MICROFLUIDIC EXTRACTION OF RNA FROM BLOOD

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ABSTRACT

We demonstrate a novel assay for purification of RNA (16S rRNA) from whole human blood infected with *Pseudomonas Putida (P. Putida)*. This assay is unique in that the extraction chemistry provides a method which both lyses bacteria cells and protects RNA from degradation when isolating from matrices rich with ribonuclease (RNase) (such as blood). The method can also be integrated with on-chip RNA purification using isotachophoresis (ITP). We demonstrate that the extracted 16S rRNA is compatible with reverse transcription polymerase chain reaction (RT-qPCR) and detect *P. putida* infected whole blood at clinically relevant bacterial cell concentration of 30-30,000 bacteria per microliter of blood.

KEYWORDS

Isotachophoresis, RNA, blood, microfluidic extraction, Rnase inactivation, sample preparation

INTRODUCTION

16S rRNA is a universal constituent of bacterial ribosomes. This sequence is present at high copy numbers (10^3 to 10^4 per actively growing cell)[1] and so targeting the RNA can potentially increase assay sensitivity compared to assays targeting the corresponding DNA. However, RNA is a challenging blood biomarker due to its extreme lability. RNA rapidly degrades at elevated temperature (>65°C), at high pH, and/or in the presence of ribonucleases (RNases). As one example, without sufficient RNase inactivation, free RNA is non-amplifiable after 15 s of incubation in plasma or serum.[2] Furthermore, whole blood samples can have high viscosity, insoluble cell debris, serum proteins, endogenous phospholipids and anticoagulants, which further compromise extraction efficiency and specificity of microfluidic isolation methods.[3] We know of only two other studies which have reported microfluidic-based isolation of RNA from blood or blood product. Witek *et al*.[4] extracted RNA from bacterial cells suspended in blood using photoactivated polycarbonate solid-phase reversible immobilization microfluidic chip. However, their assay did not address adequate RNase control. We attribute their high RNA integrity to the extraordinarily high bacterial density of 18E6 ¢/µL-blood used, a density 10^6 higher than our work. Root *et al*.[5] employed an oligonucleotide polymer capture matrix for RNA isolation, but offered no lysing strategy; instead, their assay spiked free RNA into serum already containing a powerful RNase inhibitor. There is, therefore, still a significant need for a microfluidic assay which (1) has adequate RNase control, (2) can enable direct integration of lysis and nucleic acid purification (particularly RNA), and (3) provide nucleic acid samples compatible with amplification methods.

Isotachophoresis (ITP) offers an alternative to solid-phase or liquid-phase extraction and purification of nucleic acids.[6,7] The input reagents used in ITP can be compatible with lysis and RNase mitigation strategies, and output buffers can be compatible with enzymatic amplification assays. ITP does not require specialized geometries, specific surfaces, or pumping of reagents. In ITP, the applied electric field extracts and preconcentrates the target analytes whose electrophoretic mobility is between the anions of the trailing (TE) and leading electrolytes (LE). While the anionic inhibitors with mobilites lower than of the TE do electrophorese into the microchannel, they do not focus. The separation distance, ΔL, between the ITP zone containing the nucleic acid, and the diffuse zone front of potentially PCR-inhibiting contaminants can be expressed as $\Delta L = (1 - \mu_i/\mu_{TE})L$, where L is the channel length and μ_i, μ_{TE} are the mobilites of the inhibitor and TE, respectively.

Successful ITP extractions were demonstrated for small RNA from cell culture lysate,[8] micro-RNA from total RNA[9] genomic DNA (gDNA)[6] and pathogenic DNA (malaria)[10] from whole blood lysate; and rRNA from bacteria in urine lysate.[11] However, no previous nucleic acid extraction protocols using ITP have been designed for adequate RNase mitigation and RNA integrity. In this study, we offer a novel lysing and RNAse control chemistry which is compatible with ITP-based isolation of total nucleic acid from gram-negative bacteria suspended in whole human blood. The work we describe here was very recently published as Rogacs et al.,[12] and we here provide a summary of this work as well as additional information and data which was published as part on the Supplementary Information document of Rogacs et al.

EXPERIMENT

To our knowledge, this work is the first to report extraction of RNA from blood or blood lysate in a microfluidic system at clinically relevant RNA copy numbers. Our assay (Fig. 1A) addresses the inherent challenges by performing rapid alkaline lysis of bacteria-infected blood in the presence of dithiothreitol (DTT) reducing agent and Triton X-100 non-ionic surfactant. We attribute the success of this approach to adequate destabilization and destruction of disulfide bonds of the normally very stable RNases. RNase activity requires its disulfide (S-S) bond be intact. While NaOH can reduce these S-S bonds, the half-life of RNase in 0.2 M NaOH is ~30 min.[13] 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, denaturants and/or reducing agents such as DTT.[14] In our assay, we found that the addition of a reducing agent alone, 40 mM DTT to 125 mM NaOH, in the presence of 1% non-ionic detergent (Triton X-100), results in adequate mitigation of

RNA degradation. We hypothesize that this mixture adequately destabilizes and covalently destroys disulfide bonds of the normally stable RNases even during 1 min of incubation.

We also 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 1B summarizes example preliminary experiments we performed in part to support our hypotheses concerning the combined effect of both DTT and carrier RNA on target RNA stability and recovery**.**

After 1 min incubation in the lysis cocktail, we "quenched" the high pH with ice-cold TE buffer, and then directly pipetted the combined lysate and TE into the input well of a microfluidic chip pre-filled with LE. We placed platinum wire electrodes into the wells, and applied +1000 V to the extraction well and grounded the TE well using Keithley 2410 sourcemeter. We monitored the decreasing current signal which plateaued near t = 220 s, coincident with the time at which the focused NA eluted into the extraction well. We gently mixed the content of the extraction well with a standard pipette, and collected 4 µl aliquots for each of the off-chip RT-qPCR and PCR assays. (Fig. 1A)

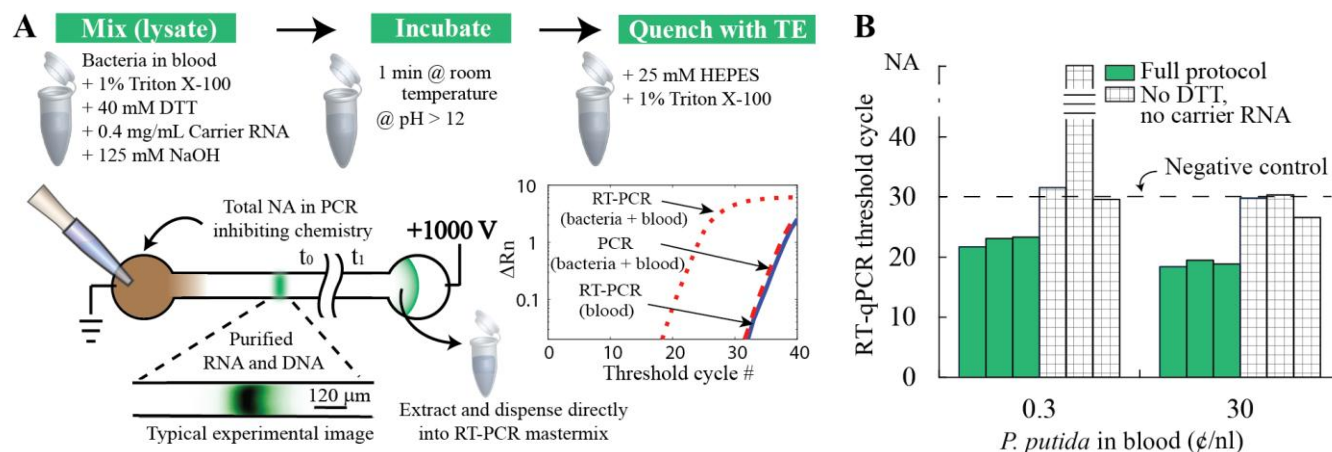

Figure 1. (A) Summary of protocol with one mixing and two dispensing steps for otherwise automated on-chip RNA extraction from whole blood. Lysate (brown) containing nucleic acid (green), proteins, and PCR-inhibiting chemistries is mixed with the TE. Appropriate selection of the ITP buffers facilitates selective extraction and focusing of nucleic acid from the PCR inhibiting chemistry. The detail view at the bottom is an experimental fluorescence image of RNA stained with SYBR Green II, directly extracted from whole blood lysate, and focused into a concentrated zone. The amplification plot shows sample data summarizing the result of alkaline based lysing (enhanced with Triton X-100, DTT and carrier RNA) of total NA from whole blood spiked with *P. Putida at* 30 ¢/nL, followed by ITP-based purification of total NA. RT-qPCR and qPCR were used to verify successful extraction of 16S rRNA (red dotted) and 16S rDNA (red bold dashed). Extractions from negative control template (uninfected blood) amplified (blue solid line) above 30 cycles in RT-qPCR and did not amplify within 40 cycles in qPCR. **(B)** Experimental demonstrations of the combined effect of reducing agent (DTT) and carrier RNA on the ITP-based RNA extraction and purification assay. Lysing in presence of DTT and carrier RNA yields consistent and low threshold amplification cycles at two different bacterial cell densities. In their absence, bacterial RNA extractions and their subsequent amplification either failed or were significantly compromised. NA indicates no amplification within 40 cycles. Negative controls amplified above 30 cycles.

The microchannel was filled with leading electrolyte, LE1, containing 0.1% Triton X-100, 1 U/µL Rnasin Plus, 1% 1.3 MDa poly(vinylpyrrolidone) (PVP) and 1X SYBR Green II in 100 mM Tris hydrochloride (Tris-HCl) at pH 7.5. The extraction well was filled with PCR-compatible leading electrolyte, LE2, containing 1 U/µL Rnasin Plus, and 1% 1.3 MDa PVP in 20 mM Tris-HCl at pH 7.5, which provided a good balance between PCR compatibility and buffering capacity. PVP and Triton X-100 served to suppress electroosmotic flow and aid solubility of the denatured proteins, respectively.

Pseudomonas Putida cells were purchased from ATCC (#12633), and cultured in Luria Broth (Invitrogen) at 37°C to a final concentration of 3e6 ¢/mL, and quantified by the plate count method. Bacteria suspensions were pelleted before diluting them in blood (collected in heparin tubes at the Stanford Blood Center) at $3x10^7$, $3x10^6$, $3x10^5$, and $3x10^4$ ¢/mL concentrations, and were stored at -80°C. 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).

Our ITP-based NA extraction and purification assay requires limited manual handling and automates the extraction, preconcentration, and buffer transfer steps in under 5 min. The extracted 16S rRNA was purified of PCR inhibitors and was detected in *P. putida* infected whole blood at bacterial cell concentration of 30-30E3 ¢/µL-blood (Fig. 2A and 2B). We have

also successfully performed RNA extraction from *E. coli* infected whole blood (data not shown). Together, our results suggest our protocol can be successfully adapted to RNA extraction and detection from many gram-negative bacteria in challenging matrices by simply changing the primer sequence and RT-qPCR protocol.

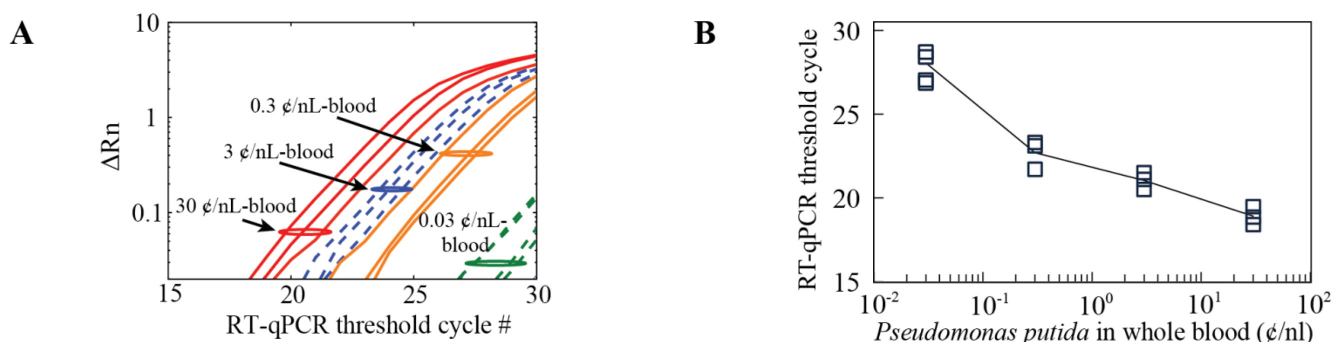

Figure 2. (A) Shown are raw RT-qPCR amplification curves for 16S rRNA purified from *P. putida* infected whole human blood at bacterial cell concentration of 0.03, 0.3, 3, and 30 ¢/nL. We performed RT-qPCR using Power SYBR Green RNA-to-CT 1-Step Kit from Applied Biosystems with 150 nM forward (5'CAAAACTGGCAAGCTAGAGTACG) and 150 nM reverse (5'TAAAATCTCAAGGATTCCAACGGCT) primers. The thermal profile was set to: 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. **(B)** RT-qPCR threshold cycles of amplification curves shown in Figure 2A. Plot summarizes the 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 (uninfected blood) amplified above 30 cycles (not shown). Threshold amplification cycles for PCR reactions targeting the 16S rRNA gene were above 30 for all experiments, demonstrating sensitivity to RNA versus DNA.

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