Integrated bacterial cell lysis and DNA extraction using paper-based isotachophoresis – towards a versatile sample preparation module for point-of-care diagnostics

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Abstract

Bacterial infections remain a global threat, particularly in low-resource settings, where access to accurate and timely diagnosis is limited. Point-of-care nucleic acid amplification tests have shown great promise in addressing this challenge. However, their dependence on complex traditional sample preparation methods remains a major challenge. To address this limitation, we present a paper-based sample preparation module that integrates bacterial cell lysis, DNA purification, and concentration using an electrokinetic technique called isotachophoresis (ITP). This is the first device that i) integrates electrochemical bacterial lysis with ITP, and ii) demonstrates the focusing of whole bacterial genomic DNA (gDNA) in paper. Characterization with buffers showed that the paper-based ITP sample preparation module (*p*-ITPrep) concentrated bacterial gDNA with an average concentration factor of 12X, and DNA could be extracted from a sample containing as few as 10^2 CFU/mL *Mycobacterium smegmatis* (*Msm*). From complex biological matrices – human saliva, human blood serum, and artificial urine, *p*-ITPrep extracted DNA from samples containing 10^2 CFU *Msm*/mL saliva or artificial urine and 10^3 CFU *Msm*/mL serum within 20 minutes. The extraction procedure involved only 3 user steps, in contrast to conventional solid phase extraction kits that require more than 10 user steps. *p*-ITPrep may provide a simple, inexpensive, and versatile alternative to conventional multi-step nucleic acid extraction protocols for point-of-care diagnostic.

Introduction

Bacterial infections remain the second leading cause of deaths worldwide, accounting for 7.7 million deaths, which was 13.6% of the global total in 2019, and for more than half of all sepsis-related deaths as reported by a recent study published in The Lancet.[1] Some of the most common bacterial infections include respiratory infections (pneumonia and tuberculosis), UTIs (urinary tract infections), and skin, blood, and gastrointestinal infections. Early diagnosis of bacterial infections is essential to prevent the spread of infection, for effective treatment, and for reducing the risk of serious complications such as sepsis.

Rapid diagnostic tests (RDTs) such as nucleic acid amplification tests (NAATs) have revolutionized the field of molecular diagnostics. Compared to traditional culture-based methods, NAATs offer several advantages, particularly rapid turnaround times (< 2 hours), higher clinical sensitivities, and comparable specificities. However, NAATs such as the polymerase chain reaction (PCR) have been restricted to centralized diagnostic laboratories because of their dependence on instrumentation, uninterrupted power supply, and qualified technical staff. In recent years, a large amount of effort has been put into developing affordable and user-friendly point-of-care (POC)-NAATs for decentralized testing. However, the primary focus has remained on nucleic acid (NA) amplification and detection strategies,[2] while sample preparation at the POC has received less attention.[3] Crude biological samples contain components that could impede the ability of polymerases to amplify NAs or suppress the fluorescence of intercalating dyes.[4,5] Sample preparation generally involves pathogen lysis followed by separation processes to concentrate and purify the target NAs from the potential inhibitors in the sample, such as proteins, lipids, and polysaccharides. Unlike NA amplification, sample preparation is not a one-pot 'batch' process but

involves multiple intermediate steps and remains the most challenging operation in any NAAT workflow.[6]

Most POC-NAATs still rely on conventional solid phase extraction (SPE) of NAs for sample preparation. In SPE, a solid sorbent material (e.g., silica, cellulose)[7–9] or polymer-based material (chitosan)[10,11] selectively binds and retains target NAs while contaminants and other unwanted materials are washed away; it is an inherently multi-step process. Several researchers have explored using inexpensive paper membranes as a substitute for the solid substrate in SPE to perform NA sample preparation.[12–16] Our group has recently reviewed these paper-based nucleic acid extraction techniques.[3] Although many of these studies have successfully reduced the use of ancillary equipment, they still require multiple timed steps. There remains a void for simple sample preparation with single or minimal user steps; therefore, NA sample preparation remains a significant roadblock in the progression of POC-NAATs.

One promising alternative to SPE is an electrokinetic separation and concentration method known as isotachophoresis (ITP). ITP uses an electric field and a discontinuous buffer system consisting of a fast-moving leading electrolyte (LE) and a slow-moving trailing electrolyte (TE). Anionic ITP exploits the negative charge of NAs (DNA in this case) to focus and concentrate NAs at the LE/TE interface, while excluding proteins and other PCR-inhibitory molecules. The ITP procedure relies on the free-solution electrophoretic mobility of NAs, which is independent of fragment length (in the range of 400 bp to 48.5 kb) or sequence.[17,18] The electrolyte buffers are strategically selected such that the effective electrophoretic mobilities, μ, of the anionic part are of the order: $\mu_{LE} > \mu_{DNA} > \mu_{TE} > \mu_{impurities,}$ thereby imparting selectivity to the extraction. Microchannel-based ITP

has been used to extract and concentrate NAs from different biological samples like blood,[19,20] urine,[21] and whole milk,[22] and to carry out (amplification-free) NA hybridization reactions.[21,23,24] However, conducting microchannel-based ITP in resource-limited settings is challenging because of their reliance on complex sample and buffer loading procedures (such as vacuum assistance), limited volume liquid reservoirs, and off-chip lysis methods.[19–21,25,26]

Recently, ITP has been incorporated into microfluidic paper-based analytical devices (μPADs). Several paper-based ITP studies have investigated the extraction and concentration of charged-analytes (ssDNA, dsDNA ladder,[27] DNA probes,[28] fluorophores,[29] and indicator dyes[30,31]) in simple buffer systems. The Posner group has shown the feasibility of DNA extraction using paper-based ITP.[32] Additionally, they demonstrated simultaneous isotachophoretic extraction and nucleic acid amplification (RPA) in paper.[33] A subsequent study from the same group detected HIV virions and MS2 bacteriophage in human serum.[34] Despite the promising results of paper-based ITP DNA extraction for molecular diagnostics, many studies required a high DC power source (>100V)[30,33,34] to execute ITP. Subsequently, Li et al.[27] demonstrated a low-voltage (18V) paper-based ITP system using two 9V batteries for the concentration of synthetic ssDNA and dsDNA samples. This was accomplished by reducing the distance between the two electrodes using a concertina or accordion paper folding technique.

In this article, we build upon the accordion paper folding technique to develop a low-voltage paper-based ITP sample preparation module (*p*-ITPrep) that, for the first time, integrates bacterial lysis and DNA extraction. We also demonstrate the ITP-based concentration of bacterial whole genomic DNA (4.4 million base pairs) in paper for the first time. Previous demonstrations of paper-based

ITP have been restricted to the concentration of shorter DNA oligomers of less than a few thousand base pairs. For demonstration, we chose a hard-to-lyse gram-positive bacterium, Mycobacterium smegmatis (*Msm*), which has a thick cell wall. We show that *p*-ITPrep can perform *Msm* bacterial lysis and DNA purification and concentration from human serum, human saliva, and artificial human urine for downstream PCR testing with either no or minimal intermediate user steps in 20 minutes using an 18V power supply. By demonstrating ITP-based NA extraction directly from a complex human matrix spiked with gram-positive bacteria, this work presents the most advanced demonstration of ITP-based sample preparation yet. The *p*-ITPrep prototype is a standalone instrument-free NA sample preparation device that may be ideal for use in low-resource settings but could also be used to automate or minimize the number of user steps in NA sample preparation protocols in any laboratory performing NA research or testing.

Results and Discussion

Design and workflow of *p*-ITPrep

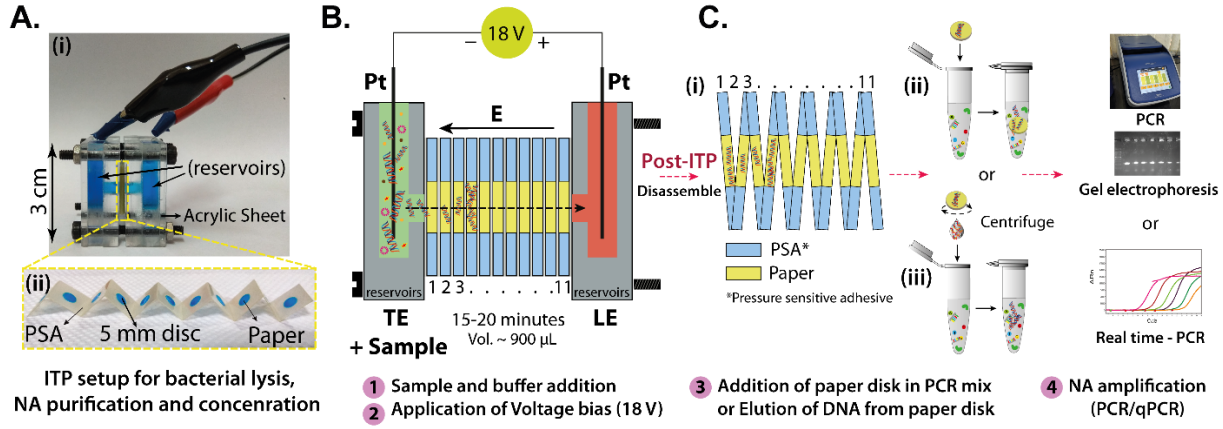

Fig. 1 Design and workflow of paper-based ITP sample preparation module (*p*-ITPrep) for NAATs. A. Image of the *p*-ITPrep containing (i) two electrolyte reservoirs and (ii) an 11-layer accordion structure with filter paper discs (containing a blue dye for visibility) adhered to each layer with pressure-sensitive adhesive (PSA). B. A schematic illustration of *p*-ITPrep depicting LE and TE reservoirs, an 11-layer foldable paper structure, Pt electrodes, and a voltage source. C. Workflow: Post-ITP, the module is disassembled to access the paper discs (containing the concentrated gDNA) (i), which could be directly

inserted into a tube (ii) or the DNA could be eluted from the paper discs and used for PCR or real-time PCR (iii).

The design of *p*-ITPrep is inspired by a paper-based ITP device featuring an accordion-like paper structure previously described by Li et al.[27]. *p*-ITPrep is fabricated by assembling two parts: 1) two acrylic buffer reservoirs for the leading electrolyte (LE) and trailing electrolyte (TE) (Fig. 1A(i)), and 2) an 11-layer foldable paper structure (Fig. 1A(ii)) sandwiched between the two reservoirs. Details of the design are provided in the electronic supplementary information (ESI Fig. S1). Briefly, the foldable structure is made of double-sided pressure-sensitive adhesive (PSA) and circular discs (diameter 5 mm) of 180 µm thick Whatman filter paper grade 1 (Fig. 1A(ii)). Both reservoirs are fabricated using three acrylic layers of varying thicknesses that are stacked, aligned, and bound together using dichloromethane (DCM). Both reservoirs have a 3 mm hole on one side, which connects to the paper discs in the folded structure (Fig. 1B). The horizontal stack of paper layers forms a fluidic channel for charged molecules to migrate in response to a voltage bias (Fig. 1B). The paper layers and the two reservoirs are secured tightly using four adjustable screws at the corners of the acrylic reservoirs to avoid liquid leakage.

The workflow for sample preparation using *p*-ITPrep is illustrated in Fig. 1B-C. The LE reservoir is first filled with 900 µL of the leading electrolyte, which saturates all paper discs. Subsequently, the TE reservoir is filled with a 900 µL solution containing the sample from which DNA is to be extracted mixed with the trailing electrolyte. Thus, the initial LE/TE interface is at paper layer 1 (PL-1). Subsequently, platinum wire electrodes are inserted into each reservoir, and a voltage bias of 18 V is applied for 15-20 minutes between the electrodes (Fig. 1B). The TE reservoir contains the cathode (negative electrode) and the LE reservoir contains the anode (positive electrode). During isotachophoresis, negatively charged NAs migrate towards the positively charged anode

(from PL-1 to PL-11). Post ITP, the Pt electrodes and buffers are removed from the reservoirs and the device is disassembled to analyze the contents of each paper layer (Fig. 1C (i)). A paper disk is either directly placed into a PCR tube (Fig. 1C (ii)), or the extracted DNA is eluted from the paper disk and added into a PCR tube (Fig. 1C (iii)) for DNA amplification.

Concentration of gDNA in *p*-ITPrep

Because previous demonstrations of DNA concentration using paper-based ITP have been restricted to DNA of less than a few thousand base pairs, it was important to determine whether whole bacterial gDNA could be concentrated using paper-based ITP. In order to assess this, purified *Mycobacterium tuberculosis* (Mtb) whole gDNA of varying concentrations was spiked in the TE buffer (sample), and ITP was performed for 15 min. After ITP, the device was disassembled, the accordion structure was unfolded, individual paper layers (discs) were removed, and the fluid in each layer was removed by centrifugation (referred to as the elute). The elute from each paper layer was then subjected to qPCR for quantification. Two types of No-ITP controls were included – **L**: the liquid sample that did not undergo ITP, and **P**: fluid centrifuged out of a paper disc that was dipped in the liquid sample that did not undergo ITP. Similarly, the two post-ITP controls included fluids remaining in the leading ($\mathbf{LE_C}$) and trailing electrolyte ($\mathbf{TE_C}$) reservoirs after ITP was performed. DNA concentrations measured from each paper layer were normalized by the DNA concentration in the No-ITP **L** control to evaluate concentration factors.

p-ITPrep successfully concentrated bacterial gDNA. Concentration factors in each paper layer for samples containing 10^3, 10^2, and 10^1 gDNA copies/µL are shown in Fig. 2A-C (concentration from a sample containing 10^0 gDNA copies/µL was also tried, but it was unsuccessful (ESI Fig. S2)).

In all cases, the variation of concentration factors with the paper layer number had a characteristic pattern with the maximum concentration factor in paper layer PL-1, followed by a Gaussian-like distribution over PL 2-7. The Gaussian-like distribution is characteristic of DNA concentration using ITP. The anomalous high concentration of DNA in PL-1 suggests that large gDNA molecules get entangled in the pores of PL-1. The gDNA that does not get entangled in PL-1 migrates electrophoretically and gets concentrated in subsequent layers by ITP. In all cases, the maximum DNA concentration was obtained in PL-1; concentration factors of 10.5, 13.7, and 12.0 (average 12 ± 1.6) were obtained in PL-1 for samples containing 10^3, 10^2, and 10^1 gDNA copies/μL, respectively (Fig. 2A-C). In all cases, the **P** controls had concentration factors < 1, which shows that some DNA is lost when eluting from discs by centrifugation. For post-ITP controls, the $\mathbf{TE_C}$ controls had concentration factors less than 1 because DNA migrated away from the sample in the TE reservoir, and the concentration in $\mathbf{LE_C}$ was undetectable, indicating that DNA did not migrate as far as the LE reservoir at the other end of the fluidic channel. Note that all results presented in this article are from using Whatman Filter Paper Grade 1 for the discs. However, the use of Standard 17 glass fiber discs was also investigated, but it did not produce any significant DNA concentration (ESI Fig. S3).

The concentration factors obtained here (~12X; Fig. 2A-C) are lower in comparison with other paper-based ITP demonstrations,[28,29,35] which are of the order of 1000X. However, all previously reported concentration factors are for smaller molecules like fluorescent tracers (DyLight 650, Alexa Fluor 488) or 80 bp DNA oligonucleotides. This is the first report of the concentration of whole bacterial gDNA (4.4 million bp). The reason for a lower concentration factor is not clear, but is most likely related to enhanced interactions of these very large molecules with the pore

walls, causing deviations from free solution electrophoretic mobilities. A large fraction of the gDNA likely gets entrapped in the pores. Nonetheless, the obtained concentration factors of ~12X are practically useful, as demonstrated subsequently in this article.

In order to ensure that the voltage bias and the ensuing ITP were the cause of DNA concentration, a zero voltage control experiment was conducted with a sample containing 10^2 gDNA copies/µL. No DNA concentration was observed in the absence of a voltage bias (Fig. 2D), which shows that the concentration of gDNA was indeed induced by electrokinetic phenomena. Additionally, instead of the discontinuous-buffer ITP system, a single buffer electrophoresis system was tested, which led to insignificant concentration of DNA (ESI Fig. S6). This shows that it is the discontinuous-buffer ITP system and not conventional electrophoresis that leads to DNA concentration.

In the proposed ITP workflow, because elution of accumulated DNA by centrifugation constitutes an additional user step, the direct addition of post-ITP paper discs into PCR tubes was also tested. PCR tubes containing paper discs performed just as well as eluted DNA for both end-point PCR and real-time qPCR (ESI Fig. S4, S5). To enable direct PCR amplification from paper discs, bovine serum albumin (BSA) and Tween 20 had to be introduced into the PCR mix, which, we hypothesize, reduced the non-specific adsorption of enzymes onto the paper discs.

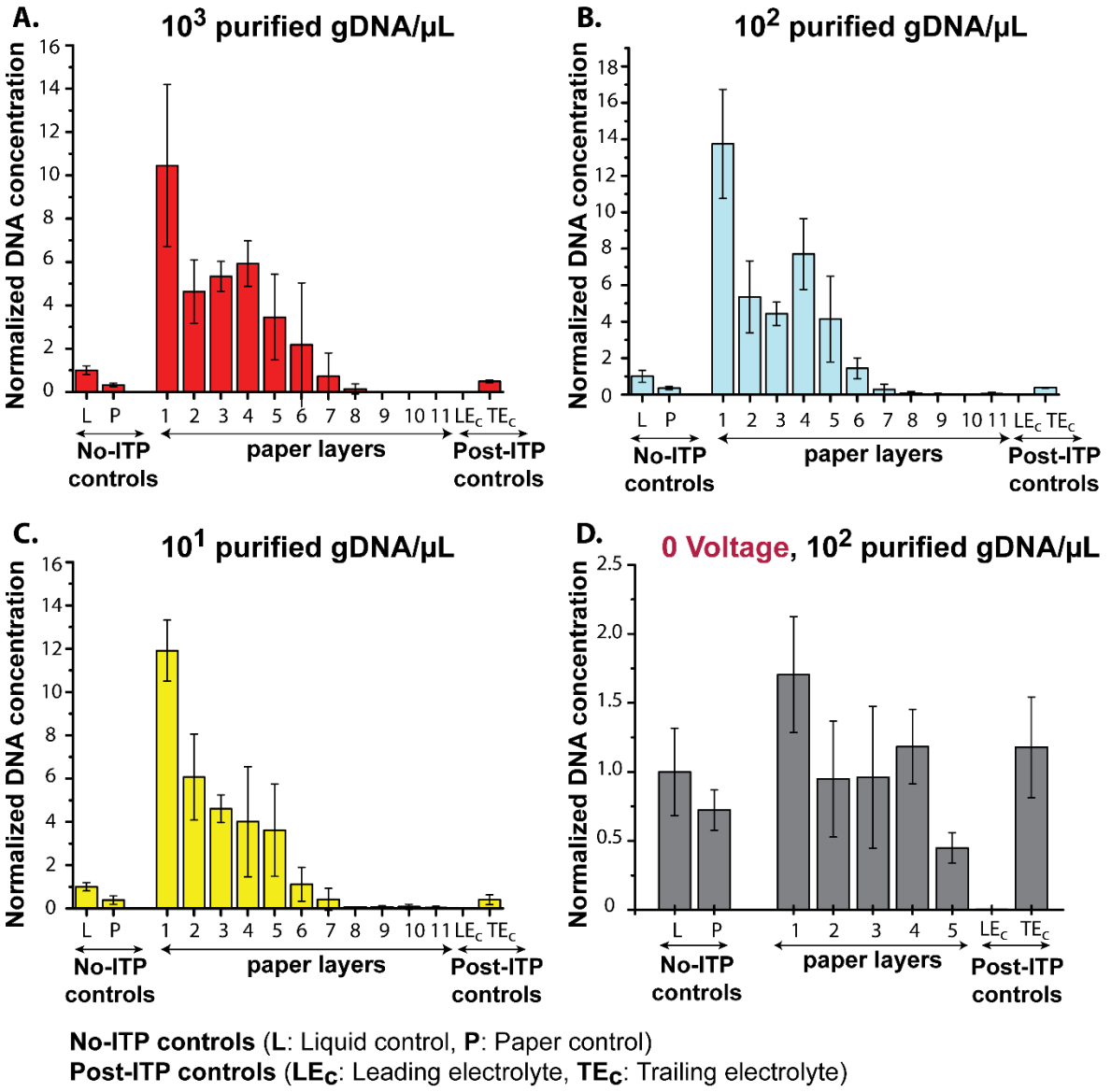

Figure 2. Concentration of purified gDNA in *p*-ITPrep. A-C: Plots of normalized DNA concentrations (concentration factors) in the paper layers for samples containing 10^3 (A), 10^2 (B), and 10^1 (C) gDNA copies/µL. D. Plot of normalized DNA concentration in paper layers when no voltage bias is applied for a sample containing 10^2 gDNA copies/µL. All DNA concentrations are normalized with the DNA concentration in the liquid No-ITP control (L). All error bars represent standard deviations (N=3 ITP devices).

Combined bacterial lysis and gDNA concentration in *p*-ITPrep

The use of electrochemically generated hydroxide, which produces a localized high pH, as a means of inducing cell lysis has been reported in literature.[36–38] Unlike electroporation-based lysis that

requires a high-voltage power supply, electrochemical cell lysis occurs at lower voltages. Based on this, we hypothesized that *p*-ITPrep could simultaneously conduct electrochemical bacterial cell lysis and genomic DNA concentration. To test this hypothesis, *Msm* bacteria were spiked in the trailing electrolyte (TE) buffer and ITP was conducted. In all cases where bacteria were spiked into the sample, a 0.45 µm mixed cellulose ester (MCE) filter membrane was used to replace the first paper layer in direct contact with the TE reservoir to restrict the electro-migration of unlysed bacterial cells into subsequent paper layers. This modification was necessary because bacterial cells have a negative surface zeta potential and could migrate towards the anode in the presence of an electric field.[39–41]

Combined bacterial lysis and DNA concentration in *p*-ITPrep was tested using *Msm*, a gram-positive bacteria having a thick cell wall. *Msm* bacteria were spiked in the TE buffer at different concentrations (10^5-10^2 CFU/mL); 900 µL of *Msm*-spiked TE buffer and 900 µL of the LE buffer were introduced into the respective reservoirs of *p*-ITPrep, and a voltage bias of 18 V was applied for 20 minutes. To test for bacterial lysis, the bacterial suspension introduced into the TE reservoir that had not undergone ITP (No-ITP control; **L**), and the solution collected from the TE reservoir post-ITP (post-ITP control; $\mathbf{TE_C}$) were plated on agar plates. In addition, PL 1-4 were directly streaked on agar plates to determine whether viable bacteria migrated into the paper. For 10^5 CFU/mL, compared to the No-ITP control, **L,** (Fig. 3A (i)), there was a significant reduction in the number of bacterial colonies in the post-ITP control, **TEc**, (Fig. 3A (ii)), which suggests that bacteria were lysed during the process. However, a large number of viable bacteria were found in PL-1 (Fig. 3A(iii)). Similar trends were observed in bacterial samples with lower CFUs (ESI Fig. S7). Based on this data, it was not possible to conclude whether bacterial lysis indeed occurred in

the TE reservoir, or whether unlysed bacteria from the TE reservoir migrated into PL-1 under influence of the electric field. PL2-4 did not show any bacterial growth, which shows that the 0.45 μm filter layer at PL-1 was effective at preventing any further migration of bacteria into the paper layers.

To conclusively determine whether bacterial lysis occurred during the process, the concentration of DNA in all paper layers was measured using qPCR. Compared to the No-ITP control, **L**, the mean DNA concentration in PL-1 was significantly higher for all bacterial concentrations (10^5 – 10^2 CFU/mL) spiked in the TE buffer (Fig. 3B-E). Because there was an accumulation of live bacteria in PL-1 (Fig. 3A(iii)), the measured DNA concentration in PL-1 could be from whole bacteria which get lysed during the high temperature PCR cycles. Therefore, this does not conclusively point to cell lysis within the ITP device. However, DNA was detected by qPCR in subsequent paper layers also: PL 2-8 for 10^5 CFU/mL (Fig. 3B), PL 2-5 for 10^4 CFU/mL (Fig. 3C), and PL 1-4 for 10^3 CFU/mL (Fig. 3D). Because no bacterial growth was observed from any of the subsequent layers, this conclusively points to partial lysis of bacteria and migration of gDNA from lysed bacteria into subsequent paper layers during the ITP process. Note that while for bacterial concentrations of 10^5 and 10^4 CFU/mL, low concentrations of DNA were detectable directly from the native samples (No-ITP controls), for concentrations of 10^3 and 10^2 CFU/mL, qPCR was not successful at detecting DNA from the No-ITP controls, but successfully detected DNA in the paper layers after the ITP process. This shows the utility of this method in conducting molecular diagnostics from samples containing low concentrations of the pathogen.

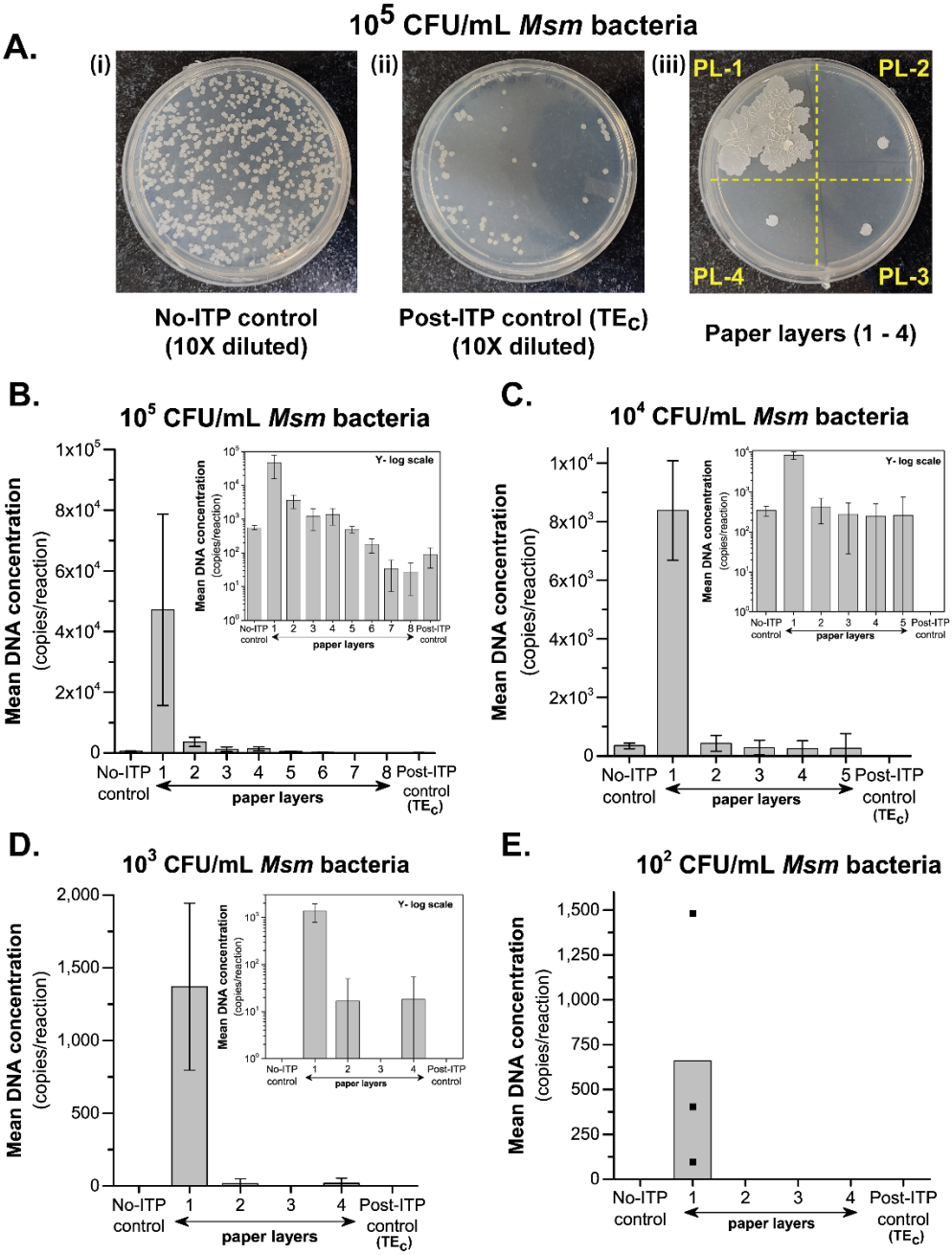

Figure 3. Combined bacterial lysis and gDNA concentration in *p*-ITPrep A. Images of agar plates showing *Msm* colony growth when the ITP device was spiked with 10^5 CFU/mL *Msm* bacteria (i) No-ITP control, **L** (diluted 10X for ease of visualization) (ii) Post-ITP control, TE_c (diluted 10X for ease of visualization), and (iii) paper layers (1-4), post-ITP. B-E: Plots of mean DNA concentration (N=3 ITP devices) in the paper layers and in No-ITP and Post-ITP controls for samples with different initial *Msm* bacteria concentrations 10^5 (B), 10^4 (C), 10^3 (D), and 10^2 (E) CFU/mL. Insets show data on a logarithmic y axis. Error bars represent standard deviations (N=3). DNA concentration of only those paper layers with detectable DNA is plotted.

Extraction of DNA from complex biological samples in *p*-ITPrep

After confirming that *p*-ITPrep lyses bacteria and releases gDNA for ITP purification, the technique was tested with complex human matrices – pooled human saliva, human blood serum, and artificial urine. Varying numbers of *Msm* bacteria were spiked in the matrix and samples were diluted 5X in the TE buffer before loading into the sample chamber of *p*-ITPrep. ITP was conducted for 20 min at 18V followed by elution of DNA from each paper layer and qPCR. Two types of No-ITP controls were included: undiluted control (UD), which was the *Msm*-spiked matrix, and diluted control (D), which was the UD control diluted 5X in TE buffer. *p*-ITPrep successfully extracted bacterial gDNA from the various complex samples.

Pooled human saliva: Results of DNA extraction from three different starting bacterial concentrations spiked in human saliva are presented. For the highest bacterial concentration of $5x10^5$ CFU/mL, while *Msm* DNA was directly detectable from both the No-ITP controls, D and UD, the DNA concentrations detected in PL-1 and PL-2 were significantly higher than those in the controls (Fig. 4A). The increased concentration is likely due to a combination of ITP-induced gDNA focusing and its separation from PCR-inhibitory molecules in the saliva sample. Nonetheless, from a diagnostics perspective, if *Msm* DNA can directly be detected from the sample without ITP, then the value of nucleic acid extraction is limited. Therefore, samples containing lower bacterial concentrations were tested. For a saliva sample containing $5x10^3$ CFU/mL *Msm* bacteria, *Msm* DNA was detectable in the undiluted sample (UD) but not in the diluted sample (D) (Fig. 4B). A significantly increased DNA concentration was detected in PL-1 (Fig. 4B). For a further lower concentration of $5x10^2$ CFU/mL *Msm* bacteria, *Msm* DNA was undetectable in both

controls, but was detectable in PL 1-4 (Fig. 4C), which demonstrates one-step DNA extraction from complex samples that are not directly fit for PCR testing.

Artificial human urine: Results of DNA extraction from two different starting bacterial concentrations spiked in artificial human urine are presented. For both $5x10^3$ CFU/mL and $5x10^2$ CFU/mL *Msm* bacteria spiked in urine, *Msm* DNA was undetectable from either No-ITP control (D and UD), but was detectable in PL 1-4 for $5x10^3$ CFU/mL (Fig. 4D) and in PL-1 and 3 for $5x10^2$ CFU/mL, demonstrating effective one-step DNA extraction for PCR testing.

Human serum: Compared to saliva and urine, serum is a more complex matrix, and the one-step DNA extraction *p*-ITPrep protocol followed thus far did not produce any detectable DNA in any paper layer, even with a high sample bacterial load of $5x\ 10^5$ CFU/mL (ESI Fig. S8). This is most likely due to the presence of high amounts of IgG in human serum, known to be inhibitory to PCR[42], not being separated efficiently from the gDNA. To address this, the bacteria-spiked serum sample was pre-incubated with proteinase K and Triton X-100 to degrade the PCR-inhibitory proteins Proteinase K and Triton X-100 were added during the 5X TE buffer sample dilution step. In a tube, the diluted sample was first incubated at 56 ^oC for 10 min to activate proteinase K, and then at 95^oC for 10 min to deactivate proteinase K, before loading into the ITP device. Heat deactivation of proteinase K was necessary as it is a PCR-inhibitory molecule (ESI Fig. S8). Inclusion of this pre-incubation step enabled the extraction of gDNA from human serum. For a sample containing $5x10^3$ *Msm* CFU/mL serum, *Msm* DNA was undetectable from both No-ITP controls (D and UD), but was detected in PL 1-4 (Fig. 4F). Note that the maximum DNA concentration was observed in PL-2 instead of PL-1 (Fig. 4F). This was most likely because

significant amounts of cell debris accumulated in PL-1 which was in direct contact with the sample reservoir, which could have a PCR-inhibitory effect. Serum sample containing $5x10^2$ CFU/mL *Msm* bacteria was also tested, but *Msm* DNA could not be detected from it.

The effect of *p*-ITPrep from complex biological samples on bacterial lysis was assessed by bacterial culture. For each *p*-ITPrep run, the various control samples (No-ITP controls D**,** UD and post-ITP control) and PL 1-4 were plated on agar plates (ESI Fig. S9). The culture results resembled those from the experiment with bacteria spiked in TE buffer (Fig. 3A). While No-ITP controls showed bacterial growth in most cases, the post-ITP controls did not show any bacterial growth. There was significant bacterial growth in PL-1 which acts like a bacterial filter, except in the case of serum samples, where proteinase K and heat treatment lysed all bacteria (ESI Fig. S9).

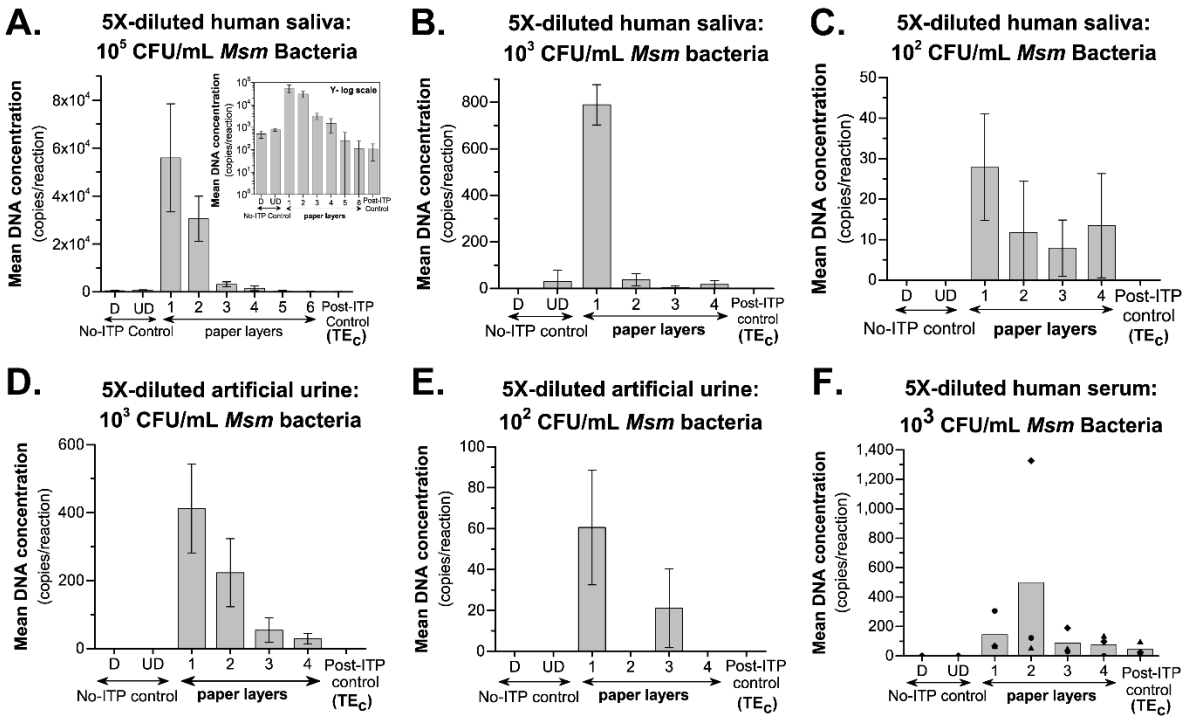

Figure 4. Integrated bacterial lysis and DNA isolation in *p*-ITPrep from complex biological samples. A-F: Plots of mean DNA concentration eluted from each paper layer (N=3 *p*-ITPrep devices) for human saliva containing 10^5 CFU/mL (A), 10^3 CFU/mL (B), and 10^2 CFU/mL (C)

Msm bacteria; for artificial human urine containing 10^3 CFU/mL (D) and 10^2 CFU/mL (E) *Msm* bacteria; and for human serum containing 10^3 CFU/mL *Msm* bacteria. All samples were diluted 5X before loading into *p*-ITPrep. The mentioned bacterial load in CFU/mL is for the 5X-diluted biological samples. All error bars represent standard deviations (N=3 *p*-ITPrep devices).

Comparison between *p*-ITPrep and a Qiagen DNA extraction kit

The nucleic acid recovery from bacteria-spiked human saliva, human blood serum, and artificial urine was compared between the newly developed *p*-ITPrep and a commercial Qiagen QIAamp DNA kit. 200 µL of *Msm*-spiked ($5x10^4$ CFU/mL) human saliva, human blood serum, and artificial urine were processed using *p*-ITPrep and Qiagen QIAamp DNA kit. For *p*-ITPrep, the crude sample underwent a 5X dilution before being subjected to the developed protocol. For the Qiagen kit, the manufacturer's recommended procedure was followed. An overview of the user steps and their durations involved in both extraction methods is provided in ESI Table S1, which highlights the large number of user steps (16 steps; 45-60 min) involved in Qiagen DNA extraction (details in ESI Table S2) in contrast to only a few user steps (3 steps; 20-30 min) in *p*-ITPrep sample preparation. Nucleic acids extracted from both methods were quantified using qPCR. A two-tailed t-test was performed to compare the DNA yield between the two methods. Even with considerably fewer user steps, nucleic acid recovery from PL-1 of *p*-ITPrep was comparable to the Qiagen DNA kit for both saliva (ns; N=3, p=0.14; Fig. 5A) and urine (ns; N=3, p=0.13; Fig. 5B) samples. However, for blood serum samples, the Qiagen kit outperformed *p*-ITPrep; DNA concentration obtained from Qiagen extraction was higher than that in PL-2 of *p*-ITPrep (*; N=3, *p*=0.04; Fig. 5C), which had the maximum detectable DNA concentration. Yet, gDNA concentration detected in PL-2 was significantly higher than the corresponding No-ITP control (UD; *; N=3; p=0.05; Fig. 5C), showing that *p*-ITPrep enables DNA extraction from human serum using minimal user steps.

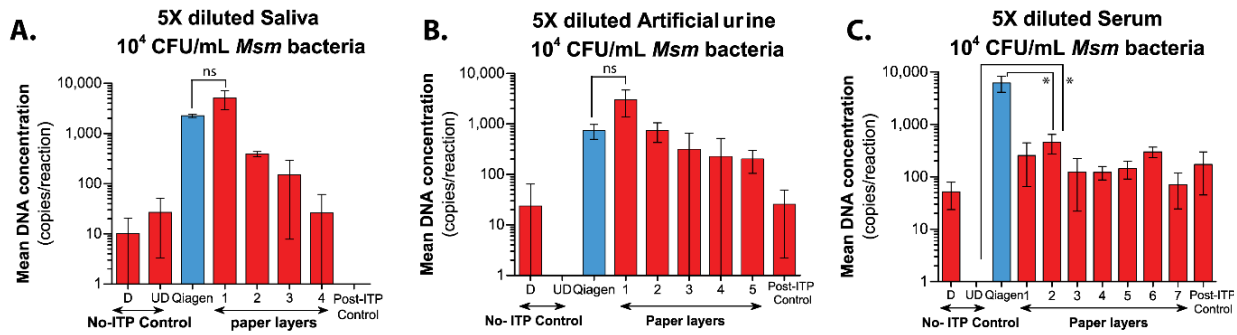

Fig. 5 Comparison of DNA recovery using QIAGEN DNA kit and *p*-ITPrep. A-C: Mean DNA concentrations in the various paper layers of *p*-ITPrep (red bars) and from a Qiagen DNA kit (blue bar) for human saliva (A), artificial human urine (B) and human serum (C), spiked with 10^4 CFU/mL *Msm* bacteria. For both saliva and urine samples, the concentration of extracted DNA from the Qiagen kit was not statistically significantly different from PL-1 of *p*-ITPrep (ns = not significant). For serum, the maximum DNA concentration was obtained in PL-2 of *p*-ITPrep, and the Qiagen kit had a higher DNA recovery compared to PL-2 (*; N=3; $P<0.05$). However, concentration of DNA in PL-2 was higher than for the undiluted No-ITP control (UD; *; N=3; $P=0.05$). All error bars represent standard deviations (N=3 ITP devices).

Cost and power requirements of *p*-ITPrep

The total cost of parts required to fabricate a single *p*-ITPrep device that can accommodate 900 µL sample volume is USD 1.23 (ESI Table S3). In addition, the reusable platinum electrodes cost USD 21. For all ITP experiments reported here, the same pair of platinum electrodes was used, which shows its longevity and reusability. A DC voltage generator was used to generate the required 18V voltage bias for all experiments. The energy required for each *p*-ITPrep run was calculated as the area under the curve of the current vs time plot (ESI Fig. S10) and varied between 0.29 to 2.64 mAh for different biological matrices. Commercially available 9V lithium ion batteries are rated for 1200 mAh. Therefore, a pair of two 9V batteries connected in series will theoretically provide sufficient energy to complete ~1000 *p*-ITPrep runs. If powered by batteries, there would be no permanent instrumentation associated with *p*-ITPrep. In contrast, conventional

DNA extraction kits like Qiagen kits require ancillary equipment such as a centrifuge and a vortexer.

Overall, these findings suggest that *p*-ITPrep could be used as a simple alternative to commercial solid phase nucleic acid extraction kits, especially when there is limited access to equipment and trained staff. *p*-ITPrep enables simultaneous bacterial lysis and purification of gDNA from complex biological samples and has the following desirable features: i) processing of large sample volumes (~1 mL), beneficial for dilute samples/lower pathogen loads, ii) concentration of genomic DNA without introducing any bias towards fragment length or sequence, and iii) bacterial lysis without introducing any harsh or PCR-inhibitory chemicals (as opposed to the Qiagen DNA kit). Although processing of blood serum involved proteinase K pretreatment to digest plasma proteins, once this step was completed, no additional intermediate user steps were involved. Conventional solid phase extraction methods like Qiagen kits, in stark contrast, are tedious, time-intensive, and involve many user steps. Note that in this study, DNA from all 11 paper layers of *p*-ITPrep was quantified, but during actual usage, only the first few layers containing the maximum DNA concentration need to be analyzed.

One of the primary novelties of this work is the integration of lysis with ITP. In order to challenge the system, a gram-positive bacterium with a thick cell wall, *Msm*, was chosen. While we have not tested the method with gram-negative bacteria, the electrochemical lysis of gram-negative bacteria has been shown to be at least as efficient as that of gram-positive bacteria.[43] Note that only partial lysis was accomplished in this embodiment of *p*-ITPrep, likely because of the low electric field strength (3000 V/m) utilized. Existing literature on cell lysis based on irreversible electroporation

reports pulsed field strengths as high as 100-1000 kV/m.[44,45]. The extent of lysis in *p*-ITPrep could be improved by using a higher voltage bias or reducing the distance between the electrodes, but it could come at the cost of a higher power requirement; this optimization may be worked on in future studies. Versatility of *p*-ITPrep is demonstrated here by successful bacterial lysis from multiple biological matrices. Independent of lysis, ITP is known to be a versatile nucleic acid purification technique compatible with different types of nucleic acids like DNA,[22,46] RNA,[26,34] and miRNA.[24,47] Because *p*-ITPrep combines versatile cell lysis with ITP, it is a versatile solution for nucleic acid extraction from complex samples.

Conclusion

Nucleic acid extraction from biological samples is one of the most cumbersome procedures in any molecular diagnostics workflow and is often neglected while developing POC-NAATs. In a recent review article published by our group[3], we point out the need for the development of standalone nucleic acid sample preparation devices that can extract nucleic acids from a wide variety of samples, such that the extracted DNA is compatible with a large number of downstream nucleic acid analysis techniques. In this article, we address this need by the development of *p*-ITPrep. *p*-ITPrep is enabled by two key findings: i) that the voltage bias applied across electrodes for paper-based ITP is sufficient to cause electrochemical lysis of bacteria, and ii) that large genomic DNA molecules released during lysis migrate through paper and can be concentrated within paper using ITP. *p*-ITPrep accomplishes nucleic acid extraction from complex biological samples in < 30 minutes, does not use any ancillary instruments, and utilizes only 3 user steps. *p*-ITPrep can accommodate large sample volumes (900 µL in the current embodiment, but can be increased further), which enables extraction of DNA from samples with low bacterial loading. Compared to

conventional solid phase extraction kits, *p*-ITPrep significantly reduces the number of user steps, time, and the cost associated with nucleic acid extraction. *p*-ITPrep is a versatile nucleic acid extraction technology which could be used not only for molecular diagnostics at the point-of-care, but also for rapid and hassle-free nucleic acid extraction in any laboratory conducting nucleic acid analyses.

Experimental Section

Chemicals and membrane materials

For ITP experiments, the LE and TE buffers were 1M Tris-HCl (pH 7.53) and 2.0 mM tris-taurine (pH 8.7), respectively. Both the buffers were prepared in DI water, followed by autoclaving for sterilization. Tris Buffer (AR 71033) and taurine (15711) were purchased from Sisco Research Laboratories (SRL), while HCl (90239) was obtained from Thomas Baker. Proteinase K from the QIAamp DNA mini kit (51304) was used for protein digestion in serum samples. Qiagen QIAamp DNA mini kit (51304) was used for the comparison study between *p*-ITPrep and Qiagen.

Whatman filter paper grade 1 (1001-150) was purchased from Cytiva, Bengaluru, India. MF-Millipore mixed cellulose ester (MCE) membrane filter (0.45 µm pore size, HAWP04700) was procured from Merck. The double-sided PSA film was obtained from 3M (9731). Acrylic sheets and platinum wire were purchased from a local vendor. All membranes and acrylic sheets were cut using a 50 W CO_2 laser cutter (VLS 3.60; Universal Laser Systems, Scottsdale, AZ).

Genomic DNA and bacterial culture

Mycobacterium tuberculosis H37Rv (*Mtb*) purified genomic DNA was generously provided as a gift from Prof. Amit Singh (Indian Institute of Science, Bangalore). *Mycobacterium smegmatis* mc^2 155 (*Msm*) culture was obtained as a kind gift from Prof. Rachit Agarwal (Indian Institute of Science, Bangalore). *Msm* was routinely grown in Middlebrook 7H9 broth (HiMedia, M198) with 0.05% Tween 80 and 0.5% glycerol for 24 hours at 37 °C, 180 RPM. *Msm* colonies were plated on Middlebrook 7H9 agar (with 0.5% glycerol) plates. Glycerol (072438) and agar powder (014042) were obtained from SRL. For plating bacteria from paper layers, the paper discs were rehydrated with 5 µL 1X Tris-EDTA buffer before streaking the discs directly on agar plates. The paper discs were left on the agar plates during culture (white circular discs; Fig. 3A(iii)). All the agar plates were imaged approximately 72 hours after plating using a mobile phone camera: Redmi Note 10 pro max.

Biological matrices

Pooled human saliva was purchased from Innovative Research (IRHUSL250ML, Novi, MI, USA). Human serum (human male AB plasma, USA origin, sterile-filtered, H4522) was obtained from Sigma-Aldrich. Sigmatrix Urine Diluent (Sigma Aldrich, SAE0074) was used as synthetic urine.

***p*-ITPrep device operation**

The *p*-ITPrep device consists of two fluid reservoirs – LE reservoir and TE reservoir. Initially, the LE reservoir was filled with 900 µL of LE buffer and the device was gently tapped to remove any air bubbles, ensuring that all Whatman filter paper discs were saturated with the electrolyte solution. Subsequently, the TE reservoir was loaded with 900 µL of the sample, which comprised of a mixture of TE buffer and gDNA or bacterial cells spiked in buffer or a biological matrix. A

voltage bias of 18 V was then applied for 15-20 minutes between the Pt electrodes (dia. 0.5 mm and length 3 cm) inserted into each reservoir. A Rohde & Schwarz (HMC 8042) DC power supply was used to power the device. After the ITP process, the Pt electrodes and buffers were removed from the reservoirs with a pipette and the device was disassembled to analyze the contents of each paper layer. The paper disk were either used directly as a PCR/real-time PCR template, or DNA was eluted from the paper disk by centrifugation for DNA amplification. For reusing the Pt electrodes, the electrodes were cleaned with 70% ethanol and 1% sodium hypochlorite (bleach) before and after performing ITP experiments. All *p*-ITPrep runs were conducted using an 18V voltage bias: 15 min for samples containing purified gDNA and 20 min for samples containing bacteria.

Elution of DNA from paper disks

Post ITP, the concentrated DNA entrapped in the paper discs was eluted by centrifugation. A 5 µl 1X Tris-EDTA buffer solution was pipetted into each paper disc. The fluid from each disc was centrifuged at 6000 RPM for 90 seconds and introduced as template into PCR/qPCR reactions.

qPCR assays

All qPCR reactions were performed using an Applied Biosystems QuantStudio 3 instrument with Takara TB Green Premix Ex Taq II (Tli RNase H Plus, RR820A). In all cases, it was ensured that the qPCR efficiency was between 90 and 100%. *Mycobacterium tuberculosis:* Primers for the *rpob* gene of *Mtb* were adapted from Agarwal et al.[48] - reverse: 5'-GTTTCGATCGGGCACATCC-3' and forward: 5'-ATCACACCGCAGACGTTGATC-3' (230 bp product). The qPCR reaction mixture contained Takara TB Green Premix (10 µL, 1x), 0.5 µM each of forward and reverse

primers, 0.8 mg/mL bovine serum albumin (BSA, Sigma-Aldrich) + 0.002% tween 20, 2 µL target nucleic acids, and sterile water to reach a final volume of 20 µL. The qPCR cycle was as follows: initial denaturation at 95 °C for 5 minutes followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 20 s. Fluorescence readings were acquired during the extension step. *Mycobacterium smegmatis: Msm* bacteria were quantified using a qPCR assay adapted from Hümpel et al.[49] and Dsouza et al.[50] Primers for the sigA gene of *Msm* were – reverse: 5'- GACTCTTCCTCGTCCCACAC-3' and forward: 5′-GAAGACACCGACCTGGAACT-3′ (185 bp product). The assay composition was identical to that of the *Mtb* assay, except 0.3 µM each of forward and reverse primers were used to avoid forming primer dimers. The qPCR cycling conditions were as follows: 5 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s. Fluorescent readings were acquired during the extension step. For both *Mtb* and *Msm* qPCR reactions, melt curve analysis was conducted post PCR by heating the samples from 50°C and 98°C at a rate of 0.05 °C/s; fluorescent readings were acquired every 1 s.

Author Contributions

Shruti Soni: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft; **Bhushan J. Toley**: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Funding Acquisition

Conflicts of Interest

The authors do not have any conflict of interest to declare

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