Point-of-care HIV-1 diagnostic with integrated nucleic acid extraction and amplification from whole blood

Mark D. Borysiak, Andrew T. Bender, David S. Boyle, and Jonathan D. Posner

Abstract— **The HIV/AIDS epidemic continues to be a major global health challenge, and accurate, affordable HIV-1 viral load testing is increasingly needed at the point-of-care (POC). Nucleic acid amplification tests (NAATs) provide high diagnostic accuracy of infectious diseases, yet most systems are restricted to central laboratories due to assay and instrumentation complexity. Here we describe a NAAT with integrated sample preparation and amplification using isotachophoresis and recombinase polymerase amplification. We argue that this approach has the potential to reduce the cost and complexity of point-of-care (POC) NAATs. We demonstrate the detection of HIV-1 nucleic acids in whole blood. Preliminary data demonstrate linearity at low copy number which may provide valuable quantitative data on the HIV viral load of a patient.**

I. Introduction

The HIV/AIDS epidemic is a major global health challenge and a significant cause of mortality over the last decade.[1] The World Health Organization (WHO) estimates that there were 35 million people living with HIV (PLHIV) as of 2013.[2] Of those diagnosed HIV+, nearly 15 million PLHIV receive antiretroviral therapy (ART), a figure that falls short of the WHO/UNAIDS targets to provide lifelong ART for all 35 million PLHIV.[3] Initiation of ART can maintain durable viral suppression, slowing progression from HIV infection to acquired immune deficiency syndrome (AIDS).[3] Viral load (VL) tests are needed to monitor the ongoing effectiveness of ART therapy, so that treatment regimens can be adjusted to maintain viral suppression if needed.[3] The WHO defines ART failure as two consecutive VL tests above 1000 cp/mL after receiving ART for >6 months.[4]

By 2020, UNAIDS aims to have 90% of PLHIV aware of their status, 90% to receive sustained ART, and 90% of those on ART to have sustained VL suppression (90-90-90).[5] To achieve these goals, there is a need for >70 million global HIV VL tests per year. However, performing routine laboratory-based VL testing for the 15 million people receiving ART has been a challenge, particularly in resource limited settings, where lack of VL testing leads to undiagnosed virological failure, late treatment switches, and potential spread of HIV drug resistance.[6]

Current tests for measuring HIV VL rely on molecular amplification methods that detect HIV nucleic acids from whole blood samples. Nucleic acid amplification tests (NAATs) are the gold standard for HIV-1 viral load testing. Despite the clear advantages of NAATs, most systems are restricted to central laboratories due to assay, instrumentation, and/or protocol complexity that requires skilled personnel to operate. The logistics around specimen collection, transport, and returning of results for laboratory NAATs typically delay obtaining diagnoses—often negating patient management benefits. These core restraints have motivated bringing NAATs toward the point-of-care (POC), as demonstrated by recent CLIA-waived tools such as the Alere i, Roche Liat, and Cepheid Omni. These devices represent a step forward, but still have cost, complexity, and deliverability limitations for primary care and global health applications due to complex mechanical actuation and instrumentation required for sample preparation and amplification from clinical samples.

We report on the development of an innovative NAAT format that is capable of simultaneously extracting and amplifying nucleic acids from whole blood using only buffers, paper substrates, and an electric field. This device can decrease the cost and complexity of NAAT because it requires minimal equipment (*e.g.* no heater), has no mechanical actuation, and only requires two user steps—adding sample/buffer and applying an electric field.

II. Methodology and Innovation

Our device integrates two novel technologies—isotachophoresis (ITP) and recombinase polymerase amplification (RPA)—to rapidly extract, amplify, and detect target nucleic acids on a strip of porous glass fiber. ITP is a powerful electrokinetic separation and concentration technique that extracts nucleic acids from complex bio-samples such as whole blood or serum, and concentrates the purified nucleic acids at the interface between two buffers known as the *leading electrolyte* (LE) and *trailing electrolyte* (TE).[9–11] ITP separates ions based on their respective electrophoretic mobility, μ_i, where migration velocity is directly proportional to mobility at a given electric field strength. Nucleic acids have high electrophoretic mobility due to a highly negatively charged phosphate backbone, making ITP an advantageous method for purifying and concentrating nucleic acids from complex samples. [9] Therefore, our ITP system is designed with LE and TE mobilities that bracket the mobility of the nucleic acids. Our device consists of two

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reservoirs that hold TE and LE buffers, with a porous glass fiber strip creating a fluidic connection. Upon application of an electric field, the nucleic acids and RPA reagents concentrate at the interface of the TE and LE on the porous substrate, providing rapid extraction and amplification in a single step (Figure 1A).

RPA is an isothermal amplification strategy that is ideal for use as a POC NAAT due to its speed (<15 minutes), low constant incubation temperature (25–43 °C), under 10 copy sensitivity, and reagent stability.[12–14] Figures 2A-2D illustrate the RPA mechanism. RPA uses recombinase enzymes to coat single-stranded DNA, *i.e.* oligonucleotide primers, to form filaments, which can then scan double-stranded DNA sequences for homology. After finding the homologous sequence, the nucleoprotein filament can invade and create a short hybrid with a displaced strand bubble known as a D-loop. Single-stranded binding proteins bind to the displaced strand to stabilize it and prevent ejection of the inserted primer. Strand-displacement polymerase then performs primer extensions and continuation of this process results in exponential amplification of the target. TwistDx (Cambridge, UK) has commercialized the RPA technology and offers amplification kits that contain all of the necessary reagents to perform RPA. [14]

This integrated ITP-RPA technique is self-heating in order to catalyze the isothermal nucleic acid amplification. When a constant electric field is applied, a significant amount of current is passed through the glass fiber substrates during ITP-RPA reactions (approximately 1–3.5 mA depending on the location of the reaction plug) that results in significant Joule heating. We leverage this effect to heat the ITP plug to the optimal temperature for RPA (35–40 °C). Different temperature ranges can be achieved depending on the applied electric field strength, the composition of the ITP electrolytes, and the dimensions of the glass fiber strip. This combination of internal Joule heating and highly concentrated reactants inside the ITP plug allows for RPA to proceed within 10–15 minutes. We apply the complex sample, either serum or filtered whole blood, to the glass fiber substrate near the TE well, followed by ITP extraction and concentration from

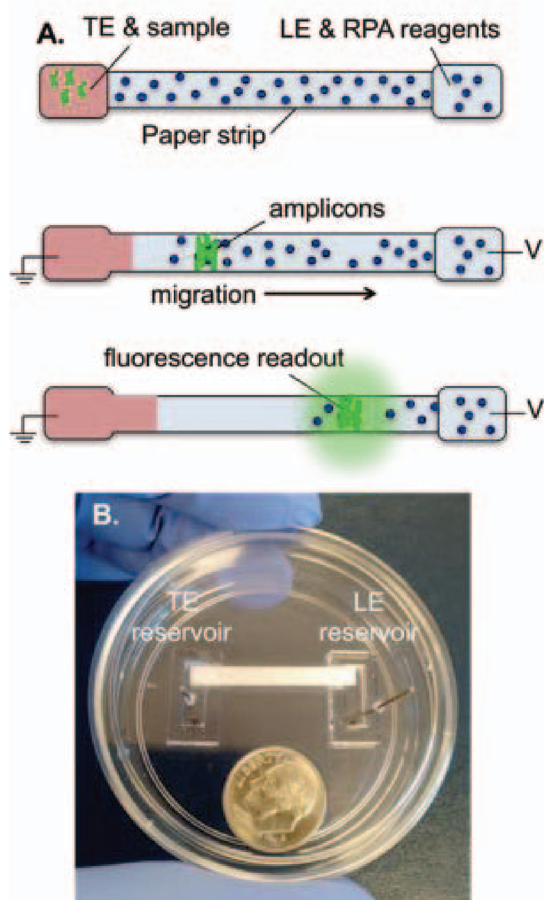

Figure 1. (A) Schematic of ITP-RPA operation. TE contains nucleic acids (NA) and blood, while LE/RPA reagents fill the strip. Applying an electric field extracts and focuses NA with RPA reagents to create amplicons and fluorescence. (B) Image of the sealed electrokinetic paper device used for preliminary experiments. The devices are completely sealed and disposable in order to mitigate contamination issues common with nucleic acid amplification work.

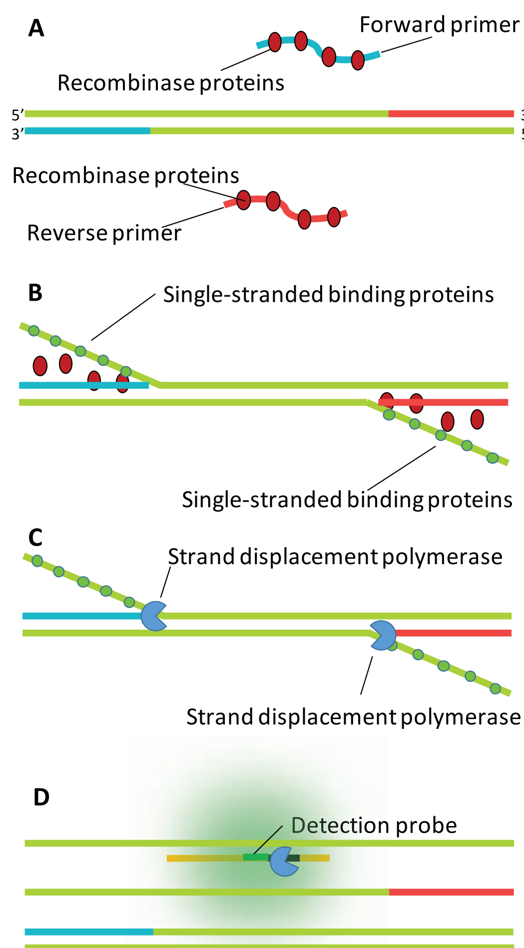

Figure 2. RPA Reaction Mechanism. (A) Recombinase proteins bind to the forward and reverse primer before scanning the target for the homologous sequence. (B) The recombinase proteins insert the primer into the target sequence and the displaced strand is stabilized by single-stranded binding proteins. (C) Strand displacement polymerase extends the primers to create a copy of the target nucleic acid strand. (D) A fluorescent probe binds to the produced amplicon, and upon binding, is cut by an exonuclease protein to remove the quencher and cause fluorescence for detection.

sample inhibitors inside of a sealed, disposable petri dish (Figure 1B).

These two technologies differentiate our device from other POC diagnostics that simply seek to automate multi-step, bench-based solid phase extraction and PCR protocols using mechanical actuation and portable thermocyclers. Our NAAT diagnostic integrates sample preparation, amplification, and detection into a simple, single step device that requires minimal user intervention and low-cost equipment.

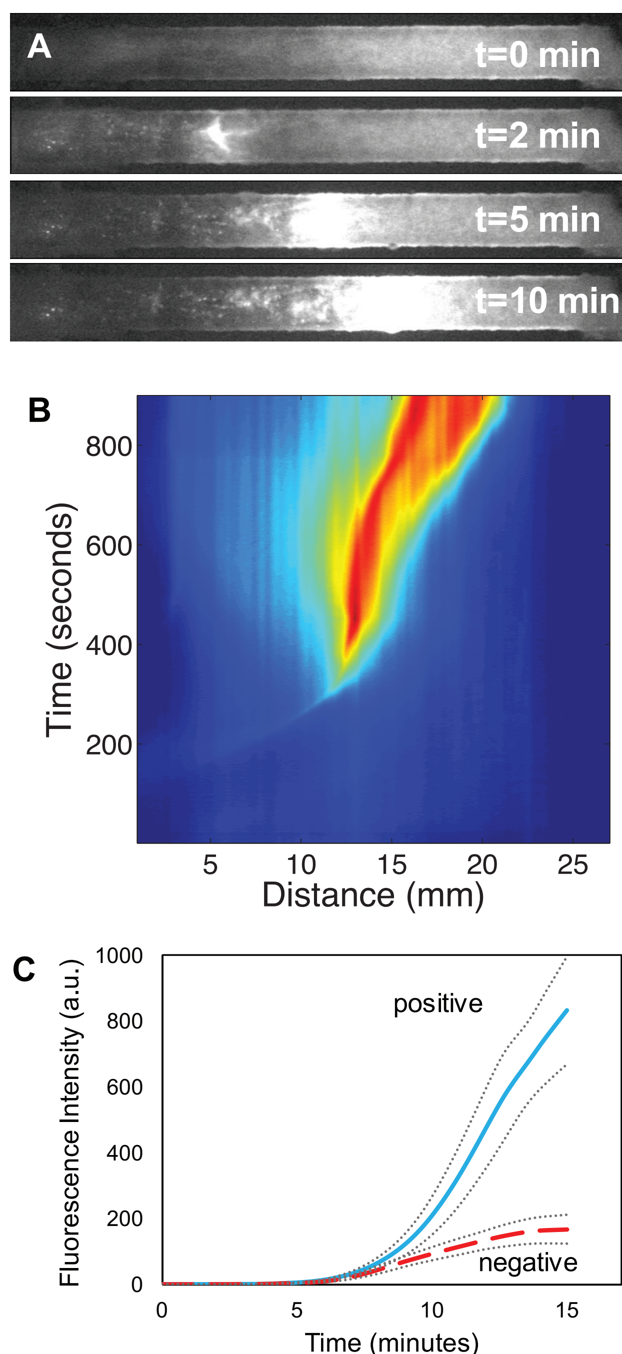

Figure 3. (A) Experimental fluorescence images of a positive ITP-RPA reaction on glass fiber from serum. (B) Spatio-temporal map that shows entire reaction progression from (A). (C) Integrated intensities of ITP-RPA experiments vs. time demonstrating HIV detection and consistent differentiation between positives and negatives. Dashed lines illustrate 95% confidence intervals.

III. Data and Results

A. *ITP Enhanced RPA Using Human Serum*

The ITP system was developed to separate nucleic acids from a serum sample, rich with confounding substances that inhibit nucleic acid amplification. As shown in Figure 1A, separated nucleic acids then focus with reagents required for RPA including enzymes, primers, and other biomolecules. However, serum proteins (e.g. proteins binding to target, RNAse or DNAse) can focus in the ITP plug with RPA reagents which deters amplification. To mitigate this effect, we apply proteinase K (PK) to the left side of the glass fiber substrate for protein digestion in the serum. The high isoelectric point of PK (~8.9) causes it to electromigrate towards the TE well, preventing PK from entering the ITP-RPA reaction plug.

We have demonstrated amplification of HIV nucleic acids spiked into 20 μL of human serum. Figure 3A shows the amplification progression of synthetic HIV DNA spiked into blood on the paper substrate. The DNA is extracted from the blood and rapidly amplified to create a bright fluorescence signal. The spatio-temporal heat map (Figure 3B) shows the RPA reaction initiates once the ITP plug migrates to the center of the paper strip. This demonstrates the need for ITP to separate reaction reagents from inhibitors present in the serum sample. Integrated fluorescence signals for positive and negative reactions in serum are plotted in Figure 3C. We show robust amplification of samples spiked with synthetic HIV-1 DNA in less than 15 minutes. Current work is focused on lowering the limit of detection (LoD) of the assay to the 1000 copies/mL clinical threshold. Optimization of ITP buffers and increasing the sample volume may reduce this LoD.

Experiments testing log dilutions of nucleic acid copies suggest that endpoint fluorescence intensity scales linearly with input nucleic acid copy number (R^2>0.98, data not shown). These results are similar to those of Rohrman *et al.* which showed quantitative HIV-1 DNA detection using a benchtop real-time RPA assay.[15] The observed linearity demonstrates the potential for quantifying HIV nucleic acids in blood samples with our device, which is a requirement for monitoring HIV viral load for ART treatment.

B. *Preliminary Whole Blood and Specificity Data*

In addition to extensive experimentation with human serum, a preliminary study showed promising results for ITP-RPA of whole blood spiked with HIV DNA. Spiked whole blood was added directly to a blood separation and passively fractionated to create plasma on the glass fiber surface. After 3 minutes of passive microfiltration, the fractionated plasma was collected on the glass fiber. ITP-RPA was run directly from this sample without further processing using the developed ITP chemistry. Preliminary experiments show ITP-RPA fluorescent outputs are similar for serum versus whole blood, yet further work is needed to improve the consistency and sensitivity of ITP-RPA with whole blood. We

aim to develop an ITP-RPA system that can amplify target nucleic acid targets in unprocessed whole blood from a finger or heel prick in under 15 minutes (3 minutes filtering + 10 minutes separation and amplification) using an integrated filter.

One concern with creating a highly concentrated reaction is increasing the likelihood of creating non-specific amplification products. However, we have shown that the system retains high specificity due to the *RecA* recombinase protein in the RPA reagents, which facilitates correct primer insertion to its complementary sequence within the target nucleic acid. We screened our HIV-1 RPA assay against a range of non-target nucleic acid sequences. Figure 4 shows preliminary specificity data in an ITP enhanced RPA system where only target HIV-1 nucleic acids are amplified.

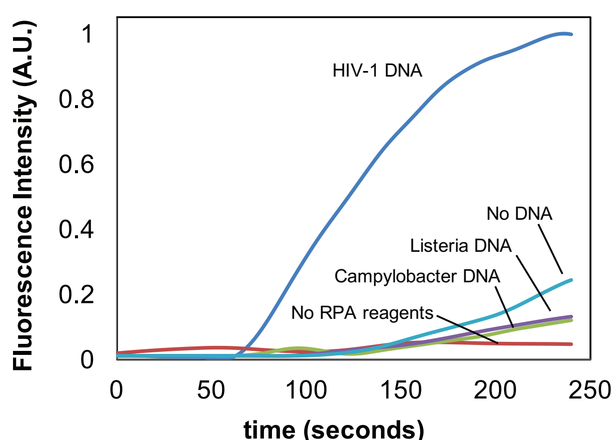

Figure 3. Specificity of the ITP enhanced RPA reaction with *pol* primers in the presence of non-target DNA. The HIV-1 DNA curve shows amplification, while the Listeria and Campylobacter DNA show fluorescence curves similar to a negative sample containing no DNA.

IV. Conclusion

We have shown that we can amplify nucleic acid targets in in 10–15 minutes by combining ITP with RPA on paper substrates with no external heating, mechanical actuation, or significant user interaction. This test has the potential to improve patient care in low resource settings, where routine VL monitoring outside the laboratory is needed. This simple, integrated approach for molecular diagnostics can be applied to numerous other diseases. Current work in our research group is focused on reducing the LoD to the clinically relevant goal of 1000 copies/mL. We are conducting comprehensive LoD and linearity studies detecting HIV-1 nucleic acids spiked into serum and whole blood.

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