Electronic Supplementary Material (ESI) for Lab on a Chip.


**Supplementary material for**

# 3X multiplexed detection of antibiotic resistant plasmids with single molecule sensitivity

G.G. Meena[1], R.L. Hanson[2], R.L. Wood[3], O.T. Brown[4], M.A. Stott[5], R.A. Robison[4], W.G. Pitt[3], A.T. Woolley[2], A.R. Hawkins[5], H. Schmidt[1a]

[1]School of Engineering, University of California, Santa Cruz, 1156 High Street, Santa Cruz, CA 95064, USA

[2]Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA

[3]Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA

[4]Department of Chemical Engineering, Brigham Young University, Provo, UT 84602, USA

[5]Electrical and Computer Engineering Department, Brigham Young University, Provo, UT 84602, USA

## S.1 Antibiotic resistance plasmid-containing *E. coli* DH5α stock preparation

*i) Extraction of clinical plasmid isolates*: The bacterial isolates used in this study were acquired from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and Intermountain Healthcare (IHC, Provo, UT) (S.2). Isolates were inoculated on Luria-Bertani (LB, Fisher BioReagents) agar containing 16 µg/mL of imipenem and grown at 37°C overnight prior to DNA extraction. Following the overnight culture, total genomic DNA was extracted using the QIAprep Miniprep kit (Qiagen) and was isolated according to the manufacturer's instructions.

*ii) Transformation of antibiotic resistance genes to E. coli DH5 α cells*: DNA sequences unique to KPC, NDM, and VIM were obtained from NCBI GenBank. The consensus sequence of KPC, NDM, and VIM were used to design primers. The primers were designed using the *PrimerQuest* algorithms from Integrated DNA Technologies (IDT). All oligo sequences were selected for proper GC content, optimal annealing temperature, and lack of hairpin structures. A thorough NCBI BLAST search and analysis of sequence alignments using Geneious were performed to ensure both primer specificity and lack of homology with sequences from other organisms. The genes of interest (KPC, NDM, VIM), were then separately amplified using PCR. After PCR was completed, the genes of interest were run on an agarose gel to confirm size, and then were cleaned up using the QIAquick PCR Purification kit (Qiagen). Each cleaned up gene product was separately cloned into pUC19, and then transformed into *E.coli* strain DH5α (New England Biolabs) following standard cloning and transformation protocols. Each bacterial stock with the respective antibiotic resistance plasmids was grown in LB broth, mixed to 50% glycerol and frozen at –80°C for further use.

*iii) KPC, NDM & VIM sequences*:

KPC-3 (CDC antibiotic resistance isolate bank reference number 0112):

ATAGCGGCAGATCAAGACGACAGAACACTTAATGGTGTAATTTCTATAGACGTAGCAGCGCCTTGGTAAGCGATTTGAGCTTGCCTTGAAAC

NDM-1 (CDC antibiotic resistance isolate bank reference number 0118):

CGTCGTGTGAAGGATAGAGCTGTACGGCCCAAAGCCCCGTCAGCGAAGGTTGCCAAACTAGCAGTCCCTAC

VIM-1 (CDC antibiotic resistance isolate bank reference number 0135):

AGCCYCTCCAGGCYCAAATGGTCTTTAATGGTGTAATTTCTATAGACGTCACAAACCAGCGTATAGCGTTGCGTCAGCAAACT

The sequences above were cloned into pUC19 plasmids individually. Only a segment of each carbapenemase gene was cloned into the plasmids, as cloning the entire gene into a susceptible bacterium is prohibited

## S.2 Sample separation, bacterial cell filtration and DNA extraction

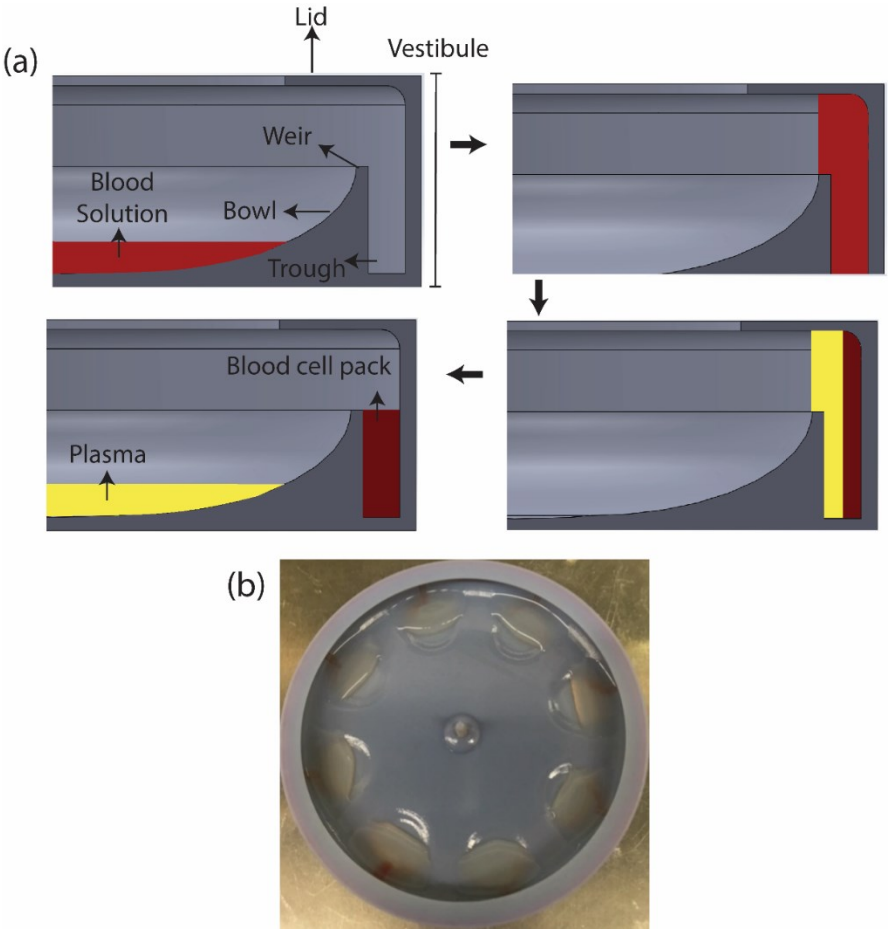


**Figure 1:** (a) Cartoon depicting the cross section of the sample separation hollow disk device showing the bowl region and the vestibule. The vestibule has an upper part covered by a partial lid and a trough in the bottom, separated from the bowl by a weir. As spinning starts, blood with the bacterial cells is spread into the vestibule. During sedimentation, plasma containing the bacterial cells is separated from the blood cells and flows down to the bowl region when spinning stops. (b) Top down image of the disk with plasma separated out. A very small amount of blood cells seeps into the collected plasma, which are chemically ruptured prior to bacterial cell lysis and DNA recovery

*i) Sample separation disk device operation*: *E.Coli* DH5α cells containing pUC19-KPC plasmids were spiked into whole human blood donated by volunteers, under an approved IRB protocol from Brigham Young University, and mixed by hand inversions (final concentration of $5x10^6$CFU/mL). Then 7mL of blood was removed and placed in a circular ring around the center of the hollow spinning disk. The hollow disks are 12 cm in diameter and built of photopolymerizable acrylates (Vero Clear TM Resin) using rapid-prototyping technology (Stratasys Objet30 Prime). Following the blood, 1.5 mL of PBS was added to the disk in a ring

inside the blood ring. Then, the disk was placed on the motor and locked into place. The disk was accelerated at 500 rpm/s until 3,000 rpm and then held at that speed for 30 seconds. During spinning, the plasma with bacterial cells get separated from WBC and RBC. Then the disk was carefully decelerated in 90 seconds for a total spin time of 126 seconds.

*ii) Blood lysing and buffer exchange*: Once the disk was stopped, plasma along with the bacterial cells flow into the bowl of the disk. The spun plasma contains a small fraction of blood cells (on the order of 1 to 5 volume %, observed visually as red streaks in the transparent plasma). The red blood cell concentration in the collected plasma solution was quantified using optical densities of the solution measured with a UV/Vis spectrometer. The spectrometer was calibrated by serial dilutions of known concentration of blood solutions measured using a hemocytometer. Separation using spinning hollow disk removes an average of 95 to 99% of the blood cells, depending on the hematocrit of the donated blood. The plasma layer that flowed down was collected by pipette (4 mL). An average of 60% of bacteria was recovered in the plasma after spinning. This number of bacteria recovered after spinning was measured through plate-counting a control (7 mL of blood with $5x10^6$ CFU/mL final concentration) and the recovered plasma. 4mL of plasma with $5.25x10^6$ CFU/mL bacteria in plasma was recovered and placed into a blood lysing solution to remove any remaining RBCs. The blood lysing solution contains 45 mL of 3% (w/v) Brij-58 and 1 M NaCl. The solution was vortexed and filtered through a 25 mm polycarbonate track-etched filter (Steriltech) with 0.4µm pore diameter, which retained the bacteria. Once all the plasma-blood-lysing solution was filtered, 3 mL of PBS were filtered through as a wash solution, and then the filter was "reverse filtered" using 2 mL of the backflush solution, 3% (w/v) Pluronic F108. The "reverse filtered" solution, which contained the captured bacteria, was collected and subjected to the DNA extraction process.

*iii) DNA extraction*: To the recovered bacteria, 400 µL of lysozyme (10 mg/mL) was added and the solution vortexed for 5 sec and incubated for 10 min on a shaker. After shaking, 0.5 mL of 6 M guanidine hydrochloride and 0.5 mL of 1% (w/v) SDS was added to the solution and vortexed and incubated for 5 min. Then, 100 µL of 1 µm superparamagnetic beads (Spherotech, SIM-05-10H) were added to the solution, followed by 2 mL of isopropyl alcohol, and the mixture vortexed for 10 sec. Following vortexing, the mixture was placed on a shaker for 3 min. Following the shaker, the solution was then placed in a magnetic holder for 2 min to allow the superparamagnetic beads to collect in one location on the side wall of the tube. The supernatant was then removed by pipetting and discarded. Next 1 mL of a washing buffer (6 M guanidine hydrochloride, 20 mM tris-HCl, 40% 2-propanol in distilled water) was added and the tube removed from the holder and vortexed for 30 seconds. The tube was then placed back in the magnetic holder for 2 min for bead collection. The supernatant was removed again and discarded. Then the DNA was allowed to air dry for 5 min by leaving the tube on the magnet with the top open. Then, 50 µL of distilled water was added and the tube removed from the holder and vortexed for 2 min. The tube was then placed back in the magnetic holder for 2 min for bead collection. The supernatant was removed and placed into an in-house-developed ultrasound chamber. The sample was sonicated using a ¼-inch-diameter probe sonicator

(Sonics and Materials, Vibra-Cell Model: VCX400) for 30 sec using 20% amplitude (1W/cm$^2$). The ultrasound tip was submerged in degassed water with a 0.1 mm polypropylene film separating the sample and the water in which the tip was submerged. The DNA solution was then pipetted out into a clean tube for subsequent use. DNA concentration of parallel samples was measured by fluorescence of pico green label. The average concentration of the 50 µL was 0.12 ng/µL, indicating that the capture by and subsequent elution from magnetic beads was only 9.6%.

## S.3 Microfluidic chip with polymer monolith structures

*i) Chip Fabrication:* The chip was fabricated using hot embossing to transfer (500µm x 500µm) microfluidic channels using a CNC machined aluminum master on a 2 mm thick polypropylene sheet. After defining inlets and outlets, the channel was thermally sealed using a 0.5 mm thick transparent polypropylene film. The functionalized monolith column was fabricated in a single step by filling the microfluidic channel completely with a polymerization mixture containing 17.6% poly(ethylene glycol) diacrylate (PEGDA), 17.6% ethylenedimethacrylate (EDMA), 13.3% 2-propanol, 40.0% n-dodecanol, 8.8% 500µM of acrydite modified capture-oligonucleotide (in 1:1 2-propanol:water) and 2.7% benzoin methyl ether (BME) photoinitiator (all w/w). The acrydite solubility limits the dissolution solution to a ~500 µM acrydite concentration. The device is then masked leaving a 5 mm gap to define the monolith column and was exposed to UV light (SunRay 600 UV lamp) for 12 min at 100mW/cm$^2$. After polymerization, the microfluidic chip was rinsed with 2-propanol for 15 min and water for 10 min to rinse out the porogens, leaving behind a porous polymer column in the fluidic channel functionalized with target-specific capture DNA sequences. SEM images of microfluidic channel containing the monolith were taken to quantify the nodule and pore size. To take the SEM measurements, the microfluidic chips with the monolith was first dried for 24hrs and then heated to 110 °C to remove any water.

*ii) Capture-oligonucleotides:* The sensitivity and selectivity of the monolith are achieved by designing three separate capture-oligonucleotides for capturing the KPC, VIM and NDM plasmids. The binding strength of the capture-oligonucleotides with the plasmids is characterized by their annealing temperature, which is dependent on the capture-oligonucleotide sequence. The sequence of the oligonucleotides was designed by querying GenBank (NCBI, NIH) to have maximum alignment with the plasmids. 50 sequences of KPC, VIM and NDM were aligned and consensus sequence were obtained. The capture probes with sequence complementary to the target plasmids were designed using PrimerQuest algorithms (IDT Inc). All capture-oligonucleotide sequences were selected for proper GC content and optimal annealing temperature.

KPC: 5’-TATCGCCGTCTAGTTCTGCTGTCTTG-3’; Tm = 68 ºC

VIM: 5’-TCGGAGAGGTCCGACTTTACCAGA-3’; Tm = 67 ºC

NDM: 5’-GCAGCACACTTCCTATCTCGACAT-3’; Tm = 65 ºC

*iii) Heater temperatures and washing buffer:* The thermoelectric heater below the serpentine channel was set at 90°C to denature the DNA. The monolith was heated at 65°C for 2 mins to

release the captured DNA and elute it in a 35µL fraction. Washing buffer used is 20mM Tris-HCl pH8 with 500mM NaCl and 50mM $MgCl_2$.

*iv) qPCR reaction confirmations:* To confirm DNA capture in eluted fractions from the plasmid samples, qPCR was carried out on the fractions obtained from the monolith chip, both before and during elution. Table 1 provides the qPCR cT values for a set of specifically captured samples, as well as the positive and negative controls. In qPCR, cT values have an inverse, logarithmic relationship to DNA concentration (detection limit of the used qPCR instrument is cT ~30). For captured plasmid fractions, cT values were all lower than the pre-elution numbers, indicating a higher concentration of the target DNA sequence during elution than before, which is consistent with specific DNA capture and enrichment. A similar trend was also observed for the positive control. However, for the negative control, the cT values were high (low DNA concentration) in both pre-elution and eluted fractions, consistent with a lack of DNA capture when the target and monolith DNA are non-complementary.

| **Target** | **Pre-elution** | | **Elution** | | |
|---|---|---|---|---|---|
| NDM plasmid sonicated | U | Heating (65 °C, 2min) | 13.4 | 16.7 | 20.5 |
| VIM plasmid sonicated | U | | 16.0 | 19.5 | 23.6 |
| KPC plasmid sonicated | 18.8 | | 14.5 | 13.9 | 16.9 |
| KPC negative control | 28.5 | | 31.5 | 31.5 | 29.1 |
| KPC positive control | 14.3 | | 12.3 | 12.5 | 12.6 |

**Table 1: cT values for qPCR on a set of pre-elution and eluted fractions collected from capture monoliths after loading different samples.** 'U' indicates undetermined, where signal did not rise above threshold.

## S.4: Device fabrication

The ARROW optofluidic biosensor device was fabricated on top of a 100 mm <100> oriented Si substrate. Six alternating layers of $SiO_2$ (n=1.47) and $Ta_2O_5$ (n=2.107), dielectric thin films of thickness 265 nm and 102 nm, respectively, were sputtered on top of the wafer. The thickness of and index of the layers were designed to form the Anti-Resonant-Reflecting layer stack which acts as the substrate on which the waveguides are fabricated. The three hollow-core microchannels (Ch1, Ch2 & Ch3) of dimension 12 µm x 6 µm (width x height), were defined on the wafer using a sacrificial patterned SU-8 layer (Microchem) [35]. SU8 photoresist and a thin nickel layer were used as a mask to selectively etch a self-aligned pedestal into the wafer using inductively coupled-plasma reactive ion etcher (ICP-RIE). The pedestal protects the sacrificial

SU-8 core while etching and provides structural integrity to the hollow cores [36]. Once the pedestals are defined, a 6µm thick $SiO_2$ layer is deposited on the wafer via plasma-enhanced chemical vapor deposition (PECVD). The MMI and the tapered collection waveguides were patterned by photolithography and 3µm tall ridges were etched into this $SiO_2$ layer using ICP-RIE to form 3 µm tall ridge waveguides intersecting the hollow-core channels at multiple points. The inlet sides of the sacrificial SU-8 layer were exposed with a wet etch through the top of the $SiO_2$ layer and SU-8 was removed using $H_2SO_4$ : $H_2O_2$ solution to form the hollow-core channel. The hollow channels are filled with buffer medium to form the liquid-core waveguides.

## S.5 Fluorescence signal from negative controls

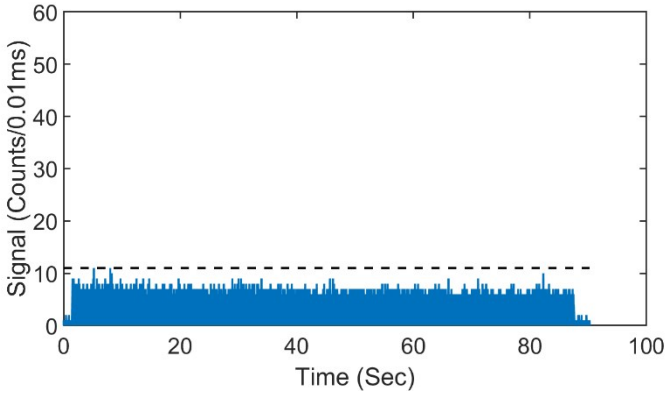


**Figure 2** Negative controls were done by running non-complementary plasmid targets in a monolith microfluidic chip (Ex: VIM plasmids run in monolith chip functionalized with KPC capture probes, KPC plasmids run in monolith chip functionalized with NDM capture probes and NDM plasmids run in monolith chip functionalized with VIM capture probes). Fluorescence trace of samples eluted from these experiments detected using ARROW optofluidic chip show no signal above the set background threshold of 11 counts/0.01ms.

## S.6 S(t) Histogram example

**Single-plex experiment:**

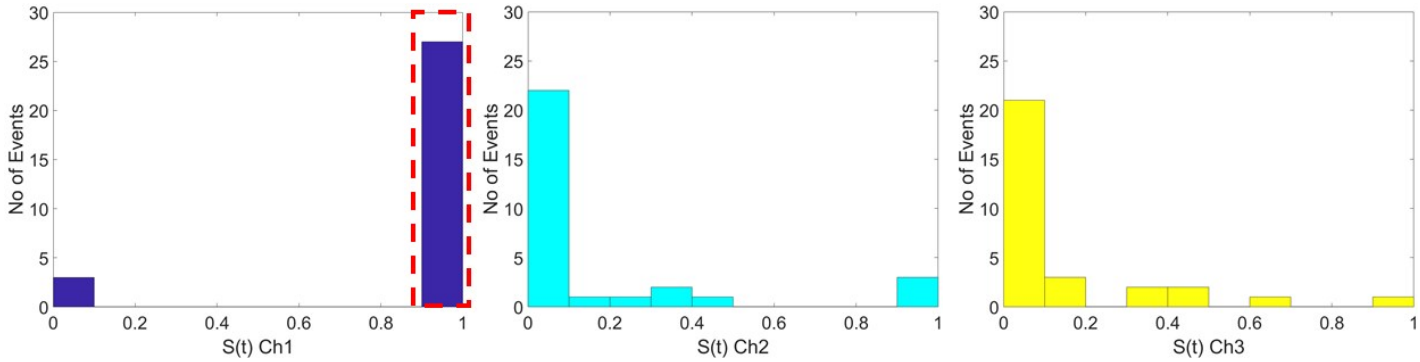


**Figure 3** (a) Distribution of $S(t)_j$ values of the events from single-plex detection of KPC plasmids show most signals have dominant $S(t)_1$ (red box) and very low S(t) value in the other two channels, enabling correct identification of signals.

**Multiplex experiment:**

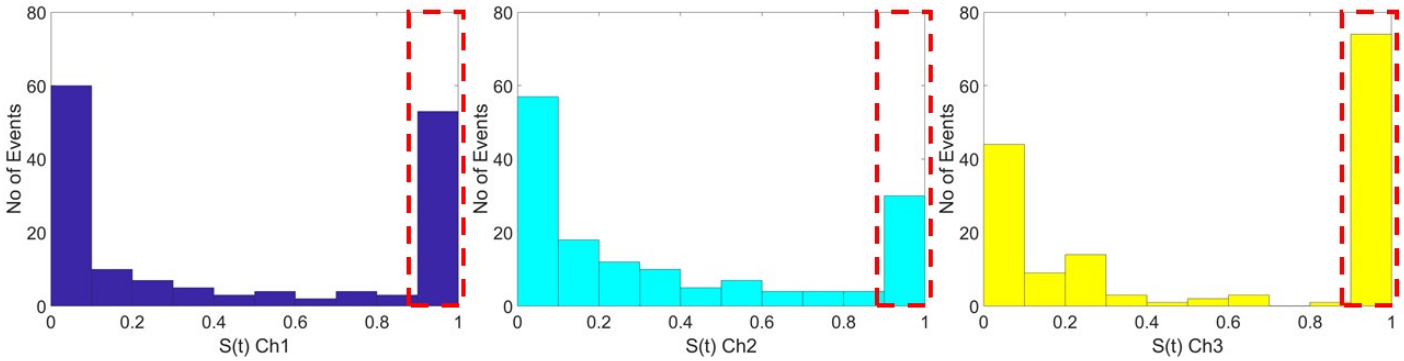


**Figure 3** (b) For multiplexed detection, KPC plasmids are introduced in Ch1, NDM in Ch2 and VIM in Ch3. Signals from all three targets are collected simultaneously by a single detector. Each signal is deciphered based on which channel S(t) value is most dominant.