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Sample pre-concentration on a digital microfluidic platform for rapid AMR detection in urine

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ABSTRACT

There is a growing need for rapid diagnostic methods to support stewardship of antibiotics. We describe an analytical platform for sample concentration to detect antimicrobial resistance (AMR) genes directly from human urine for the diagnosis of Urinary Tract Infections (UTIs) that are resistant to antibiotics. A sample-processing unit concentrates plasmid DNA directly from urine using magnetic beads, followed by isothermal amplification of target genes. The sample pre-concentration unit interfaces with a digital microfluidic platform (DMF) and scales the sample volume by 500-fold, pre-concentrating DNA from 1mL into a 2µL droplet for downstream processing. Tests with a clinical strain of *Klebsiella pneumoniae* (NCTC 13443), spiked into human urine demonstrated a limit of detection of 10⁴ cfu/mL and a “sample to answer” in approximately 30 minutes.

INTRODUCTION

Antimicrobial resistance (AMR) is a growing worldwide public health problem, and is expected to become the primary cause of death and claim 10 million lives per year by 2050 [1]. The latest guidance recommends that by 2020, no antibiotics should be prescribed to patients unless resistance to that antibiotic has been excluded by a diagnostic test [1]. Currently, routine testing is not performed and the test that are in use to detect antibiotic resistant bacteria, rely on cell culture and/or molecular detection that require dedicated laboratories, expert users and days to complete. One of the most common bacterial infections is Urinary tract infections (UTIs) that accounts for substantial health costs and morbidity [2]. UTIs are the second most common cause of bacteremia in hospitalized patients and are responsible for as many as 40% of nosocomial infections [3]. Organisms including *Escherichia coli* and *Klebsiella pneumoniae* that produce extended spectrum β -lactamase (ESBL) and carbapenemase enzymes have emerged as a major healthcare issue over the last 10-20 years [4]. Amongst the wide range of ESBL enzymes, the CTX-M enzyme family is the most prevalent, conferring resistance to key β -lactam antibiotics, including 3rd generation cephalosporins [5]–[7] in at least 26 bacterial species, residing in both nosocomial and community environments [7]. Therefore, a gene encoding for the widespread CTX-M-15 isoform, *bla*_{CTX-M-15}, that encodes a protein with enhanced catalytic activity [7] is an ideal target candidate for rapid point-of-care (PoC) detection of antibiotic resistant pathogens.

Numerous nucleic acid-based assays have been developed and implemented for pathogen detection on microdevices, particularly since the advent of isothermal amplification techniques [8]–[12]. One attractive method is Recombinase Polymerase Amplification (RPA) which is growing in popularity owing to its low incubation temperature, ease of primer

design, and sensitivity and high tolerance to impurities eliminating the need for elaborated DNA purification protocols [13], [14]. Previously, we have demonstrated a programmable Digital Microfluidic (DMF) platform to perform RPA for rapid detection of AMR encoding genes with an LoD of approximately 10 copies within 10 minutes [12], [15]. The device enables automated and simultaneous manipulation of many nanolitre (nL) droplets of liquid on an array of thousands of individually addressable microelectrodes [16]. Our previous publications used DNA samples extracted and purified using commercial kits with sample preparation separate to the analysis chip. In many diagnostic platforms, sample preparation often remains a rate-limiting step, and in the case of UTIs clinical samples of several mL must also be scaled down by at least 2-3 orders of magnitude to the μL (or nL) volumes required for PoC assays. This requires technologies for pre-concentration of target cells or the genetic material from the large volumes of clinical sample.

A number of different approaches have been developed to pre-concentrate samples, for example capture of bacterial cells from solution using aptamer or antibody coated magnetic microparticles [17]–[20] or microfluidic chip surfaces [21] or ion exchange beads [22], [23], [24], but all of these approaches are either restricted to a limited range or have variable capture efficiency depending on the species and on the composition of the sample matrix. Cell lysis to release nucleic acids into solution is the most direct way of performing molecular analysis of a whole bacterial community within a clinical sample. Nucleic acid extraction on microdevices is usually based on a miniaturized version of bench-top techniques that include cell lysis, DNA capture onto a solid surface (beads or columns), followed by washing and finally elution for subsequent analysis. Cell lysis often requires pre-concentration by centrifugation off-chip [25], [26]. Subsequent steps are then incorporated into microfluidic devices. Microfluidic sample preparation has been demonstrated extensively

for blood samples. For example, extraction of RNA [27] or DNA [28] from human whole blood has been implemented on a DMF platform, with subsequent downstream analysis off device. However, bacterial cells are generally hard to lyse and samples must be subjected to more aggressive procedures. These may include a combination of lysis by heat, enzymes or mechanical forces. Heat lysis is very simple, and has been successfully used for complete extraction and detection of DNA from *Staphylococcus* and *Streptococcus* from saliva samples [29]. However, when target cells are very dilute, heat lysis requires a pre-concentration step to achieve clinically relevant detection limits. Mechanical forces generated by microstructures have been used to improve the enzymatic lysis of bacteria in human urine, before DNA recovery on a solid phase extraction (SPE) column for purification with off-chip analyses [30]. van Heirstraeten *et al* (2014) scaled down a complete standard DNA extraction protocol utilizing an SPE column, extracting Gram-positive and negative bacteria, and virus DNA from swab samples [31]. A centrifugal Lab-on-a-Disk system analysis system demonstrated a complete “sample in – answer out” assay to detect bacterial pathogens [32]. This included DNA extraction after cell lysis, DNA capture on magnetic beads, washing and release for multiplex PCR. The authors demonstrated a LoD between 2 and 5 cfu in 200 μ L serum sample with a “sample-to-result” time of under 4 hours. Choi *et al* (2016) demonstrated an RPA assay using a centrifugal platform for identification of three different organisms in contaminated milk with a LoD of 4 cells per 3.2 μ L of milk (1250 cells per ml) within 30 min [33]. Mosley *et al* (2016) described a simple method for lysing and extracting DNA from the Gram-negative bacteria *Helicobacter pylori* in stool samples by addition of guanidine hydrochloride [34]. Magnetic microparticles were used to capture the DNA from crude lysate where the DNA bead sample was cleaned by pulling the beads through an immiscible mineral oil, through an adaptation of the IFAST (immiscible filtration assisted by surface tension) technique developed by Beebe group [35]. Recently, the Wheeler group

demonstrated a pre-concentration technique (liquid intake by paper) to allow interface of larger volumes to digital microfluidics. They concentrated protein biomarkers using magnetic beads. [36], [37].

In this publication, we describe a simple sample pre-concentration unit compatible with a DMF platform, and analyse processed urine sample for identification of AMR. Bacterial plasmids are pivotal in the transfer and acquisition of resistance gene [38], [39], therefore we ensured that the extraction and concentration method would be suitable for both plasmid and genomic DNA. Viable bacteria suspended in a urine sample (typically 1 mL) are heat lysed (on a thermomixer) to release genetic material in the presence of a chaotropic salt and plasmid binding beads. The sample with DNA immobilised on the magnetic beads is processed with the pre-concentration unit that interfaces directly with the DMF platform. The DNA loaded beads are pulled from the main sample through an immiscible oil/aqueous interface directly onto the DMF platform, reducing the sample volume from 1 mL to 2 μ L in a single step. No wash steps are performed and the entire assay takes less than 30 minutes with a LoD of 10^4 bacteria cfu/mL, which is compatible with the requirements for direct detection of bacteria causing UTIs and in line with clinical recommendations in the UK[2]. The utility of the assay was demonstrated by spiking a clinical isolate of *Klebsiella pneumoniae* into human urine followed by detection of the *bla*_{CTX-M-15} gene. This provides evidence that the technology could be applied to enable rapid detection of this problematic multidrug resistant (MDR) bacteria, as a model for detecting commonly occurring UTI pathogens direct from urine samples.

METHODS

Bacterial culture and control DNA (genomic and plasmid) extraction

Klebsiella pneumoniae NCTC 13443, isolated from a human clinical sample was obtained from the National Collection of Type Cultures (NCTC) at Public Health England (PHE). The genome of this strain has been sequenced (EBI accession number SAMEA2742597) and is known to contain genes for the ESBL CTX-M-15 and the carbapenemase NDM-1. Routine maintenance of bacteria on Tryptone Soy Agar (TSA) plates, cell inoculations and cell counts of cultures in Tryptone Soy Broth (TSB) medium were conducted as previously described [24]. Overnight cultures in TSB medium were diluted 20-fold the following morning and incubated at 37 °C for a further 1 h to ensure that cells were in early exponential growth phase. Control DNA, containing plasmids, was obtained from an overnight culture of *Klebsiella pneumoniae* NCTC 13443. For benchtop assays, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, UK) following the manufacturer's protocol. The eluted DNA was quantified on a Qubit® fluorometer using DNA assay reagents.

Test samples

Experiments were conducted using human urine spiked with *Klebsiella pneumoniae* NCTC 13443 to simulate clinical samples with an infection of antibiotic resistant bacteria. Urine samples from healthy volunteers were collected with ethical approval from the Southampton Research Biorespository Access Committee (Ref: 12/NW/0794). All experiments were performed in accordance with the Guidelines from University Hospital Southampton R & D policies, and approved by the ethics committee at University Hospital Southampton. Informed consents were obtained from human participants of this study.

Approximately 50 mL of mid-stream urine was collected into sterile polypropylene tubes. View Article Online
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Urine from four volunteers was used to evaluate the robustness of the assay to variability in sample composition, for example urine hydration.

Extraction of bacterial DNA for benchtop assay

A benchtop protocol for extraction of DNA from bacteria spiked into urine was designed that could be translated to the DMF platform. The protocol is shown in Fig 1. 525 μ L of unfiltered urine was spiked with 100 μ L of *K. pneumoniae* bacteria of varying concentrations. 375 μ L of GuHCl at 8M was then added to the sample to obtain a final concentration of 3M and final volume of 1mL. To specifically isolate plasmids DNA (rather than total genomic DNA), we used MagneSil™ Red paramagnetic particles (Promega, UK). 2.5 μ L stock solution of these beads were added to the solution and the sample incubated at 90°C for 10 minutes with continuous mixing at 800 rpm on a thermomixer (Thermomixer comfort, Eppendorf, Germany). This bacterial lysis and plasmid capture protocol was identical for both benchtop and DMF platform. The sample was allowed to cool to room temperature, following which the beads were concentrated on the side of the tube with a magnet as shown in Fig 1. Approximately 950 μ L of the supernatant was removed and discarded and after re-suspending the beads, the remaining 50 μ L volume was covered with a layer of dodecane (100 μ L). The beads were then extracted by pulling them through the oil-urine interface with a magnet (step 6). After discarding the remaining solution, the bead pellet was re-suspended in the same tube with 5 μ L elution buffer for five minutes (Promega, UK) to elute the plasmid from the beads (step 7). Finally, the beads were concentrated on the side of the tube with a magnet and the resulting plasmid (in elution buffer) used in bench-top RPA assays (step 8).

Sample pre-concentration device and DMF assembly

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The assembled DMF platform is shown in Fig 2A & B. It consists of a Thin Film Transistor (TFT) backplane that drives the electrodes, together with a transparent glass Indium Tin Oxide (ITO) top plate. The sample pre-concentration unit is attached to this top plate (Fig 2C & D). The TFT backplane comprises an active matrix array of 96 x 175 electrodes, each approximately 200 μm x 200 μm in size. The TFT backplane also has 9 large volume (2.2 μL) fluid input pads at the periphery of the active matrix array. Each of these fluid input pads is made of 7 discrete electrodes [see 12] and has a footprint of 3 mm x 9 mm. Custom electronics were used to provide electrode actuation signals to all the different electrodes. The device is described further in [12] and an image of the DMF assembly is shown in Fig 2B. The DMF platform was assembled by physically clamping the ITO top plate to the bottom with a 125 μm spacer.

To analyse urine samples directly on the DMF platform, a sample pre-concentration unit was designed to directly interface with the top plate of the device. A schematic diagram of this unit is shown in Fig 2C & D. It is designed to enable the same work-flow as the bench-top assay (Fig 1) delivering magnetic particles (with bound plasmids) to fluid input pads of the DMF device (see below) [12]. The pre-concentration unit comprises a 1.2mL volume chamber made from a 10 mm thick PMMA sheet (Techsoft, UK) by laser micromachining (Epilog Laser, UK). A thin spacer (275 μm) forms a microfluidic channel beneath the chamber (Fig 2D). The channel is 7.2 mm long and terminates with a capillary stop (diameter 1 mm) (see Fig 2D). The fluid channel crosses a 1mm opening in the ITO top plate through which the bead-plasmid mixture is extracted onto the fluid input pads. The channel was cut in 175 μm clear PMMA sheet (Goodfellow, UK) and stuck to both surfaces with 50 μm double-side adhesive (467MP, 3M, USA), total depth = 275 μm .

Sample pre-concentration protocol on DMF

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Before pre-concentration of the sample and its subsequent amplification on DMF, RPA reagent drops were pipetted on the TFT backplane. $\sim 2\mu\text{L}$ droplets were dispensed onto the fluid input reservoirs as follows: $2.2\mu\text{L}$ each of RPA mastermix, magnesium acetate, no template control (NTC, nuclease free water), positive control (DNA 100 pg/uL) and $2\mu\text{L}$ of elution buffer (Promega, UK). The elution buffer droplet was placed on the fluid input reservoir pad 4 (see Fig 2A). These pad electrodes were electrically actuated to pin the droplets to the substrate whilst the device was loaded with dodecane. The dodecane fills the gap between top plate and TFT backplane and stops at the hole in the top plate. Fig 3 and S1 shows the assay flow for plasmid capture and pre-concentration on the DMF platform. To begin an assay, 1mL of urine sample containing lysed bacteria and magnetic beads with plasmids was pipetted into the chamber of the sample concentration unit. The shallow channel at the base of the unit slowly fills with urine by capillary action until it stops at the capillary stop as shown in Fig 3 (1) & Fig S1 (1). The dodecane in the hole in the DMF top plate forms an immiscible interface with the urine. The beads in the urine were pulled to the bottom of the chamber with a magnet (Fig 3 (2) & Fig S1(2)). The bead pellet was then translated along the microfluidic channel by manually moving the magnet to bring the bead pellet over the hole in the top plate (Fig 3 (3) & Fig S1(3)) and onto an empty fluid input reservoir pad (Fig 3 (4) & Fig S1(4)). The plasmids were eluted off the beads by translating the bead pellet through the oil in the DMF device onto a neighbouring fluid reservoir pad that contained elution buffer (Fig S3(5) & S1(5)). Beads were dispersed by moving the magnet and incubated for 5 minutes in the elution buffer. The “waste” beads were pulled off the pad into the oil leaving bacterial-plasmids in the reservoir droplet (Fig 3(6)). Fig S2 shows photographs of this sequence. To initiate the DNA amplification assay, different daughter

droplets were dispensed from the respective reservoirs (including the sample) onto the DMF chip for amplification by RPA (Fig 3 (6-8)).

The efficiency of the bead extraction process was evaluated using optical absorbance measurements. A 1 mL urine sample with beads was loaded into the pre-concentration unit. The beads were pulled onto the DMF platform and then moved to the edge of the top plate (with the magnet) through the oil for recovery into an Eppendorf tube. The beads were re-suspended into 1mL of the same urine sample and absorbance measurements used to estimate bead recovery. In a separate experiment, the residual sample volume carried over by the beads through the oil phase was estimated using fluorescein dye. The beads were suspended in 1 μ M fluorescein in PBS (1 mL) and this solution was loaded into the sample chamber of the pre-concentration unit. The beads were pulled onto the TFT backplane and through the oil for recovery. The beads were then re-suspended to their original volume in PBS and fluorescence measurements were used to quantify the amount of fluorescein carried over to the TFT backplane in the interstitial space of the packed beads.

Detection of the *bla*_{CTX-M-15} gene.

Bench-top assay

The *bla*_{CTX-M-15} gene was amplified using the TwistAmp® Exo kit as described previously [12]. Lyophilised RPA proteins were reconstituted with rehydration buffer, forward and reverse primers, fluorescent probe and the DNA (or only plasmids) sample. In each 50 μ L reaction, the final concentrations of primers and Cy5 labelled probe were 0.42 μ M and 0.12 μ M, respectively. The 5 μ L aliquot of bacterial plasmid extracted from urine (Fig 1) was added to this mix. Each RPA reaction mix was transferred to the well of a non-binding, black polystyrene 96-well plate (Corning, UK). Amplification was initiated by adding magnesium acetate to a final concentration of 14 mM and mixing the reaction vigorously. The plate was

transferred to a GloMax microplate reader (Promega, UK) set to 39 °C and the fluorescence measured at 1 min intervals for 40 min. The RPA reaction was quantified by measuring the Time to Positivity (TTP) for each sample; the point at which the fluorescence exceeded a threshold value equal to three times the standard deviation of the negative controls (urine containing beads but no bacteria).

DMF assay

Assays performed on the DMF platform were essentially the same as above, except for the addition of Tween®20 to all reagents (to a final concentration of 0.1% v/v) to reduce the surface tension of the droplets. The volume ratios for the droplets on device were set to 4:1:1 for the RPA reaction mix, sample and magnesium acetate, respectively. The RPA reaction mix (enzymes, rehydration solution, primers and probe) was prepared so that the final assay component concentrations in the reaction droplet were identical to those in the 50 µL benchtop assay.

The protocol for performing the assay on the DMF chip is shown in Fig 3 (steps 6 to 8). Daughter droplets were aliquoted from reservoirs; DNA, NTC and positive control samples mixed with RPA mastermix by moving droplets back and forth along the device as described previously [15]. Magnesium acetate was then mixed and the DNA amplification initiated by heating the device to 39 °C. In order to image all droplets at the same time, a custom wide-field imaging system was developed [15], comprising a high sensitivity SLR camera (Canon 5D, Mark III) with a macro lens (55 mm focal length) and an LED illuminator (Nathaniel Group, USA). Two different filters were used, an excitation (590-650 nm, Semrock, UK) and emission filter on the camera (670-740 nm, Semrock, UK).

For a typical assay, six (45nL) droplets were used: three sample, two NTC and a positive control. These droplets were mixed with RPA mastermix (180 nL) for 30 seconds and then

with Magnesium Acetate (45 nL) for 20 seconds. The entire sequence of droplet manipulation was programmed into the DMF software. During the assay, sample droplets were mixed continuously for 20 sec and then the fluorescence signal was acquired for 2.5 seconds, repeated at 30 second intervals. Image processing and analysis was performed using a custom MATLAB script [15].

RESULTS AND DISCUSSION

Protocol optimisation: Extraction and pre-concentration on benchtop

The procedure for pre-concentration of target DNA, particularly plasmids, onto magnetic beads was adapted from [35] and based on filtration through immiscible phase. The method provides a single step purification and pre-concentration. Although this DNA isolation protocol is simple and does not involve any wash steps, beads do carry over a finite volume of both urine and guanidine in the process. Urine is known to contain several PCR inhibitors [40], so the robustness of the protocol was evaluated by measuring any possible inhibition of the RPA. Unfiltered urine was added to the RPA mix (1 μ L in 50 μ L reaction mixture) and the time to positivity (TTP) measured for the equivalent of 90,000 copies of purified DNA (genomic and plasmids). The change in TTP was compared against a reference value, obtained with the same amount of DNA suspended in 1 μ L of nuclease free water. A slight increase in TTP was observed as shown in Fig 4A (urine from four different volunteers). The conductivity of the four samples was 0.6, 0.8, 1.3 and 1.0 mS/cm. As reported previously [40], samples containing small amounts of urine can be amplified successfully. Our results show that the reaction becomes slower (evident from the increase in TTP) and does not appear to be any correlation with the amount of salt in the urine.

It is also known that denaturing reagents such as guanidine can inhibit enzyme activity during DNA amplification [41]. To assess the effect of Guanidine on RPA, and also to determine the optimal concentration of Guanidine for plasmid DNA capture, purified control DNA (genomic and plasmid) was spiked into either PBS or urine with varying concentrations of GuHCl. Plasmid DNA was captured on magnetic beads (as in Fig 1A) and eluted into 5 μ L elution buffer. The change in TTP compared with a positive control (no guanidine and no PBS/urine) was measured. The data is shown in Fig 4B. For PBS, there is a clear correlation between increasing GuHCl and increasing TTP, indicating a reduction in the capture efficiency and/or inhibition of the RPA enzymes by the carry over guanidine. A similar trend is seen for the urine samples, except that the increase in the TTP is significantly less than for PBS. Urine samples without GuHCl (data not shown) also successfully amplified, implying that DNA was captured on beads in the absence of any chaotropic agent. However the TTP was significantly increased (by ~ 4 mins). From this data, the optimal concentration of GuHCl in the final protocol was fixed at 3M to enable fast diagnosis. For 3M GuHCl, the change in TTP of ~ 1 min is within the 3 sigma of the effect produced by addition of urine (Fig 4A), suggesting excellent capture efficiency and downstream amplification of target genes.

Full bench-top assay: plasmid DNA extraction and amplification

The effect of heat on the DNA extraction efficiency was also evaluated (for two different volunteer urine samples), with the addition of GuHCl at 3M or 5M. Samples were either left to incubate at room temperature or heated (90°C for 10 mins) and the DNA processed as described previously. Table 1 shows the average TTP (mins) for replicate experiments. For a given sample, the optimal conditions are 3M GuHCl with heat. Although DNA amplification was observed for samples that were not heated, they had a much longer TTP suggesting poor DNA capture and/or sample lysis.

Fig 5A shows data from a typical bench-top experiment performed using the protocol shown in Fig 1. Consistent amplification of DNA extracted from *Klebsiella pneumoniae* was observed with a LoD of 10^3 cfu/mL (TTP ~27 mins). Fig 5B shows the TTP vs \log_{10} concentration of bacteria in urine. This data is representative for a sample, but the TTP values vary from volunteer to volunteer and also depend on the time of day the urine was taken. The plot shows a linear trend from 10^6 cfu/mL to 10^4 cfu/mL and an exponential increase in the TTP from 10^4 cfu/mL to 10^3 cfu/mL (with TTP doubling). Assuming that 1 cfu corresponds to 1 *bla*_{CTX-M-15} gene on 1 plasmid and comparing the TTP in Fig 5B to calibration plots for purified DNA (Fig S3), the plasmid capture efficiency from lysed bacteria in urine is estimated to be >80% for bacterial load $>10^4$ cfu/mL and <5% for bacterial load $<10^4$ cfu/mL.

Plasmid DNA extraction on sample pre-concentration unit

The pre-concentration protocol was designed to streamline the plasmid purification and pre-concentration process into a single step, significantly reducing assay time. The unit interfaces the macro (mL) sample volume to micro-fluidic requirements (μ L volume) and could be used on any microfluidic device. The extraction of beads through the oil will carry over a finite volume of residual guanidine and urine. For the device, this residual volume was experimentally determined (using fluorescein dye) to be ~300 nL for a 2μ L reservoir droplet volume. Based on this measurement, the guanidine in the sample should be further diluted by approximately 7x, when the beads are re-suspended in the elution buffer reservoir.

The area of the hole in the top substrate is $\sim 13 \text{ mm}^2$, and is filled with oil which prevents the urine sample flowing into the DMF device. The efficiency with which magnetic beads were pulled through the sample pre-concentration unit was experimentally calculated to be $\sim 80\%$

(n=3). Some of the beads were lost during the procedure, for example adhesion to the top glass substrate or at the circumference of the hole (see image in Fig S2).

The plasmid capture efficiency for the pre-concentration unit was evaluated as follows. Purified whole DNA (plasmid and genomic) was extracted using DNeasy Blood and Tissue Kit and spiked into PBS containing 3M GuHCl (1 mL) with 2.5 μ L magnetic beads (MagneSil Red). The beads were processed on the DMF device (Fig 3, steps 1 to 4), pulled to the edge of the device and re-suspended in 5 μ L of elution buffer. Fig S4 shows the relationship between the amount of plasmids retrieved against the amount of total DNA added, demonstrating an extraction efficiency of $\sim 13\%$ (for purified sample, no cell debris). The spiked DNA contained both genomic and plasmid DNA. The MagneSil red beads preferentially bind plasmid DNA and thus should preferentially concentrate plasmid into the elution volume. Since the approximate amount of plasmid present in total genetic content of bacterium can be around 10-20% of the total genome content in some cases [42], a capture efficiency of 13% demonstrates efficient and plasmid extraction.

Full DMF assay: plasmid DNA extraction and amplification

Before transferring the protocol to the DMF platform, the RPA assay was first implemented on the DMF device with purified DNA. Fig 6A shows time-lapse images of DNA amplification in droplets. Note the presence of background fluorescence due to a polymer coating on the TFT backplane. In this image, the 6 (270nL) droplets consisted of 3 reaction droplets, each with 800 DNA copies plus 3 NTC droplets. The three positive droplets give high fluorescence signals as seen in the image. Fig 6B shows a plot of fluorescence as a function of time with a TTP for 800 copies in approximately 3.2mins. Fig 6C shows amplification curves for a titration down to 7 copies per droplet; the TTP vs $\log_{10}(\text{concentration})$ is plotted in Fig 6D. This shows a typical LoD of 10 copies, similar to

our previous reported work where fluorescence was measured with a confocal laser imaging system. In the present case, the TTP was also improved due to optimisation of the forward primer sequence (sequence information available on request).

Finally, the functionality of the DNA extraction and pre-concentration unit integrated with the DMF device was tested using bacteria (*Klebsiella pneumoniae* NCTC 13443) spiked into urine from healthy volunteers. The sample was processed as shown in Fig 1 (1-3b) & Fig 3. The images in Fig 7B are example time lapse images of amplification of target plasmid pre-concentrated and eluted on the DMF platform from urine containing 1.7×10^5 cfu/mL (Fig 7C). The TTP for this particular reaction is less than 5 minutes. Fig 7D shows similar data for higher concentration (6×10^4 cfu/mL), with a TTP of approximately 5 minutes. Note that these two experiments were done with different volunteer samples, which explains the difference in the TTP. Nonetheless, the data demonstrates that the assay is sufficiently sensitive to detect clinically relevant levels of bacteria in urine for an uncomplicated UTI, which is in the region of 10^5 cfu/mL [3]. The combined assay is also reasonably fast, with a total test time of approximately 30 minutes, comprising 10 minutes sample incubation, 5 minutes sample processing and droplet dispensing and 10 to 15 minutes for the RPA. The LoD for the assay on the bench is 10^3 cfu/mL; the same assay performed on the DMF platform has an LoD approximately 10 times worse at 10^4 cfu/mL although the positive controls (purified DNA) could be detected down to 10 copies. This may be attributed to the low volume used for the assay (45 nL droplets from a 2 μ L reservoir). With a drop in the plasmid extraction efficiency for concentrations lower than 10^4 cfu/mL to <5% (Fig 5B & Fig S3), the number of plasmids extracted for 10^3 cfu/mL will be approximately 30 copies (assuming 1 plasmid per bacteria). With a bead recovery efficiency of ~80%, this reduces to 24 copies in an elution volume of 2 μ L. The 45 nL daughter droplet then have less than one copy per droplet. We

anticipate that an increase in sample volume (10 mL) should be able to enhance the limit of detection on the platform.

CONCLUSIONS:

We have developed a simple pre-concentration device to interface mL sample volume to μ L volume requirements of the DMF platform. The device concentrates a sample using plasmid specific magnetic beads into μ L volume for processing on a digital microfluidic platform. The system measures the presence of a target gene that confers antibiotic resistance to bacteria present in urine. The platform benefits from advantages of single step sample purification and pre-concentration at an immiscible interface, together with automated fluidic operations on the DMF platform. Using a clinical strain of *Klebsiella* as a target organism, we demonstrate a nearly complete “sample to answer” assay time of approximately 30 minutes, and sufficient sensitivity to measure resistance in uncomplicated UTIs. The RPA chemistry is robust and can tolerate a small amount of contamination with urine and GuHCl, so that only a single bead wash step in elution buffer is required. Plasmids were extracted by lysing the Gram negative bacteria using heat in a fast and simple process. Although this was done off-chip, it could be integrated following a re-design of the architecture to include a heater or IR laser. The combination of a simple sample processing cartridge with a fast programmable DMF provides a new platform technology which could have many different applications in diagnostics of microorganisms in range of sample matrices.

Conflicts of interest

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There are no conflicts of interest to declare.

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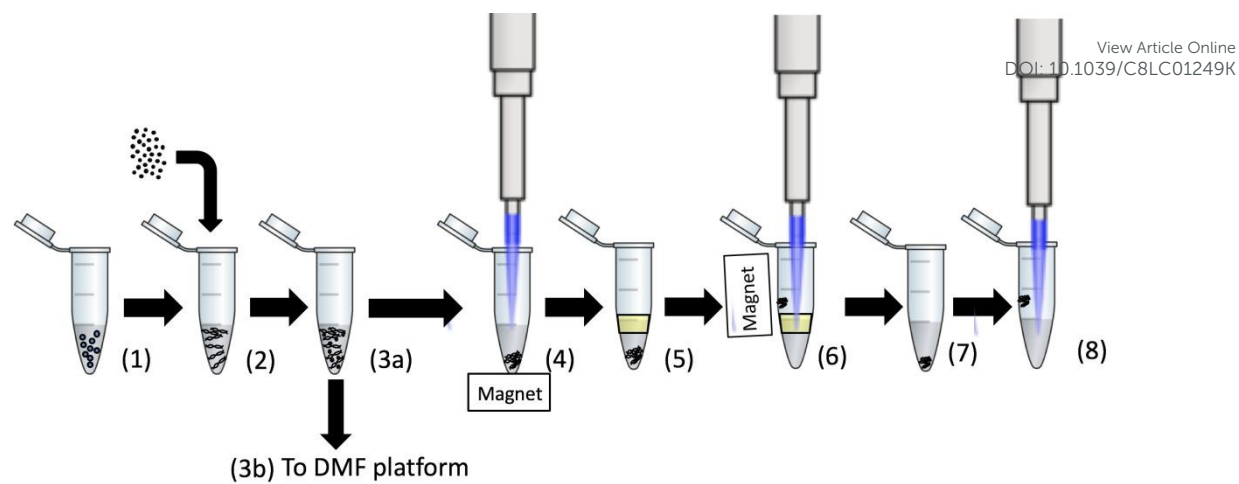


Figure 1 Schematic representation of the workflow for benchtop RPA assay on urine spiked with *Klebsiella pneumoniae* NCTC 13443. (1) Unfiltered urine sample is spiked with bacteria and GuHCl added to a final concentration of 3M in the sample (total sample volume = 1mL). (2) Magnasil Red beads (2.5 μ L) are added and the sample incubated at 90° C for 10 minutes. (3a) Sample is cooled to room temperature. (4) Beads are concentrated in a pellet at the bottom of tube and 950 μ L of supernatant removed. (5) 100 μ L of dodecane is added. (6) Beads are pulled through oil phase to avoid carry over of inhibitors. Remaining solution in the tube is removed and (7) the bead pellet is suspended in 5 μ L of elution buffer to elute plasmid from the beads. (8) Beads are pelleted on the sidewall and the entire volume of eluate is used in benchtop RPA. For the DMF system, the assay is the same except that the sample is introduced to the device at point (3b).

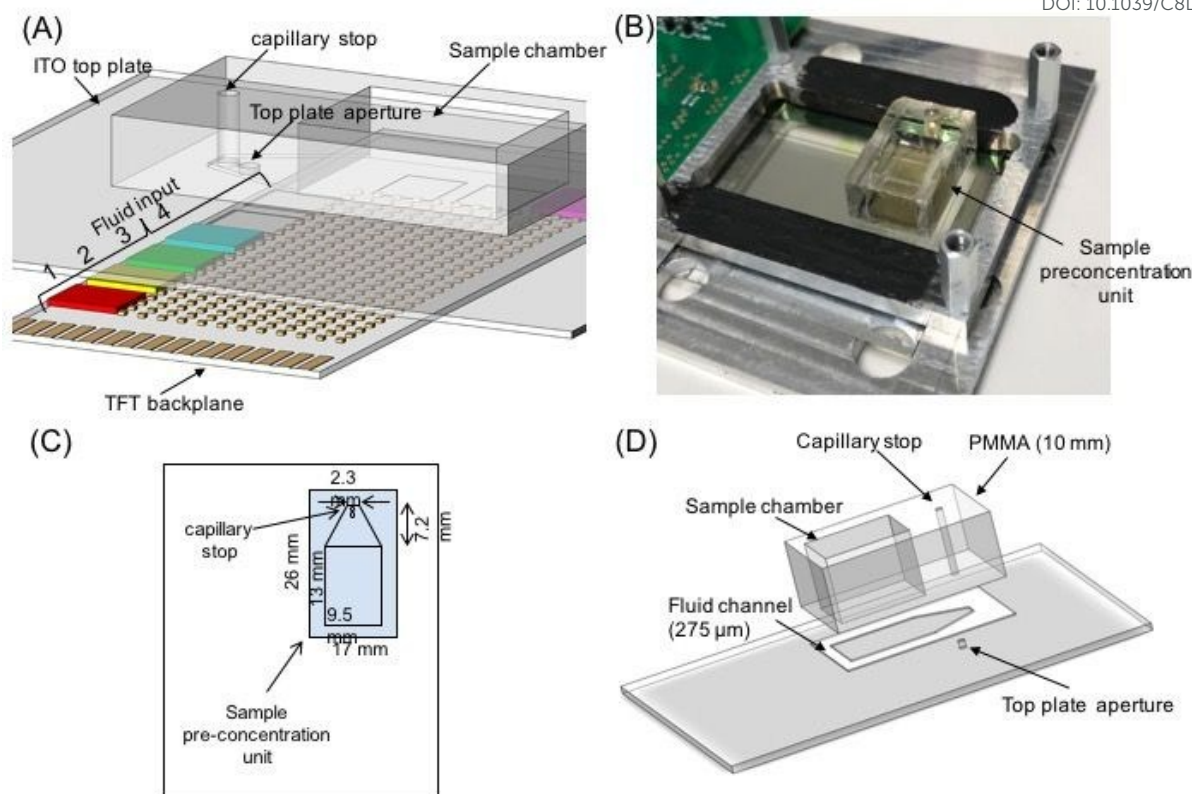


Fig 2(A) Schematic representation and (B) an image of the assembled DMF platform. The assembly consists of the driver (TFT) electronics backplane, a conducting ITO top electrode plate and the sample preconcentration unit. Custom electronics is used to provide the DMF actuation signals. (C) Plan view of the ITO top electrode plate of the DMF device with the sample pre-concentration unit. This is fabricated from PMMA using a laser cutter and bonded to the top glass using double sided adhesive tape which provides the fluidic channel. (D) Side view of the same. The bead sample is introduced into the chamber and slowly fills the channel defined by the spacer until it hits the capillary stop. A magnet is placed beneath the TFT substrate and translated to guide the beads through the aperture in the ITO top plate into the elution buffer reservoir, see Fig 3. Drawing not to scale.

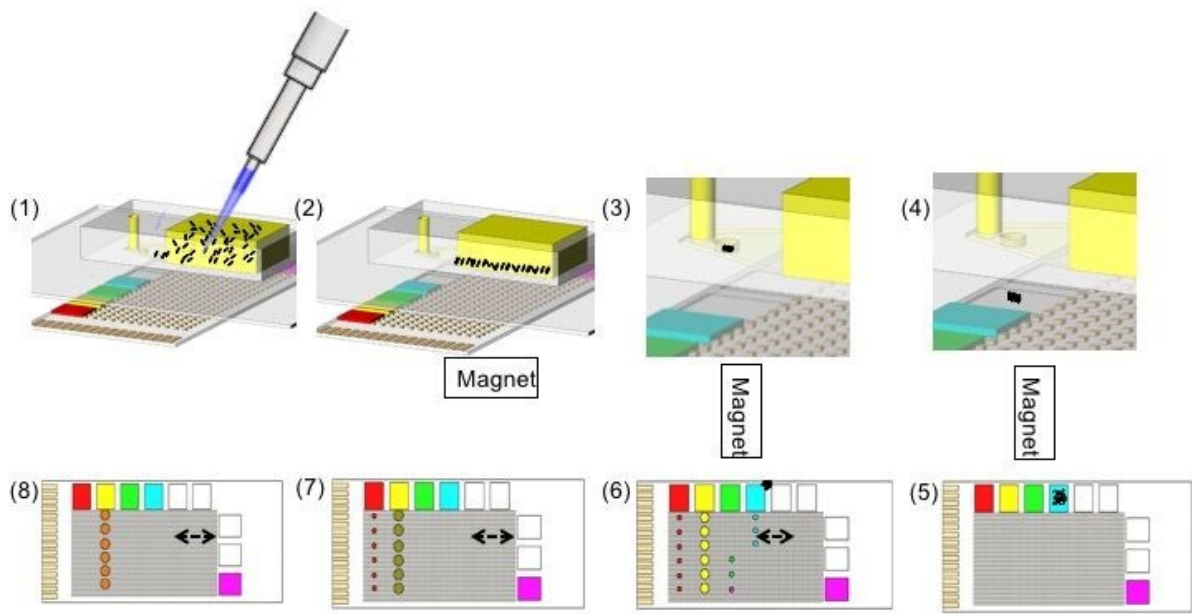


Figure 3: Protocol for sample pre-concentration and DNA amplification on the DMF platform. Reservoir electrodes are pre-loaded with reagents, represented by different colors: red magnesium acetate, yellow RPA mastermix, green NTC, blue elution buffer and purple positive DNA control (1) Urine sample spiked with bacteria (lysed) with magnetic beads as prepared in Figure 1(A) pipetted into the sample chamber. (2) Urine sample slowly fills the channel between the sample pre-concentration unit the ITO glass plate, stopping at the capillary stop. (3) Using a permanent magnet, beads are pelleted and pulled along this channel and then through the aperture in the glass top plate onto the TFT back plane (4). (5) Beads are dragged through the oil in the DMF device onto the reservoir pad with elution buffer, and incubated for five minutes to release plasmids from the beads. (6) Droplets are dispensed from the reagent reservoirs. (7) Plasmids, NTC and positive control droplets are merged and mixed with RPA mastermix. (8) Magnesium acetate droplets are dispensed and mixed. Black dotted arrows represent the direction of droplet movement for mixing. Figure not to scale.

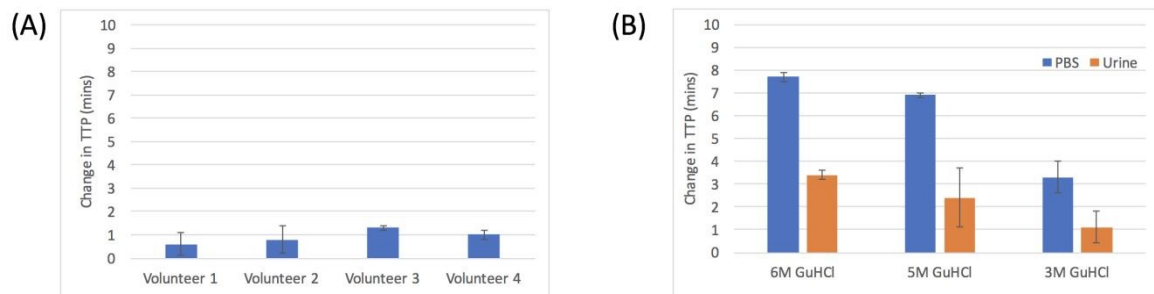


Figure 4: (A) Effect of the addition of unfiltered urine to the RPA reaction mixture (1:50 dilution) plotted as a change in TTP compared with the mean TTP of the positive control (purified *K. Pneumonia* plasmid DNA, 90,000 copies). The DNA was added to RPA reaction mix prepared as per manufacturer's recommendation, except that the 1 μ L of nuclease free water is replaced by urine. Data from duplicates. (B) Capture efficiency of plasmid DNA onto the beads at different concentrations of GuHCl in PBS and urine, quantified as a change in TTP compared with the positive control. Purified plasmid DNA (90,000 copies) was added to either phosphate buffer saline (PBS) or urine containing different concentration of GuHCl and captured on 2.5 μ L of magnetic beads. Plasmid was eluted into 5 μ L of elution buffer. Experiments were performed using the protocol shown in Figure 1A. Data from duplicates.

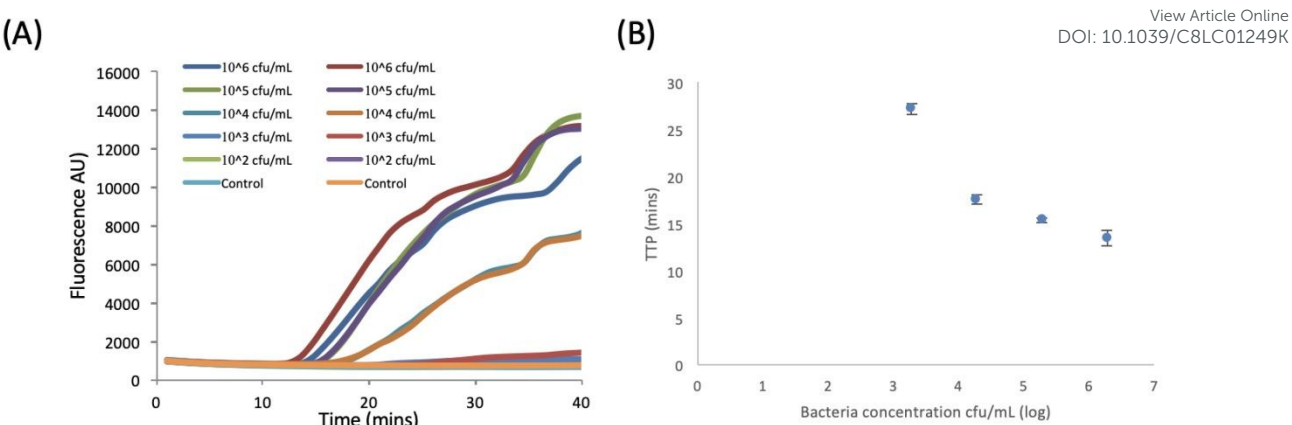


Figure 5: (A) RPA amplification curves for DNA extracted from *Klebsiella pneumoniae* NCTC 13443 using the benchtop protocol shown in Figure 1. (B) Plot of time to positivity (TTP) vs log₁₀ bacteria concentration. Data from duplicates.

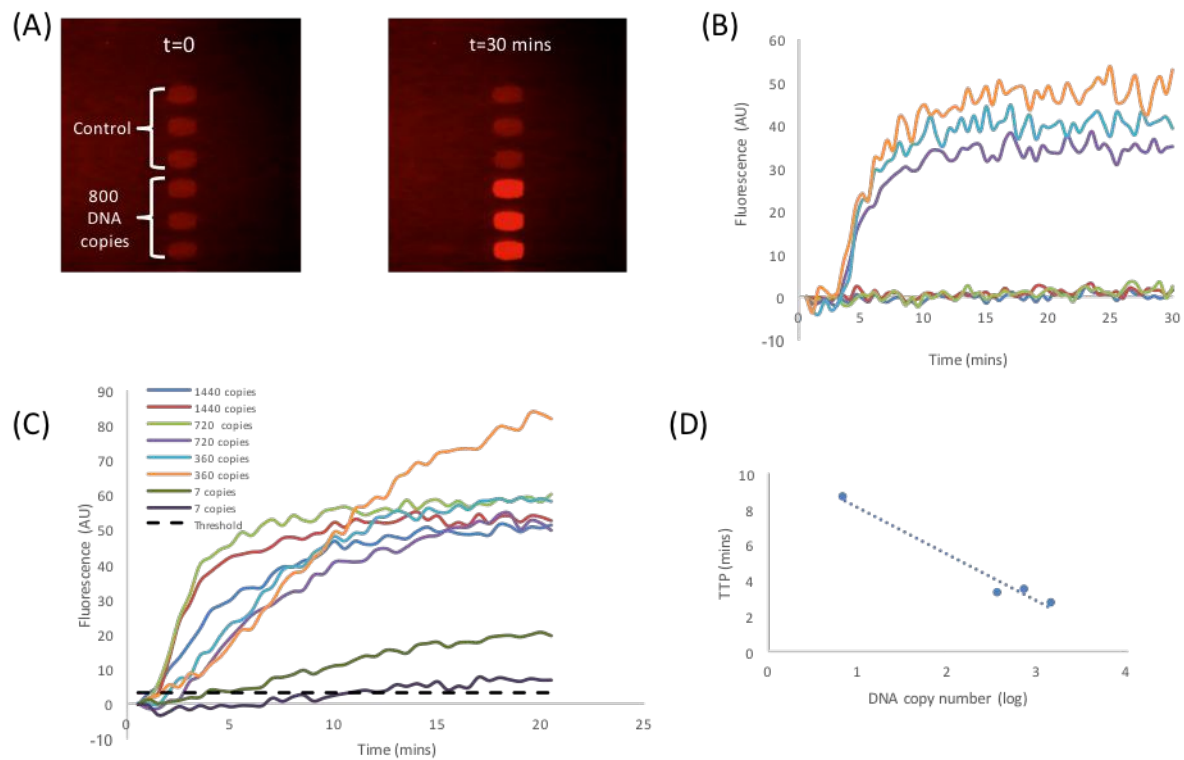


Figure 6: (A) Time lapse images showing RPA amplification for 270nL volume droplets processed on the DMF platform. Purified plasmid DNA was loaded directly onto reservoir electrodes, dispensed and mixed with RPA reagents using a custom programmed sequence. (B) RPA amplification curve for the image shown in (A). Each reaction droplet contained 800 copies of DNA (C) RPA amplification curves for purified DNA loaded directly on reservoir electrodes. (D) TTP with respect to DNA concentration. Data is average of replicates shown in Fig 6C.

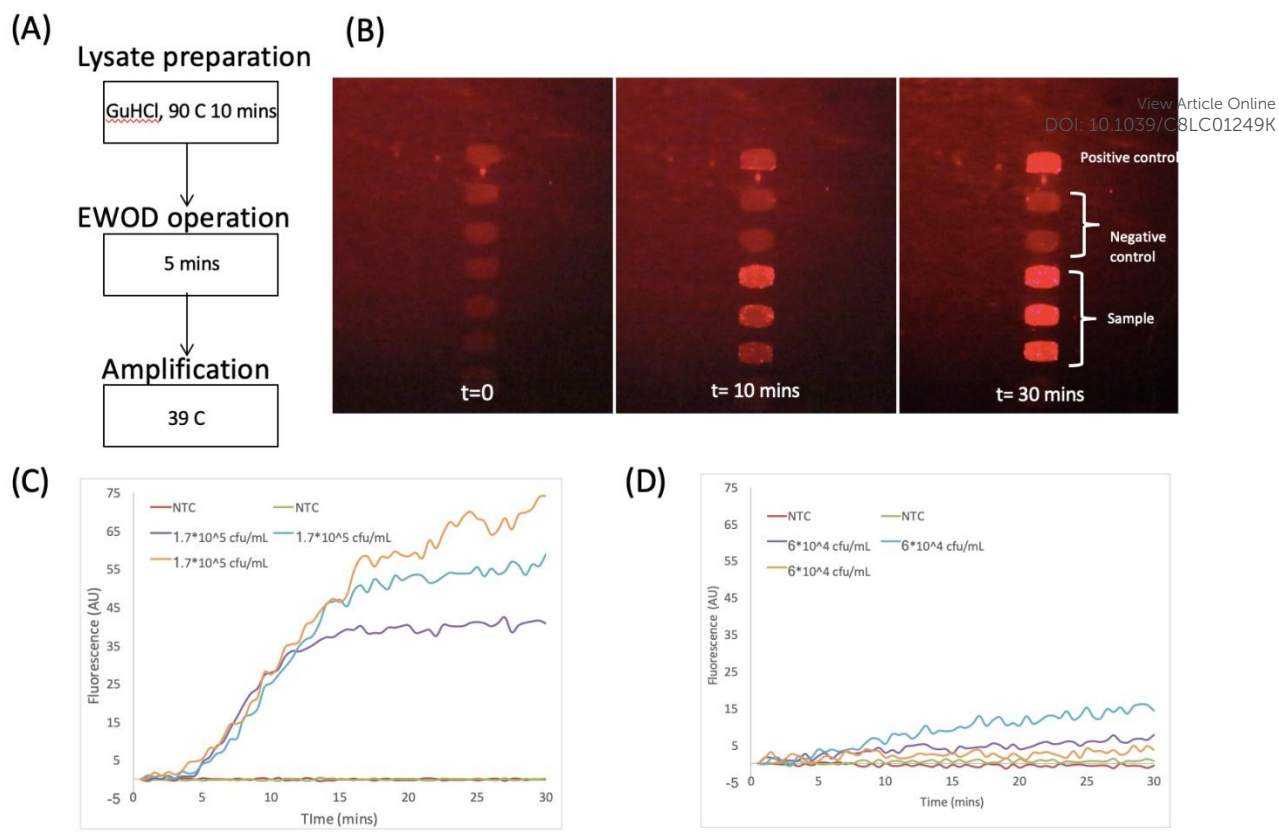


Figure 7: (A) The procedure used to determine antibiotic resistance in bacteria on the DMF platform. (B) Time lapse images showing RPA amplification in 270nL droplets. 1.7×10^5 cfu/mL is spiked into urine and processed as shown in Figure 2. (C, D) RPA amplification curve for two different bacteria concentrations in urine from two healthy volunteers.

Assay variable	TTP (mins) No heat	TTP (mins) Heat
5M GuHCl, Urine, 7.5 mS/cm, 1.3E7 cfu/mL <i>Klebsiella Pneumoniae</i>	22.7*	18.3*
5M GuHCl, Urine, 7.8 mS/cm, 1.9E6 cfu/mL <i>Klebsiella Pneumoniae</i>	**	23.8 ± 3.1
3M GuHCl, Urine, 7.5 mS/cm, 1.3E7 cfu/mL <i>Klebsiella Pneumoniae</i>	20.5 ± 3.4	16.7 ± 1.3
3M GuHCl, Urine, 7.8 mS/cm, 1.9E6 cfu/mL <i>Klebsiella Pneumoniae</i>	18.9 *	16 ± 1.1

Table 1: Effect of additional heat lysis step on plasmid extraction. *Klebsiella pneumoniae* was spiked into urine containing either 5M or 3M GuHCl and 2.5 µL of magnetic bead suspension. The sample was either used directly or heated. Plasmids were eluted into 5µL of elution buffer. Experiments performed on benchtop using protocols shown in Figure 1A. Data from duplicates. *no amplification in replicate. ** no amplification in both replicates.