230−6235.
(25) Hong, J. W.; Studer, V.; Hang, G.; Anderson, W. F.; Quake, S. R. *Nat. Biotechnol.* **2004**, *22*, 435−439.
(26) Hagan, K. A.; Meier, W. L.; Ferrance, J. P.; Landers, J. P. *Anal. Chem.* **2009**, *81*, 5249−5256.
(27) Hagan, K. A.; Bienvenue, J. M.; Moskaluk, C. A.; Landers, J. P. *Anal. Chem.* **2008**, *80*, 8453−8460.
(28) Jung, B.; Zhu, Y.; Santiago, J. G. *Anal. Chem.* **2006**, *79*, 345−349.
(29) Bahga, S. S.; Kaigala, G. V.; Bercovici, M.; Santiago, J. G. *Electrophoresis* **2011**, *32*, 563−572.
(30) Garcia-Schwarz, G.; Rogacs, A.; Bahga, S. S.; Santiago, J. G. *J. Visualized Exp.* **2012**, e3890.
(31) Boček, P. *Analytical Isotachophoresis*; Springer: Berlin/Heidelberg, 1981; Vol. *95*, pp 131−177.
(32) Jung, B.; Bharadwaj, R.; Santiago, J. G. *Anal. Chem.* **2006**, *78*, 2319−2327.
(33) Schoch, R. B.; Ronaghi, M.; Santiago, J. G. *Lab Chip* **2009**, *9*, 2145−2152.
(34) Persat, A.; Chivukula, R. R.; Mendell, J. T.; Santiago, J. G. *Anal. Chem.* **2010**, *82*, 9631−9635.
(35) Persat, A.; Santiago, J. G. *Anal. Chem.* **2011**, *83*, 2310−2316.
(36) Persat, A.; Marshall, L. A.; Santiago, J. G. *Anal. Chem.* **2009**, *81*, 9507−9511.
(37) Marshall, L. A.; Han, C. M.; Santiago, J. G. *Anal. Chem.* **2011**, *83*, 9715−9718.
(38) Bercovici, M.; Kaigala, G. V.; Mach, K. E.; Han, C. M.; Liao, J. C.; Santiago, J. G. *Anal. Chem.* **2011**, *83*, 4110−4117.
(39) Chomczynski, P.; Sacchi, N. *Anal. Biochem.* **1987**, *162*, 156−159.
(40) Heptinstall, J. *Total RNA Isolation from Bacteria: The Nucleic Acid Protocols Handbook*; Rapley, R., Ed.; Humana Press: New York, 2000; pp 47−52.
(41) Bimboim, H.; Doly, J. *Nucleic Acids Res.* **1979**, *7*, 1513−1523.
(42) Freitas, S.; Azzoni, A.; Santos, J.; Monteiro, G.; Prazeres, D. *Mol. Biotechnol.* **2007**, *36*, 151−158.
(43) Florence, T. M. *Biochem. J.* **1980**, *189*, 507.
(44) Lu, B.-Y.; Chang, J.-Y. *Anal. Biochem.* **2010**, *405*, 67−72.
(45) Chomczynski, P. *Nucleic Acids Res.* **1992**, *20*, 3791−3792.
(46) Strauss, J. H., Jr.; Sinsheimer, R. L. *J. Mol. Biol.* **1968**, *34*, 453−465.
(47) Winslow, S. G.; Henkart, P. A. *Nucleic Acids Res.* **1991**, *19*, 3251−3253.
(48) Gallagher, M. L.; Burke, W. F., Jr.; Orzech, K. *Biochem. Biophys. Res. Commun.* **1987**, *144*, 271−276.
(49) Rio, D. C.; Ares, M.; Hannon, G. J.; Nilsen, T. W. *Cold Spring Harb. Protoc.* **2010**, *2010*.
(50) Shaw, K. J.; Thain, L.; Docker, P. T.; Dyer, C. E.; Greenman, J.; Greenway, G. M.; Haswell, S. J. *Anal. Chim. Acta* **2009**, *652*, 231−233.
(51) Doherty, E. A. S.; Meagher, R. J.; Albarghouthi, M. N.; Barron, A. E. *Electrophoresis* **2003**, *24*, 34−54.
(52) McCormick, R. M. *Anal. Chem.* **1988**, *60*, 2322−2328.
(53) Khurana, T. K.; Santiago, J. G. *Anal. Chem.* **2008**, *80*, 6300−6307.
(54) Darton, T.; Guiver, M.; Naylor, S.; Jack, D. L.; Kaczmarski, E. B.; Borrow, R.; Read, R. C. *Clin. Infect. Dis.* **2009**, *48*, 587−594.
(55) Hackett, S. J.; Guiver, M.; Marsh, J.; Sills, J. A.; Thomson, A. P. J.; Kaczmarski, E. B.; Hart, C. A. *Arch. Dis. Child.* **2002**, *86*, 44−46.

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