Electronic Supplementary Material (ESI) for Analyst.

SUPPORTING INFORMATION

HIV detection from human serum with paper-based isotachophoretic RNA extraction and reverse transcription recombinase polymerase amplification

Andrew T. Bender[1], Benjamin P. Sullivan[1], Jane Y. Zhang[1], David C. Juergens[2], Lorraine Lillis[3], David S. Boyle[3], and Jonathan D. Posner[1,2,4]

[1] Department of Mechanical Engineering, University of Washington, Seattle, USA

[2] Department of Chemical Engineering, University of Washington, Seattle, USA

[3] PATH, Seattle, USA

[4] Family Medicine, School of Medicine, University of Washington, Seattle, USA

Table of contents

This Supporting Information document describes:

Experimental validation of SPRESSO simulations

We experimentally verified SPRESSO simulations predicting the pH profile of an ITP system. We performed ITP in a paper membrane and placed a strip of pH paper on top of the ITP membrane to visualize its approximate pH profile. We compared these results to simulations of the same ITP system in SPRESSO. We examined an ITP system employing a unique TE consisting of 10 mM tris, 10 mM serine, and 10 mM ß-alanine. The LE contained 120 mM HCl and 180 mM tris. We performed paper-based ITP for 10 minutes at 1 mA constant applied current. In Figure S1, we show experimental images of the ITP device with pH paper applied to the ITP membrane both before and after ITP. We observed a two distinct pH zones to the left of the ITP plug. The first zone contains serine ions and the second contains ß-alanine. ß-alanine has a lower electrophoretic mobility than serine in our system. The serine zone is at a significantly higher pH than the ß-alanine zone. We found good agreement between the experimental results and the simulated pH profiles generated by SPRESSO. This suggests that SPRESSO is a useful tool in designing ITP systems, especially for applications where pH control is important.

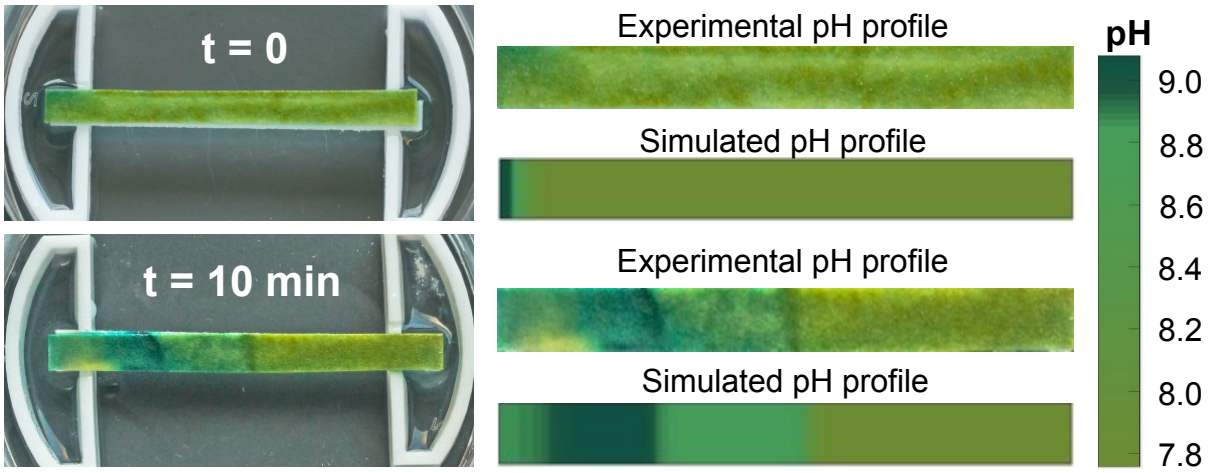
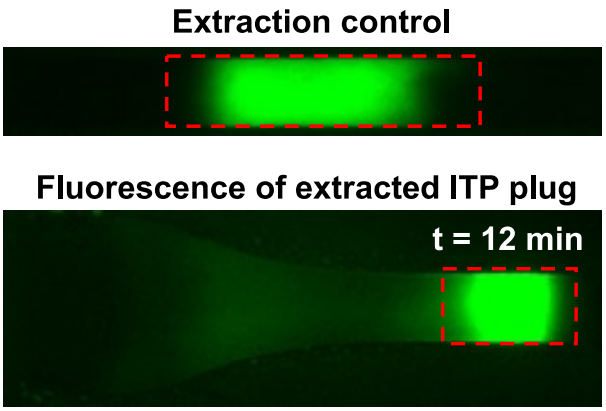


Figure S1. Simulations and experiments describing the pH profiles of an ITP system with ß-alanine and serine in the TE buffer. Experimental images of an ITP µPAD with pH paper overlaid on the separation membrane (left side). Images of pH paper were taken at t = 0 and t = 10 mins. Comparisons of SPRESSO simulations and experimental images of pH paper of the ITP system (right side). The ITP system employed a 10 mM Tris, 10 mM ß-alanine, and 10 mM serine TE buffer with 150 mM tris and 100 mM HCl LE buffer. After the LE/TE interfaces migrates 50% of

the strip length, a serine zone with pH ~8.8 develops directly adjacent to the ITP plug, followed by a ß-alanine zone at pH ~9.1.

ITP µPAD Extraction Efficiency

We investigated the extraction efficiency of the paper-based ITP system using digested serum samples that were spiked with DNA labeled with Alexa Fluor 488. The ITP extraction samples contained 36 µL of digested serum and 4 µL of 1 µM labeled DNA, totaling 4 picomoles. Experiments where the samples were diluted with LE buffer had the same DNA content (4 picomoles total). ITP extractions were imaged with epifluorescence microscopy and fluorescence intensities in the cropped region containing the ITP plug were used for the extraction efficiency calculations. We measured the control value denoting 100% extraction efficiency by imaging a wetted Fusion 5 strip of equal width that had been spiked with 4 picomoles of labeled DNA (same total DNA content as ITP samples). We calculated the bulk fluorescence of the cropped region of the control (denoted with red box in Figure S2) by summing the signal intensity of every pixel in the region. We similarly measured the bulk fluorescence of the extracted DNA using the cropped region of the ITP plug. All images were background subtracted to eliminate issues with differing areas and pixel counts between experiments. We performed replicate experiments on different days with distinct digested serum samples (no replicates collected for diluted samples). We observed that the ITP µPAD had extraction efficiencies ranging from 70% to 81% when processing undiluted serum samples. We observed that extraction efficiency increased when the serum was diluted, illustrating the challenge posed by proteinaceous samples in ITP systems.



$$Extract.Efficiency = \frac{Fluor_{ITP\ plug}}{Fluor_{control}}$$

DNA Extraction Efficiency of ITP µPAD			
Serum dilution	**Day 1**	**Day 2**	**Day 3**
No dilution	0.809	0.704	0.779
2X dilution	0.857	–	–
4X dilution	0.934	–	–

Figure S2. DNA extraction efficiency of the ITP µPAD. Epifluorescence microscopy images measured the signal intensity emanating from labeled DNA present in the paper membrane. The bulk fluorescence in the ITP plug was calculated by summing the pixel values from a cropped region of the image containing the plug (denoted by the red box). The extraction control (100% extraction efficiency) was similarly calculated. All images were background subtracted. Replicate extraction efficiency experiments were carried out on separate days with different samples. Only one set of experiments was run with diluted samples.

Membrane selection

We performed experiments using different porous membranes for ITP electromigration of DNA to identify the ideal membrane for minimizing analyte loss. Several studies have reported issues with nucleic acid adsorption to substrates or entanglement in porous membranes.[1,2] We use fluorescence imaging of labeled DNA to visualize electromigration of nucleic acids with ITP and identify paper substrates that result in DNA entanglement or adsorption. Figure S3 illustrates electromigration of DNA (70 bp) labeled with Alexa Fluor 488 through two different membranes: Fusion 5 (GE Healthcare) and glass fiber (GFCP203000, EMD Millipore). We used a TE buffer consisting of 20 mM tris and 20 mM serine and an LE buffer with 120 mM HCl and 180 mM tris. We used porous membranes cut into straight strips (35 mm by 3 mm). After 15 minutes of ITP in glass fiber, we observe pockets of fluorescence trailing behind the ITP plug. This indicates adsorption or entanglement of DNA occurs during electromigration. Due to this undesirable source of analyte loss, we explored other membranes for use in paper-based ITP. We observed similar DNA adsorption issues in several other glass fiber membranes that are commonly

used in lateral flow assays (data not shown). We found that DNA is able to successfully electromigrate through Fusion 5 membrane during ITP with no observable loss of analyte.

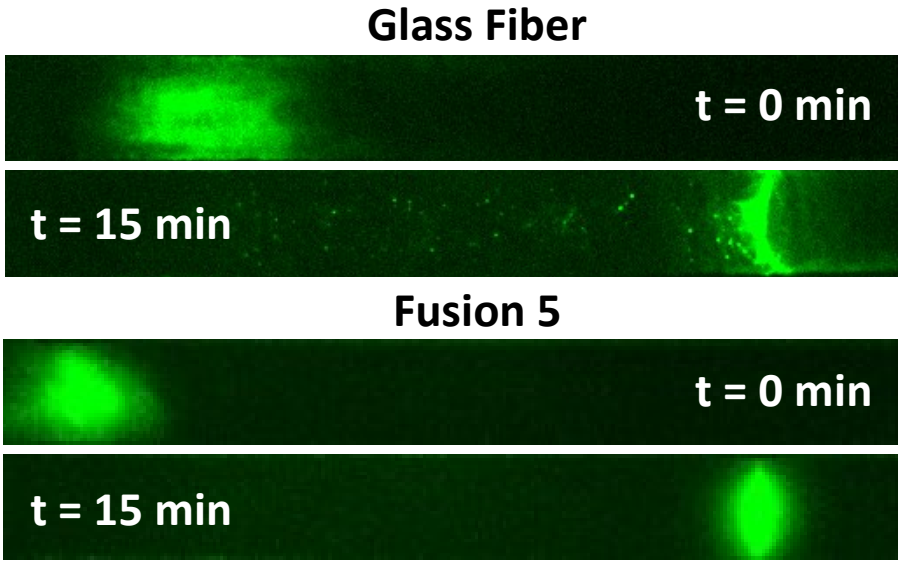


Figure S3. Electromigration of fluorescently labeled DNA through porous substrates. We transported DNA labeled with Alexa Fluor 488 across Fusion 5 and glass fiber membranes under identical conditions. Fluorescence images at t = 0 and t = 15 minutes are shown.

ITP extractions of nucleic acids from pure buffer samples

Visual observations of DNA transport are useful in designing ITP systems and selecting appropriate membranes, but do not provide information on whether low copies of nucleic acids electromigrate through paper and are viable for amplification. Using the procedure illustrated in Figure S4, we electromigrated DNA and RNA across porous membranes and detected successful transport with off-chip RPA or RT-RPA. For DNA extractions, we used 200 bp synthetic DNA of a proviral HIV gene and corresponding RPA primers and probe, as previously described.[3] We were able to consistently electromigrate 10 copies of DNA across the length of a Fusion 5 strip and detect them with RPA. Figure S5 shows fluorescence measurements over a 20-minute RPA reaction, with fluorescence over 100 arbitrary fluorescence units indicating successful amplification. We also performed experiments electromigrating HIV-1 RNA with an 8,941 bp genome length. We could consistently migrate 50 copies of HIV RNA across the Fusion 5 membrane. Figure S5 shows successful amplification of 50 copies of HIV RNA after ITP extraction (N = 3).

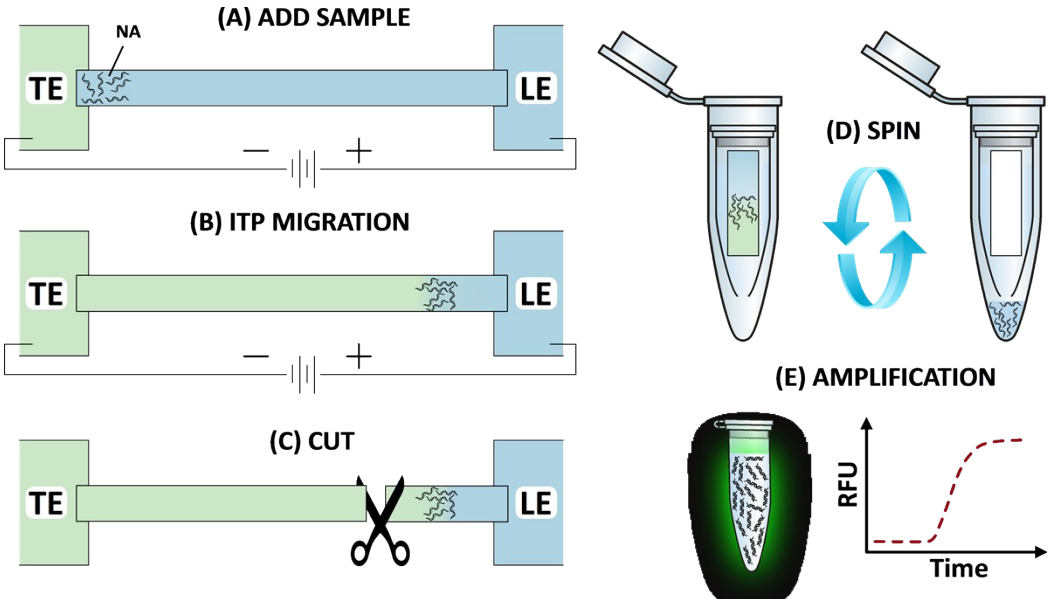


Figure S4. Procedure for detecting low copies of DNA or RNA electromigrated across a paper membrane. (A) ITP buffers are initially added to the paper strip and respective reservoirs. Initial location of LE buffer is colored in blue while initial location of TE buffer is colored in green. Nucleic acids (NA) are added to the strip directly adjacent to the TE reservoir. A 1 mA constant current initiates ITP. (B) Nucleic acids are negatively charged and electromigrate across the paper strip towards the anode. (C) The region of the paper strip containing the ITP plug is cut and placed in a 0.5 mL plastic tube with a small hole at the bottom. (D) The 0.5 mL plastic tube is placed inside a 1.5 mL plastic tube and centrifuged, removing the contents of the ITP plug (*i.e.* nucleic acids) from the paper. (E) The contents of the plug are pipetted into an RPA reaction for off-chip nucleic acid amplification.

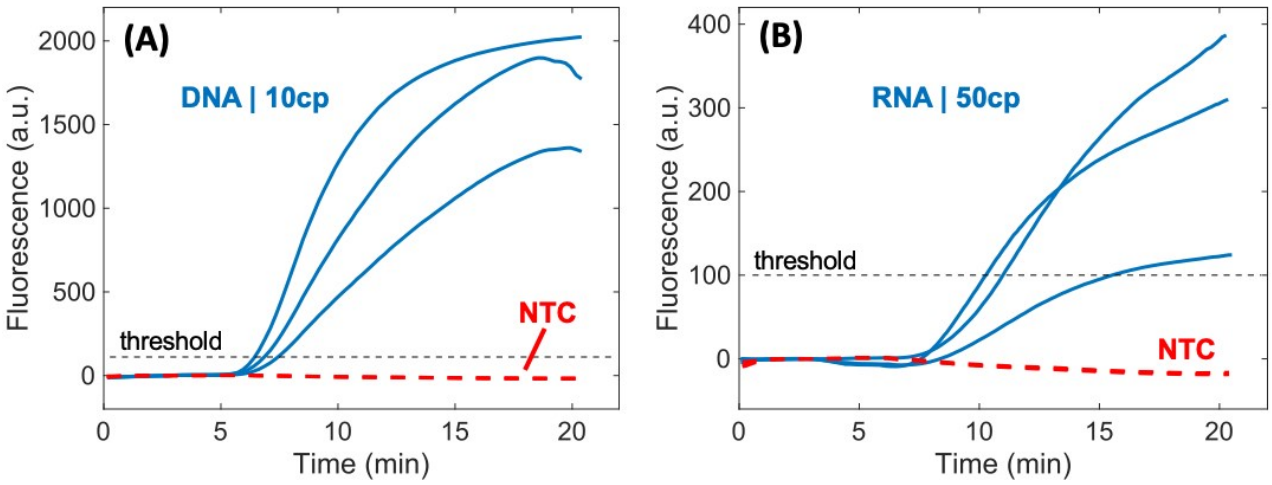


Figure S5. Fluorescence measurements of RPA reactions to detect successful electromigration of DNA or RNA across a paper membrane in a pure buffer system. (A) Triplicate experiments of 10 DNA copies extracted with ITP and amplified with RPA. RPA reactions with extracted DNA consistently amplified over the positive/negative threshold of 100 arbitrary fluorescence units. (B) Triplicate experiment of 50 HIV RNA copies extracted and then amplified for detection. Extracted RNA was consistently amplified over the positive/negative threshold. We observed that no template controls (NTCs) for both assays (N = 3 for each) were very consistent and never approached the positive/negative threshold.

Effect of TE selection on ITP plug purity

SPRESSO simulations indicated that some TE selections resulted in an elevated ATE pH, which may lead to proteinase K migrating into the ITP plug along with RNA. In Figure S6, we show data for two different ITP systems to demonstrate the importance of TE selection. The first ITP system featured only ß-alanine as the trailing electrolyte. ß-alanine can be a powerful TE for extracting analytes due to its extremely low electrophoretic mobility at most pH levels.[4] However, it is not well-suited for preventing proteinase K from electrophoresing into the plug. The ITP system processed a digested serum sample and the contents of the ITP plug were added to RPA reactions with 1,000 copies of HIV RNA. As expected, these experiments did not amplify because proteinase K was not electrophoretically removed. The second ITP system was designed to remove proteinase K from the ITP plug with a serine TE. This ITP system generated an ITP plug that had minimal effects on the amplification of HIV RNA.

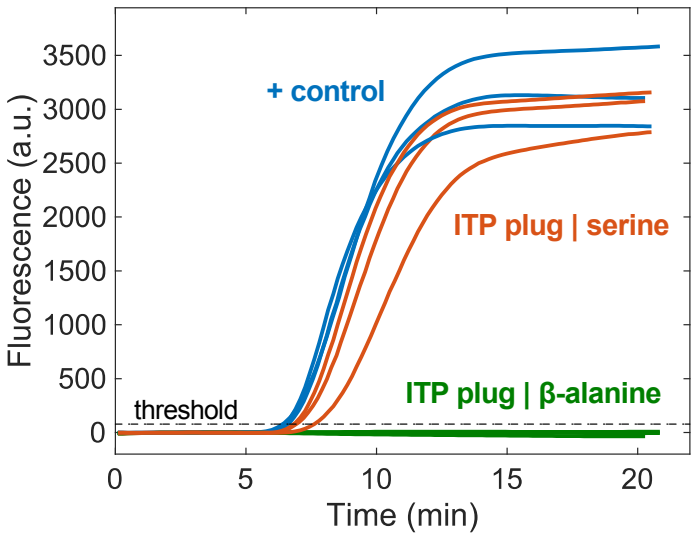


Figure S6. Purification of nucleic acids from digested serum with different TE selections. We used ITP to process digested serum (no nucleic acids added) with either serine or ß-alanine TEs. ITP plugs were added to RPA reactions with 1,000 copies of HIV-1 RNA. Fluorescence measurements of RPA indicate the purity of the ITP plugs. Positive control experiments (N = 3) simply include nuclease free water. Serine experiments (N = 3) utilized an ITP chemistry with 10 mM serine. ß-alanine experiments (N = 3) employed a TE buffer with 10 mM ß-alanine.

ITP µPAD for DNA extraction and detection from human serum

In addition to the RNA extractions from serum lysate presented in the main text, we analyzed the performance of our ITP µPAD by extracting DNA from digested serum. Figure S7 presents fluorescence amplification curves for extracted DNA and respective triplicate NTCs. The LoD of DNA (200 bp) spiked into digested serum was determined to be 500 copies per mL of serum. This is equivalent to 20 copies in the 40 µL sample volume. The LOD for extracting DNA from digested serum in our ITP µPAD is an order of magnitude better than for extracting RNA. We believe there are multiple reasons for this result. We used synthetic DNA (200 bp length) which is an easier analyte for diagnostics due to the stability of DNA and the low prevalence of harmful DNases in human serum. RNA is much more prone to degradation chemically or due to endogenous RNases. DNA that has a short length is easier for ITP extractions in porous membranes because its electrophoretic mobility is not as hindered as HIV RNA (~9 kb). Further, the RPA assay

for DNA used in this work has nearly single-copy sensitivity, while the RT-RPA assay for HIV has sensitivity down to approximately 30 copies per reaction.[5]

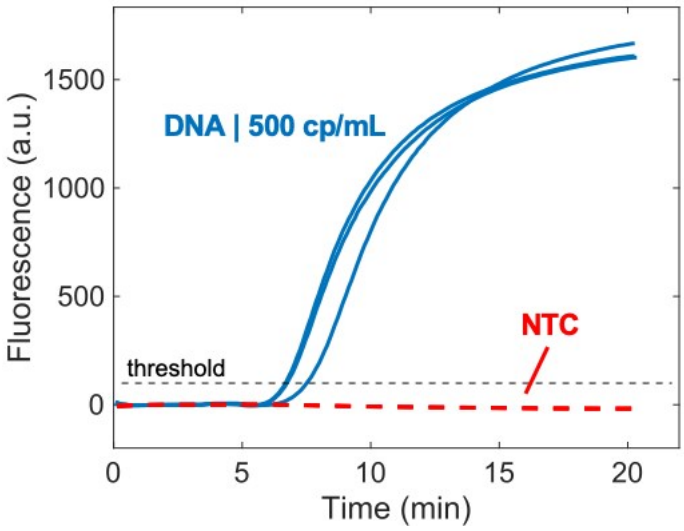


Figure S7. Fluorescence measurements for RPA reactions detecting synthetic DNA isotachophoretically extracted from 40 µL samples of digested human serum. (A) DNA at a concentration of 500 copies per mL of serum (20 copies suspended in 40 µL of serum) were extracted and consistently amplified above the positive/negative threshold with RPA (N = 3).

Extractions from proteolyzed vs non-proteolyzed serum

We demonstrate the importance of extensive proteolytic digestion of serum proteins in enabling ITP extraction of nucleic acids. Figure S8A shows fluorescence images of DNA extraction from serum digested with 1 mg/mL proteinase K (1 hr incubation at 50 °C), with corresponding y-averaged intensity profiles as a function of strip length. The experiment shown in Figure S8B is identical to that of Figure S8A, except serum was not proteolyzed with proteinase K. Digestion of serum proteins with proteinase K clearly alters the behavior of ITP-based DNA extraction. Other reports on DNA extraction from serum and plasma have stated that ITP cannot adequately separate DNA without deproteinization of the sample.[4,6] Our findings are consistent with these reports. We observe undigested serum hinders the electromigration of DNA through porous membranes, likely due to nucleoprotein complexes that form between serum proteins and DNA. We conclude that thorough digestion of serum proteins is critical to efficient DNA extraction.

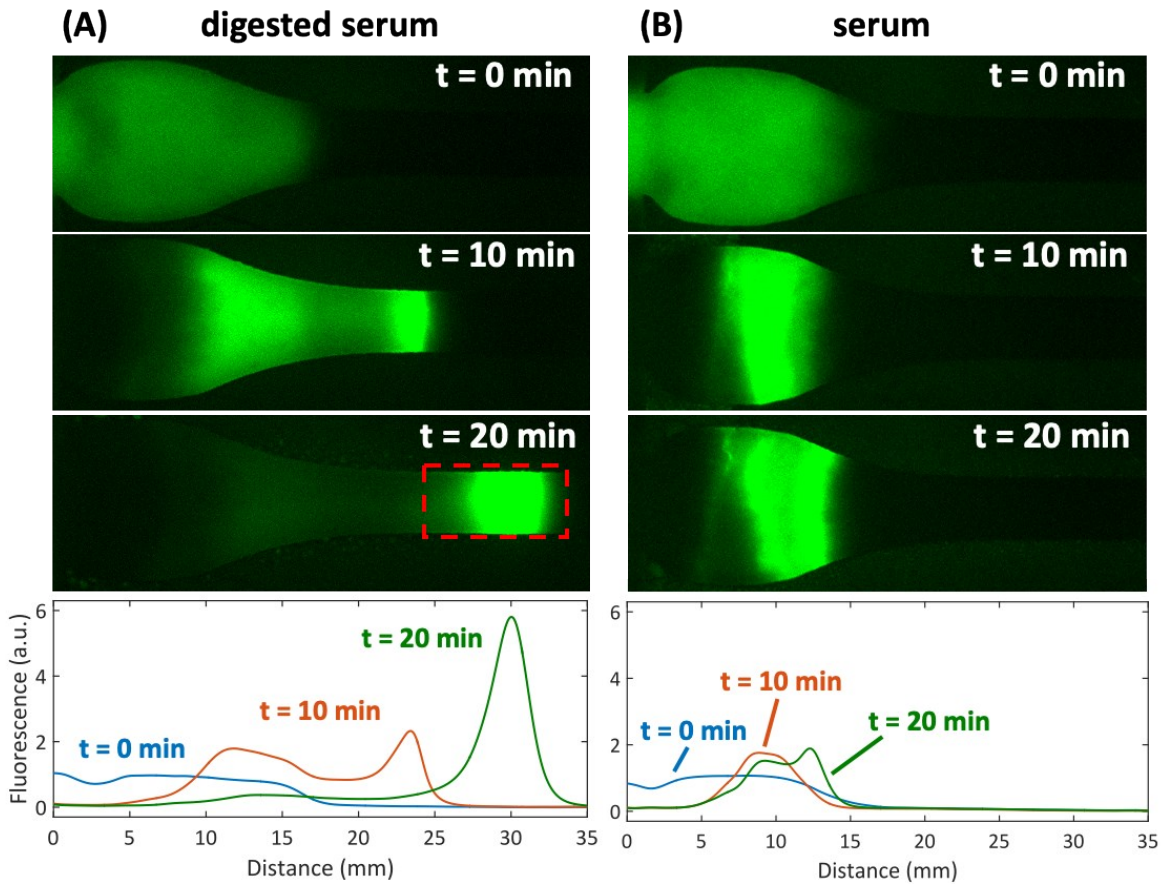


Figure S8. Experimental fluorescence images of ITP extraction of nucleic acids from digested serum and untreated serum. (A) DNA labeled with Alexa Fluor 488 is mixed with digested human serum and is initially located in the wide sample zone of the Fusion 5 membrane. After 20 minutes of ITP, DNA focuses into a concentrated plug in the straight portion of the strip. Pixel intensities of the images are y-averaged, creating normalized fluorescence distribution with respect to distance along the membrane for each time point (0, 10, and 20 minutes). (B) ITP extraction of labeled DNA from untreated serum is presented for comparison. Without serum digestion, DNA migration is hindered and does not electromigrate across the length of the strip.

REFERENCES

1 L. K. Lafleur, J. D. Bishop, E. K. Heiniger, R. P. Gallagher, M. D. Wheeler, P. Kauffman, X. Zhang, E. C. Kline, J. R. Buser, S. Kumar, S. A. Byrnes, N. M. J. Vermeulen, N. K. Scarr, Y. Belousov, W. Mahoney, B. J. Toley, P. D. Ladd, B. R. Lutz and P. Yager, *Lab Chip*, 2016, **16**, 3777–3787.

2 S. A. Byrnes, J. D. Bishop and P. Yager, *Analytical Methods*, 2017, **9**, 3450–3463.

3 A. T. Bender, M. D. Borysiak, A. M. Levenson, L. Lillis, D. S. Boyle and J. D. Posner, *Analytical Chemistry*, 2018, **90**, 7221–7229.

4 V. N. Kondratova, I. V. Botezatu, V. P. Shelepov and A. V. Lichtenstein, *Analytical Biochemistry*, 2011, **408**, 304–308.

5 L. Lillis, D. A. Lehman, J. B. Siverson, J. Weis, J. Cantera, M. Parker, O. Piepenburg, J. Overbaugh and D. S. Boyle, *J. Virol. Methods*, 2016, **230**, 28–35.

6 V. N. Kondratova, I. V. Botezatu, V. P. Shelepov and A. V. Lichtenstein, *Biochemistry (Moscow)*, 2009, **74**, 1285–1288.