Supporting information

Amplification-Free Detection of *Escherichia coli* Using an Acidic Deoxyribozyme-based Paper Device

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Section A: Materials and Instruments

A1. Oligonucleotides and Materials.

The DNA oligonucleotides (Table S1) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China), and purified by 10% denaturing (8.0 M urea) polyacrylamide gel electrophoresis (dPAGE) or high-performance liquid chromatography (HPLC). T4 DNA ligase, Thermus thermophilus DNA polymerase, adenosine triphosphate (ATP) and deoxyribonucleoside-5'-triphosphate (dNTPs) were purchased from Sangon Biotech. T4 polynucleotide kinase (PNK) was obtained from Thermo Fisher Scientific. Streptavidin agarose beads were produced by Med Chem Express (MCE). Streptavidin-horseradish Peroxidase (HRP) was purchased from Shanghai Beyotime Biotechnology Co., Ltd. 3,3',5,5'-tetramethylbenzidine (TMB) and Technology Co., Ltd. H_2O_2 (30%) were purchased from Beijing Solarbio Science and Beijing Hua Teng, respectively. All other chemicals were purchased from Sigma-Aldrich and used without further purification.

A2. Bacterial cells.

The *Escherichia coli BL21* (EC, CICC 23796), *Bacillus subtilis* (BS, CICC 10002), *Bacillus cereus* (BC, CICC 10352), *Burkholderia gladioli* (BG, CICC 10574), and *Klebsiella pneumoniae* (KP, CICC 13883) were purchased from China Center of Industrial Culture Collection. The *Staphylococcus aureus* (SA, ATCC 6538) and *Pseudomonas aeruginosa* (PA, ATCC 9027) were purchased from Agricultural Culture Collection of China.

A3. Instruments.

The fluorescent images of gels were obtained using a Typhoon 5 variable mode imager (GE Healthcare, US) and analyzed using Image Quant software (Molecular Dynamics). The UV-vis absorbance was monitored on a microplate reader (Tecan, Switzerland). The photos were taken using the iPhone 12 mobile phone camera and analyzed with ImageJ software.

A4. Buffers.

1× PNK buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM Dithiothreitol (DTT), and 0.1 mM spermidine, pH 7.6 at 25°C,

10× T4 DNA ligase buffer: 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, pH 7.8 at 25°C.

1× reaction buffer (1× RB): 50 mM sodium citrate, 150 mM NaCl, 50 mM KCl, 20 mM EDTA, and 0.01% Tween 20, pH 5.3.

1× PCR buffer: 75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$.

2× urea PAGE loading buffer: 80 mM EDTA (pH 8.0), 16 M urea, 180 mM Tris, 180 mM boric acid, 20% sucrose (w/v), 0.05% xylene cyanol, and 0.05% bromophenol blue.

TMB colorimetric reaction system: 10 mM TMB, 2 mM H_2O_2, 25 mM sodium citrate, 50 mM Na_2HPO_4, pH 5.3

1× Binding buffer for probe immobilization: 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0.

Section B: Experimental details

B1. Preparation of crude intracellular mixture (CIM) from bacterial strains

E. coli BL21 cells were cultured in 30 mL of Luria Bertani (LB) with continuous shaking at 37°C. 1 mL of the culture was centrifuged at 8,000 rpm for 10 min at 4 °C. The cell pellets were re-suspended in 300 μL of 1× SB, sonicated for 30 s, put on the ice for 2 min, and repeated 6 times. The cell suspension was centrifuged at 13,000 rpm for 10 min at 4 °C. The clear supernatant was collected as the CIM-EC and heated at 90 °C for 5 min before storing at -20 °C.

The CIM from every other bacterium was prepared as described above except that: 1) *Klebsiella pneumoniae* cells were cultured in Tryptic Soy Broth (TSB); 2) *Burkholderia gladioli* cells were cultured at 30 °C; 3) each bacterium was cultured for a different period of time until the OD_{600} (optical density at 600 nm) reached ~ 1.

B2. Preparation of sensing material for the colorimetric test.

Ligation of fluorogenic substrate (FS) to MaRCD-EC1. MaRCD-EC1 (300 pmol) was phosphorylated at 37 °C with 10 units (U) of PNK in 50 μL of 1× PNK buffer with 2 mM ATP for 40 min. 10 μL of 10× T4 DNA ligase buffer, FS (360 pmol) and MaRCD-EC1 (360 pmol) were added and the volume was adjusted to 98 μL with ddH_2O. The mixture was heated to 90 °C for 2 min, and then cooled at RT for 10 min. This was followed by the addition of 2 μL of 10 U T4 DNA ligase and incubation at room temperature (RT) for 2.5 h to obtain the ligation product of MaRCD-EC1. The obtained MaRCD-EC1 was purified by 10% dPAGE and used for the following experiments.

Preparation of adapter DNA (aDNA)-HRP conjugate. 10 μL of 15 μmol streptavidin-HRP was mixed with 1 nmol aDNA and adjusted to a final volume of 100 μL with 1× PBS buffer. The mixture was incubated at RT for 2 h, and passed through a membrane-based molecular sizing centrifugal column with a molecular weight cut-off of 30 kDa (NANOSEP OMEGA, Pall Incorporation) to remove the non-conjugated

DNA. The column was washed with 50 μL of 1× PBS buffer 3 times and the obtained aDNA-HRP conjugate was re-suspended in 50 μL of 1× PBS buffer.

MaRCD-EC1 and HRP immobilization. The average size of the agarose beads was 100 microns with a range of 45 to 165 microns. 50 μL of the stock suspension of streptavidin-coated agarose beads was transferred to a microcentrifuge tube and washed twice with 300 μL of binding buffer. And the obtained agarose beads were re-suspended in 300 μL of binding buffer. 500 pmol of MaRCD-EC1 was then added to the bead suspension, and the mixture was mildly shaken by DNA mixer at RT for 1 h. The beads were sedimented by brief centrifugation using a bench top mini centrifuge, and washed 3 times with 300 μL of binding buffer. Through the streptavidin-biotin interaction, the obtained MaRCD-EC1/beads were then re-suspended in 300 μL of binding buffer. This was followed by the addition of 50 μL aDNA-HRP conjugate. The mixture was then heated to 55 °C for 5 min and then cooled to RT. After incubation at room temperature for 2 h, the beads were sedimented by the brief centrifugation using a bench top mini centrifuge. The beads were washed until the color of the supernatant did not change any more in the presence of TMB and H_2O_2. Finally, the beads were suspended in 500 μL of 1× RB noted as HRP@ MaRCD-EC1/beads.

B3. MaRCD-EC1 activity assay. The MaRCD-EC1 ligation product was prepared according to the protocol described above and stored at -20 °C. Kinetic analysis of MaRCD-EC1 to CIM-EC was carried out as follows (**Figure S1**): 50 μL of 1× SB containing 200 nM MaRCD-EC1 were heated at 90°C for 2 min and cooled at RT for 10 min. 50 μL of CIM-EC (10^6 CFU mL^{-1}) was added and incubated with the above mixtures at RT for 1, 3, 5, 10, 20, 30, 60, 120 min. After ethanol precipitation, the resultant products were analyzed by 10% dPAGE (8 M urea). Observed rate constants were determined by curve-fitting the percent cleavage of MaRCD-EC1 in the presence of CIM-EC vs reaction time using Origin 2021 where $Y = Y_{max}\,[1\text{-}e^{-kt}]$, Y_{max} represents the maximal cleavage yield and k is the observed first-order rate constant (k_{obs}).

B4. Selectivity and sensitivity by dPAGE assay. For the selectivity tested by dPAGE, 50 μL of 1× RB containing 200 nM MaRCD-EC1 was heated at 90°C for 2 min and cooled at RT for 10 min. 50 μL of CIM (10^6 CFU mL^{-1}) prepared from various bacteria was added and incubated with the above mixtures at RT for 5 min. After ethanol precipitation, the resultant products were analyzed by 10% dPAGE (8 M urea). For the sensitivity test, the only changes made were a 30-minute incubation time and using various concentrations (10-10^7 CFU mL^{-1}) of EC to prepare the CIM, while the else procedure was the same as described above. For comparison, we also performed the dPAGE analysis of MaRCD-EC1 to CIM-EC prepared from 10-10^7 CFU/mL *E. coli* cells using 10 min reaction time (**Figure S2**).

B5. Native HRP catalytic activity in various pH values. To determine whether the native HRP exhibits the optimal activity in the acidic environment of MaRCD-EC1, we investigated the TMB oxidation at different pH values. Briefly, the colorimetric reaction was performed using 0.7 nM HRP in TMB colorimetric buffer under varying pH conditions. Subsequently, the k_{obs} were estimated to confirm the optimal pH range for the colorimetric reaction (**Figure S4**). The buffer solutions were prepared as following: 200 nM Glycine hydrochloride buffer for pH 2.0, 100 nM sodium citrate and 200 nM Na_2HPO_4 for pH 3.0-5.0, 200 nM Na_2HPO_4 – NaOH for pH 6.0-8.0, 200 nM Glycine sodium hydroxide buffer for 9.0-10.0.

B6. HRP release rate from assembled beads. 50 µL of the HRP@MaRCD-EC1/beads and 50 µL of CIM-EC (10^6 CFU mL^{-1}) were mixed and incubated for different time (1, 3, 5, 10, 20, 30, 60, 120 minutes) at 25 °C with occasional mixing using a pipette to prevent the sedimentation of beads. By centrifugation for 5 min, the beads were sedimented using a bench top mini centrifuge. The supernatant was carefully transferred to a microcentrifuge tube for backup. 10 µL of the stocked supernatant was dispensed in a well of the 384-well transparent plate containing 30 µL of TMB colorimetric reaction system and recorded the UV-vis absorption at 652 nm. The signal was acquired every 6 sec for 6 min duration, and shaken for 3 sec before measurement (**Figure S5**). HRP was quantified by comparing the k_{obs} of the test samples to a standard curve (**Figure S6**). The formula for calculating the relationship between HRP concentration and the catalytic rate is $Y_c = 150/(10^a \text{ꝏ} x^b)$, where Y_c is the concentration of HRP and x is the k_{obs}, 150 is the initial concentration constant of HRP without dilution, a = 3.15696, b = -1.56282.

B7. Specificity and sensitivity of the colorimetric assay in solution. 50 µL of HRP@MaRCD-EC1/beads and 50 µL of CIM-EC (10^6 CFU mL^{-1}) were mixed and incubated for 30 min at RT with occasional mixing using a pipette to prevent sedimentation of the beads. By centrifugation for 5 min, the beads were sedimented using a bench top mini centrifuge. 20 µL of the supernatant was carefully transferred to a PCR tube containing 60 µL of the TMB colorimetric reaction system. The color change was observed and recorded using a mobile phone camera. The k_{obs} was estimated as the same as described in the B6 (**Figure S7**). For the sensitivity test, the procedure was the same as described above, except for various concentrations (10-10^7 CFU mL^{-1}) CIM-EC instead of 10^6 CFU mL^{-1} CIM-EC.

B8. Design of paper-based device. The paper-based device was designed using Microsoft PowerPoint with a black background, which provided 2 spherical zones interconnected through a flow channel (zones and the connecting channels are white). The diameter of the sensor zone is 10 mm, while the detection zone is 5 mm. The

connecting channel was 2 mm wide and 3 mm long. Wax was printed using a Xerox ColorQube 8570N solid wax printer followed by heating at 120°C for 2 min to diffuse the wax through the Whatman filter paper (Grade 1) to create a uniform hydrophobic barrier (black region). After immersion into the blocking buffer (50 mM sodium citrate containing 1% BSA, 0.02% Tween-20, pH 5.3) for 10 min, the obtained bioactive paper was dried at RT. The obtained paper was then attached to a backing card. To create a sensor film, the HRP@MaRCD-EC1/beads were homogeneously suspended in 500 μL of 1× RB. Concurrently, 500 μL of 5 wt% pullulan was also prepared in 1× RB. 100 μL of HRP@MaRCD-EC1/beads suspension was mixed with 100 μL of the pullulan solution (final concentration of pullulan: 2.5 wt%) in a tube. 30 μL of the suspension was evenly dispensed on the Z1 and dried at RT. To form a reporting film, a 2.5 wt% pullulan solution including 10 mM TMB and 10 μL of the mixed solution was dispensed on the Z3 and dried at RT. Finally, the Z2 was covered evenly with 2.5 μL of 5 wt% pullulan and dried to form a transparent film.

B9. Selectivity and sensitivity of the paper-based device. 50 μL of CIM prepared from each bacterium or with different concentrations of *E. coli* was added onto Z1 and incubated for 30 min. After the pullulan film was dissolved, the buffer containing released HRP was migrated to Z3. 10 μL of TMB color developing buffer containing 2 mM H_2O_2 was added to Z3. After 2 min reaction, the color change was captured using an iPhone 12 mobile phone camera and analyzed with ImageJ software. Tests were run in triplicate to obtain error bars and calculate a limit of detection based on 3 σ/slope.

B10. Pullulan membrane dissolution time and fluidity. The Z1 and Z3 were evenly encapsulated with 30 μL and 10 μL of 2.5 wt% pullulan, respectively. The Z2 was covered with 2.5 μL of 5 wt% pullulan. After being dried, 50 μL of the red colored solution as the indicator was added to the Z1 for monitoring the dissolution time of the pullulan film (**Figure S8**).

B11. Feasibility of the paper-based device. We conducted a comparative analysis to determine the colour change of Z3 in the presence of absence of HRP, MaRCD-EC1, and CIM-EC (**Figure S9**).

B12. Stability Test of the paper-based device. We prepared a series of paper devices and stored at RT for 1 month. At the 1st ,15th and 30th day, three paper devices were used to detect CIMs prepared from 10^6 CFU mL^{-1} EC. The color intensities were quantified using Image J. The negative control was performed by adding the reaction buffer on Z1. (**Figure S10**).

B13. *E. coli* detection using the paper device in clinical urine samples. To eliminate the impact of the interferences in urine, such as divalent metal ions (e.g., Ca^{2+}, Fe^{2+}, Zn^{2+} and so on) and DNase, for *E. coli* detection, the bacteria in urine were precipitated

by centrifugation and re-suspended in the reaction buffer that only contains monovalent ions and EDTA for the following cell lysis. Briefly, 1 mL of clinically obtained urine samples was centrifuged at 8,000 rpm for 10 min at 4 °C. The cell pellet was washed with 60 μL of 1× SB, and re-suspended in 60 μL of 1× SB. The obtained mixture was sonicated for 30 s, put on the ice for 2 min, and repeated 2 times. The cell suspension was centrifuged at 13,000 rpm for 5 min at 4 °C. The collected CIM was used for *E. coli* detection. In a typical experiment, 50 μL of CIM was applied onto the Z1 and incubated for 30 min. After the pullulan film was dissolved, the buffer containing the released HRP was migrated to Z3. Then 10 μL of TMB color developing buffer containing 2 mM H_2O_2 was added onto Z3. After 1 min, the color change was captured using a mobile phone camera and analyzed with ImageJ software to record the average optical density. Paper-based detection devices and curve fitting enable qualitative and semi-quantitative analysis of E. coli counts in positive samples (**Figure S11**). All of urine samples were acquired from the Dalian Municipal Central Hospital. This study was approved by the Ethics Committee of Dalian University of Technology.

B14. *E. coli* detection using culture method in clinical urine samples. The human urine samples were obtained and processed in accordance with approved protocols from the Dalian Municipal Central Hospital. *E. coli* in urine samples was cultured using cystine-lactose-electrolyte-deficient agar or medium (CLED) agar plates. To determine *E. coli* in urine, the samples were cultured at 37 °C. Each sample was diluted in three gradients (10×) using a coating volume of 10 μL. Subsequently, the plates were incubated at 37 °C for 15 h. After the incubation period, yellow colonies with yellow zones were counted as *E. coli* on CLED agar plates **(Figure S12)**. These *E. coli*+/culture+ samples were further confirmed by MALDI-TOF mass spectrometry in the hospital.

Tables and Figures.

Table S1. Sequences of DNA oligonucleotides used for colorimetric tests

DNA oligonucleotide	Sequence (5'-3')
Modified aRCD-EC1 (MaRCD-EC1, 56 nt)	CAGGTCCATC GAGTGGTAGG ATGCGGCGGT CAGTCGCACT GCTCCTTTTT TTTTTT-B
Fluorogenic substrate (FS, 44 nt)	F-AAAA AAAAAAAAAA CTATGAACTG ACTrATGACCT CACTACCAAG
DNA Splint (DS, 24 nt)	TCGATGGACC TGCTTGGTAG TGAG
adapter DNA (aDNA, 30 nt)	F-AAAA AAAAAAAAAA CTATGAACTG ACTrATGACCT CAGTTCATAG TTTTTTTTTT TTTTTTTTTT-B
F-adapter DNA (FaDNA, 30 nt)	F-CAGTTCATAG TTTTTTTTTT TTTTTTTTTT-B
Cleaved DNA (cDNA, 13 nt)	F-CTATGAACTG ACT
Complementary DNA (cDNA 13 nt)	AGTCAGTTCA TAG

Note: F = fluorescein, B = Biotin.

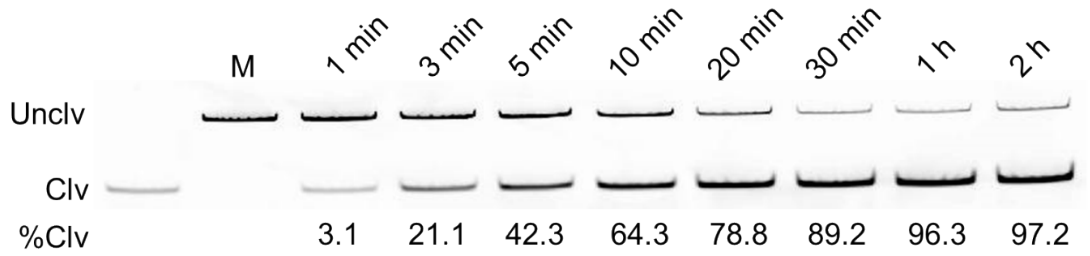


Figure S1. dPAGE analysis of the kinetic responses of MaRCD-EC1 to CIM-EC (10^6 CFU/mL) at 25 ℃. M = marker, Unclv = uncleaved, Clv = cleaved, %Clv = cleavage percentage, which is calculated based on the equation: %Clv = (clv*100)/(clv + unclv), where clv is the volume of cleaved band and unclv represents the volume of un-cleaved band.

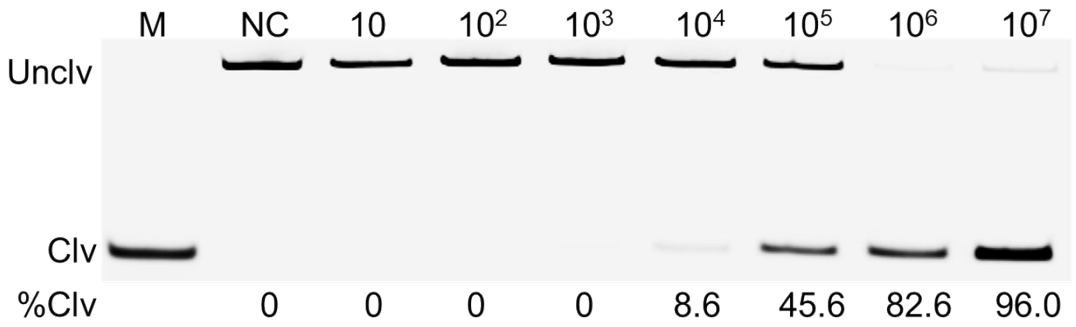


Figure S2. dPAGE analysis of response of MaRCD-EC1 to CIM-EC prepared from 10-10^7 CFU/mL *E. coli* cells after 10 min reaction at 25 °C.

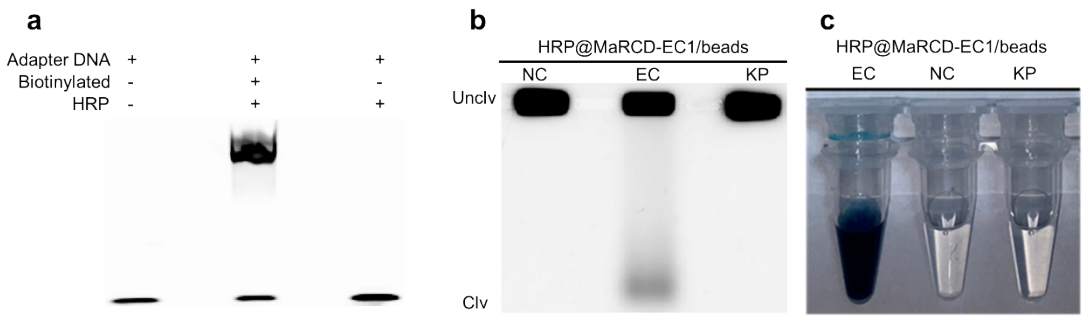


Figure S3. (a) Analysis of adapter DNA-HRP conjugation mixtures using SDS-PAGE. (b) HRP@ MaRCD-EC1/beads treated with RB, CIM-EC and CIM-KP, respectively. Clv: cleavage, Unclv: uncleaved, NC = negative control, EC = *E. coli*, KP = *K. pneumoniae.* (c) Colourimetric assay of supernatant from HRP@ MaRCD-EC/beads incubated with buffer, *E. coli* and *K. pneumoniae.*

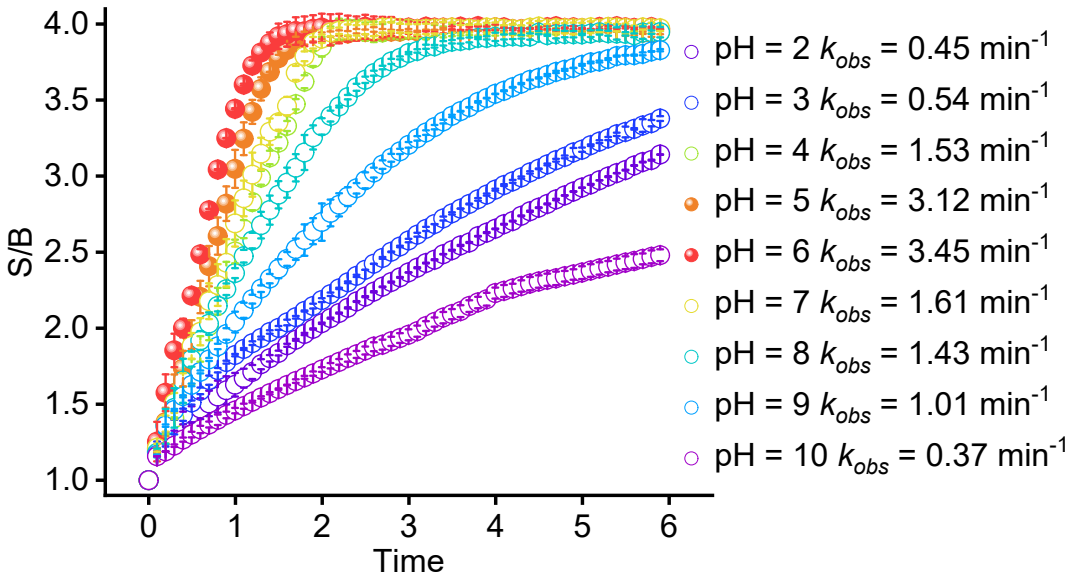


Figure S4. Kinetic of native HRP in response to TMB under various pH conditions. S/B is the signal to background ratio.

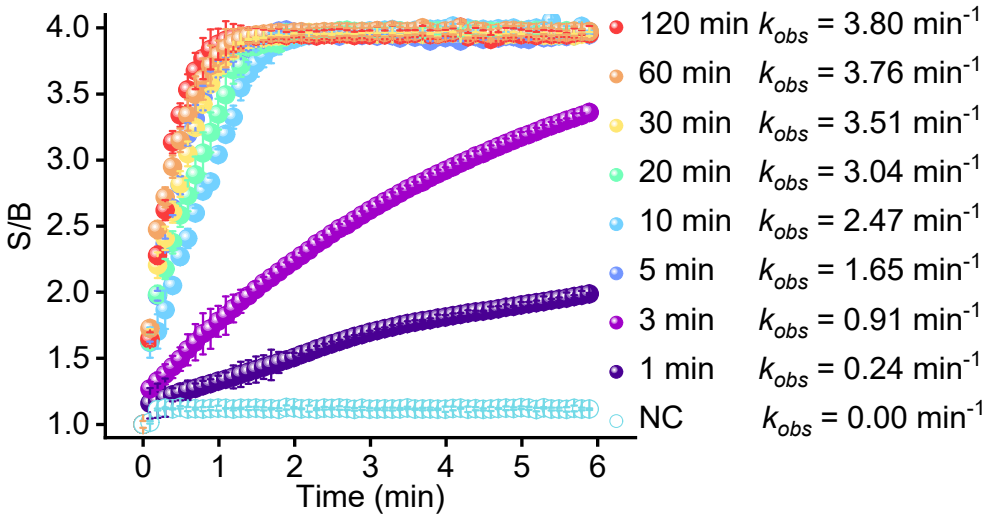


Figure S5. Kinetic of the released HRP from HRP@MaRCD-EC1/beads incubated with CIM-EC at various reaction time.

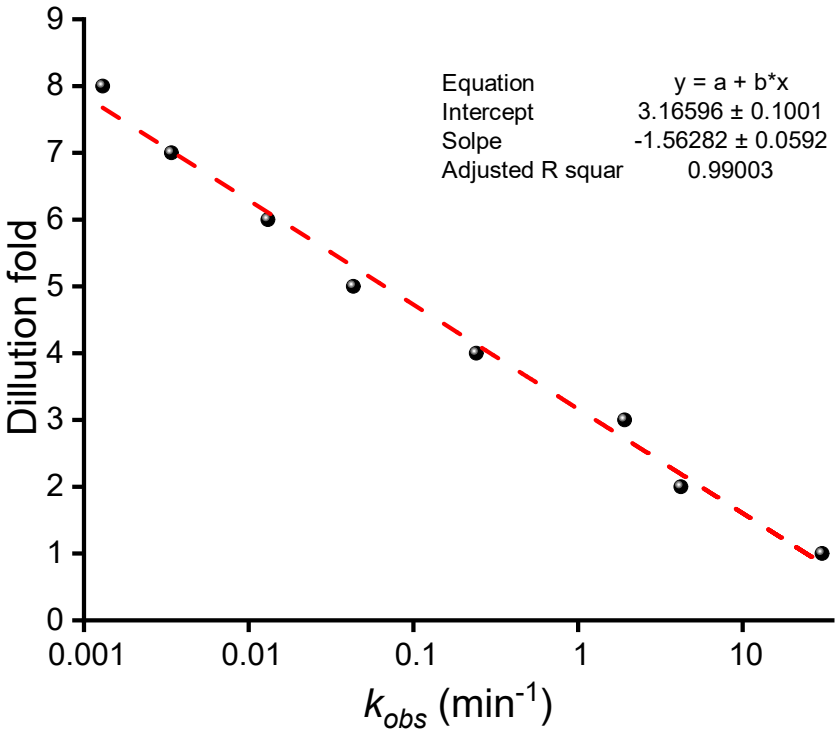


Figure S6. Standard curve of the k_{obs} to the different dilution fold of the HRP concentration.

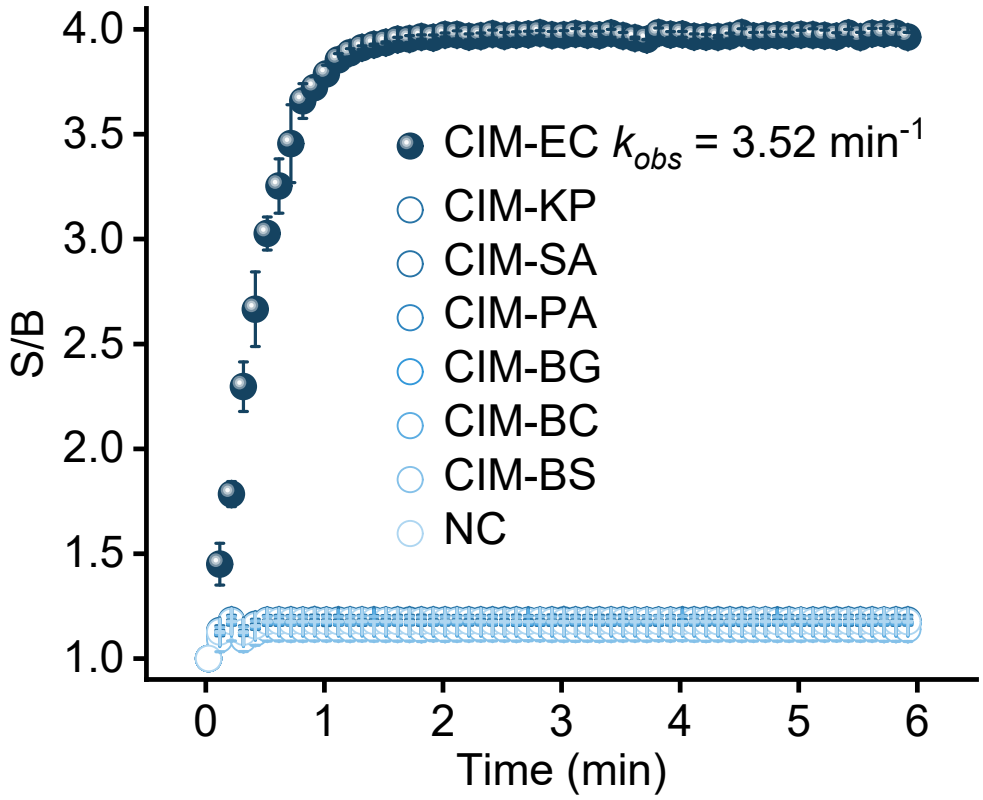


Figure S7. Kinetic of the released HRP from HRP@ MaRCD-EC1/beads incubated with CIMs prepared from different bacteria.

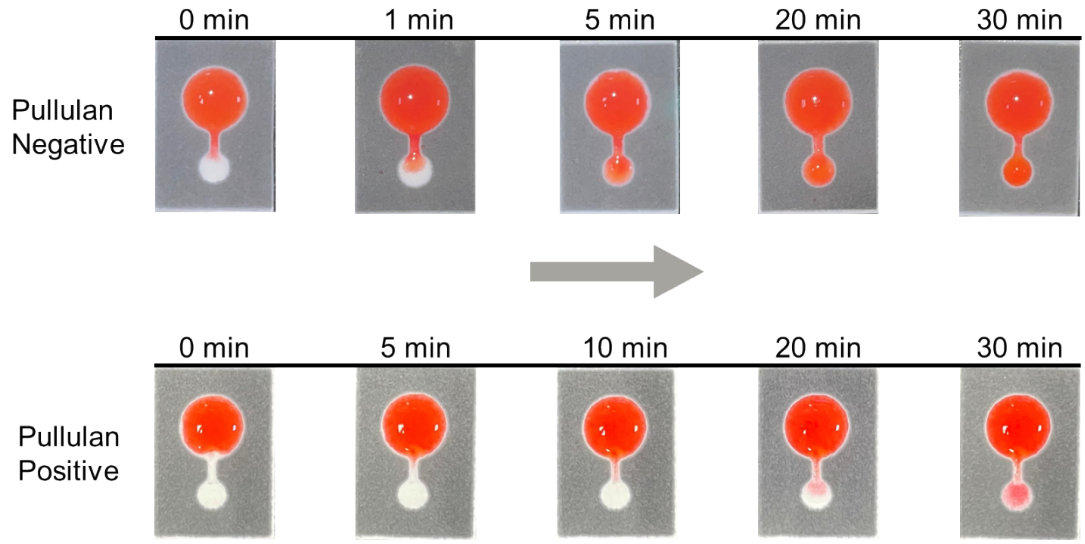


Figure S8. Flow test of the paper-based device with and without pullulan film regent after different time. The red color solution is prepared by Congo Red.

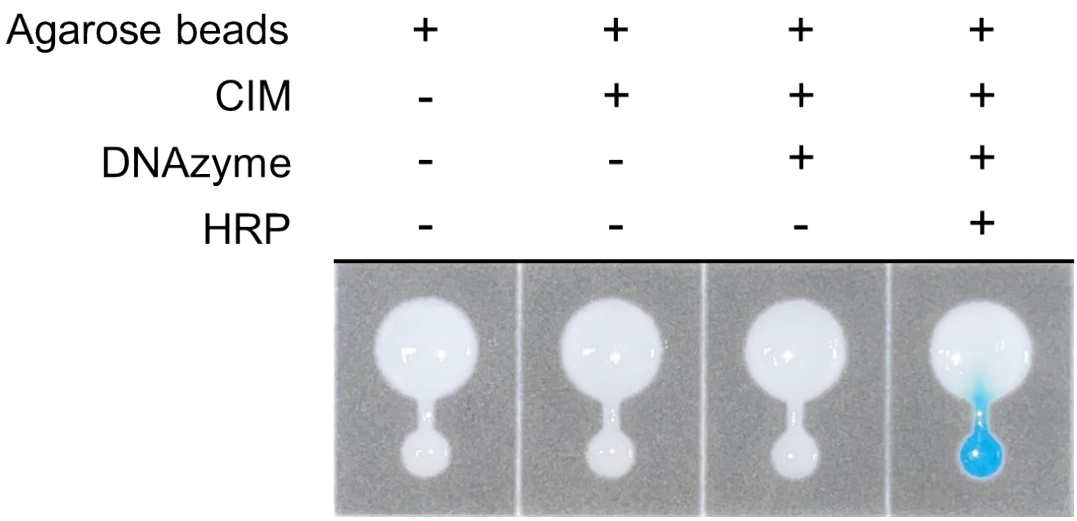


Figure S9. Images of the paper-based device with the addition of different regents onto Z1 zone after 30-min reaction.

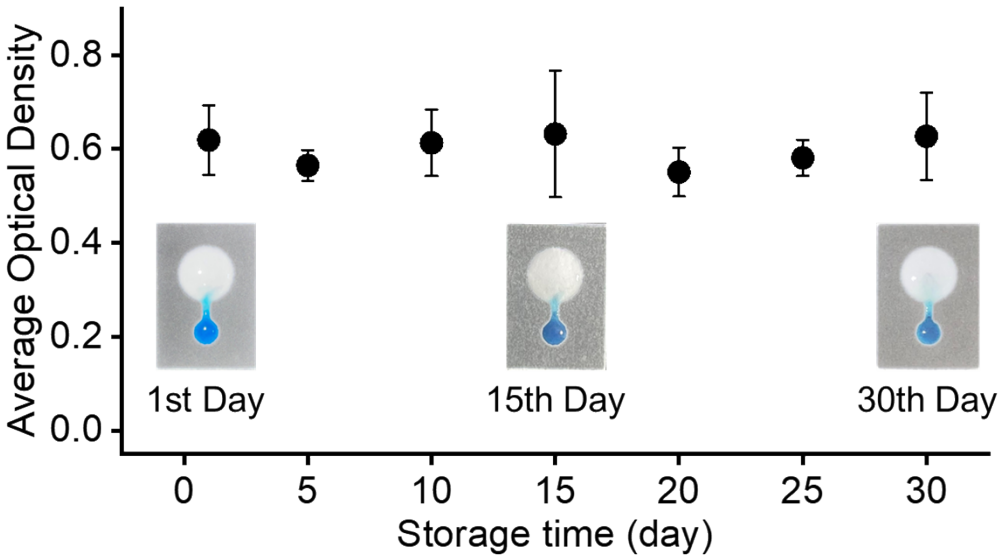


Figure S10. S/B values of the paper devices with the addition of HRP on the Z3 zones after different storage days. Inset: Images of the paper devices after storage of 1, 15 and 30 days indicated in the figure. The error bars represent the standard deviations from experiments that were carried out in triplicate.

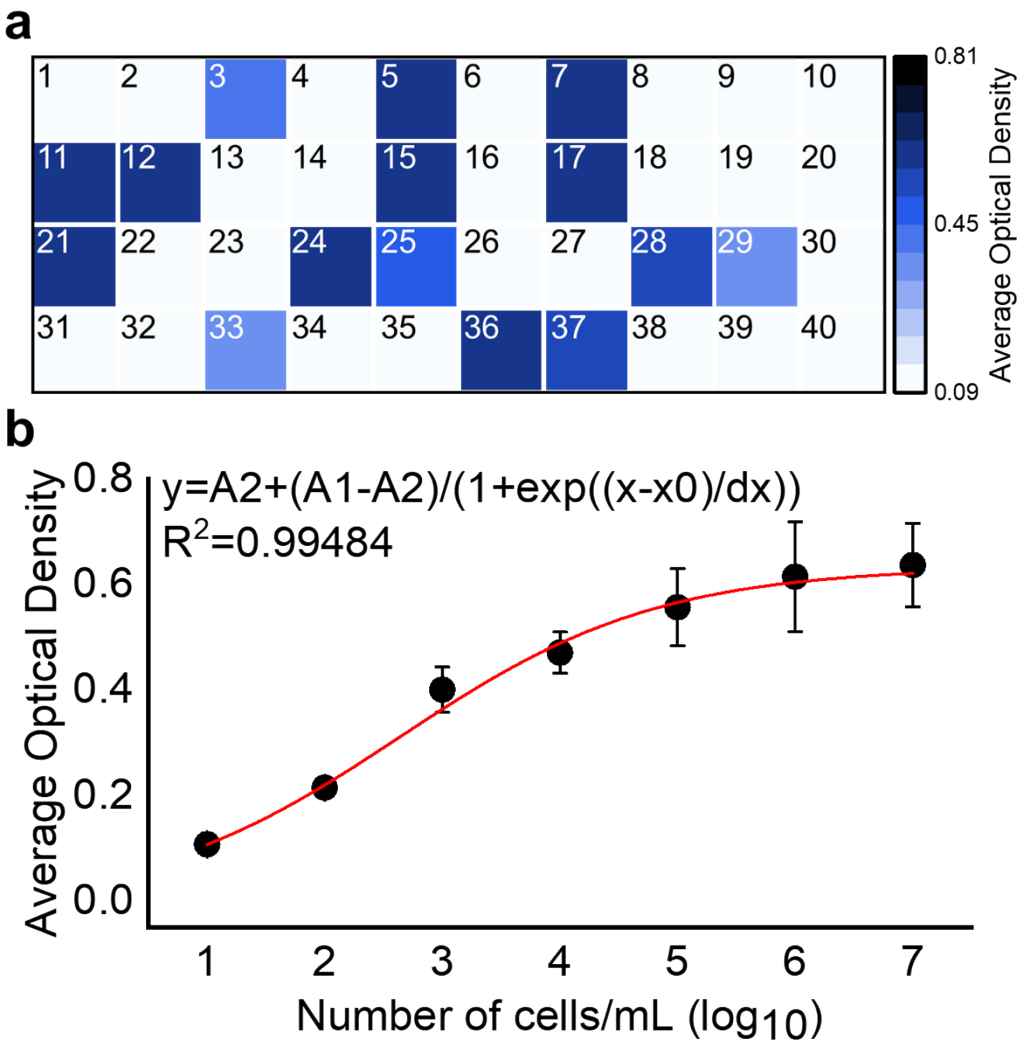


Figure S11. (a) Heat map of the average optical densityof paper-based devices. **(b)** Standard curve of the number of EC cells to the average optical density obtianed from the paper-based assay. The curve-fitting formulation is shown in the figure, where A1= -0.01033, A2= 0.63292, x0= 2.63932, dx= 1.09638.

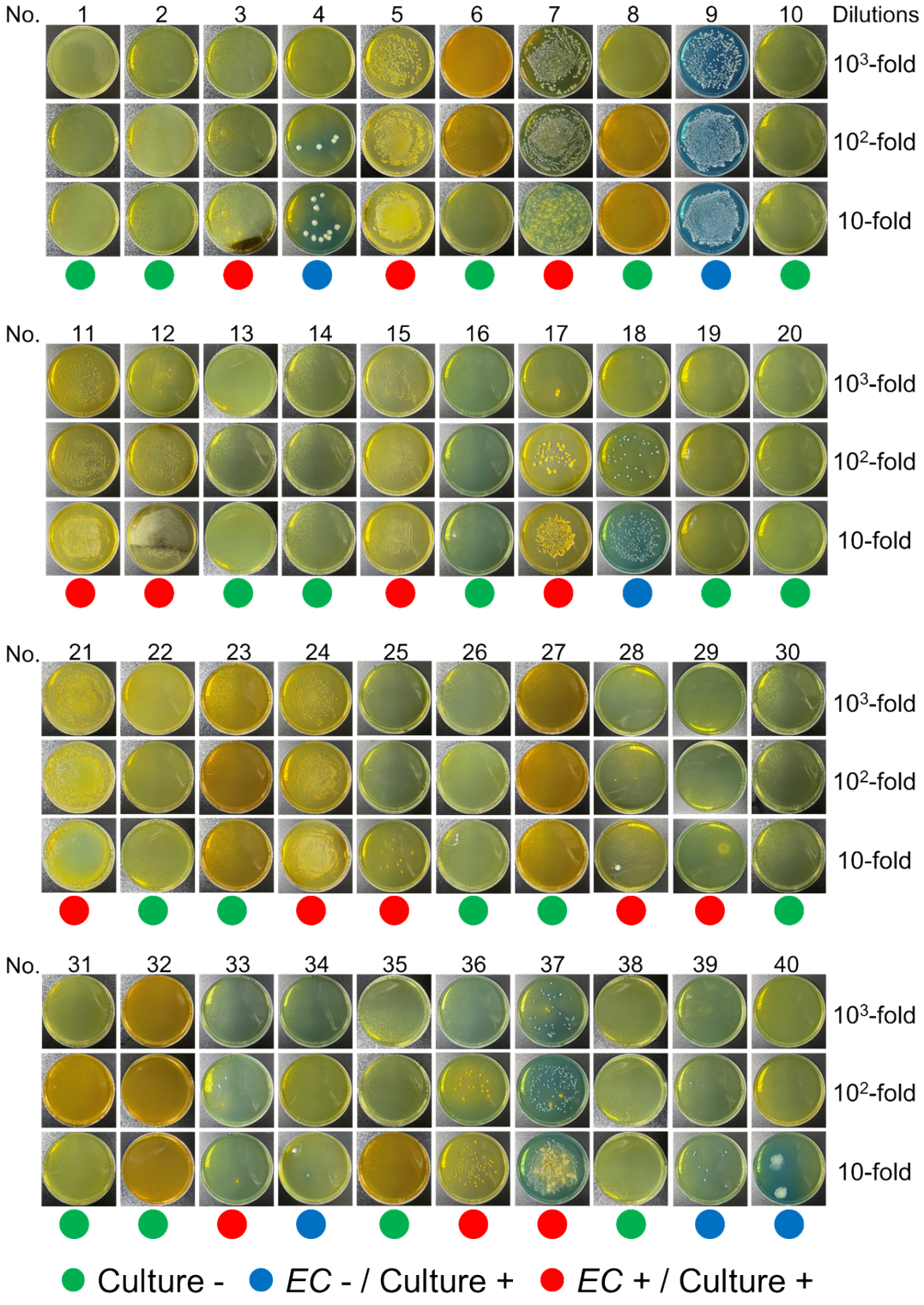
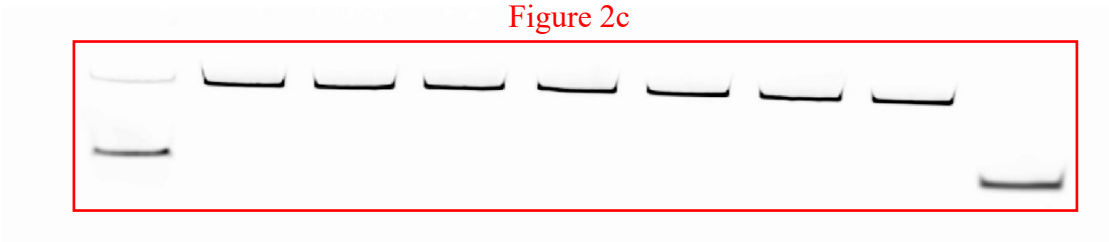


Figure S12. Images of CLED agar bacterial urine culture plates after adding different urine samples at 37°C for 15 h.

Uncropped gel images

Figure 2c



Uncropped dPAGE gel for Figure 2c. The red box indicates the region presented in Figure 2c

Figure 2d

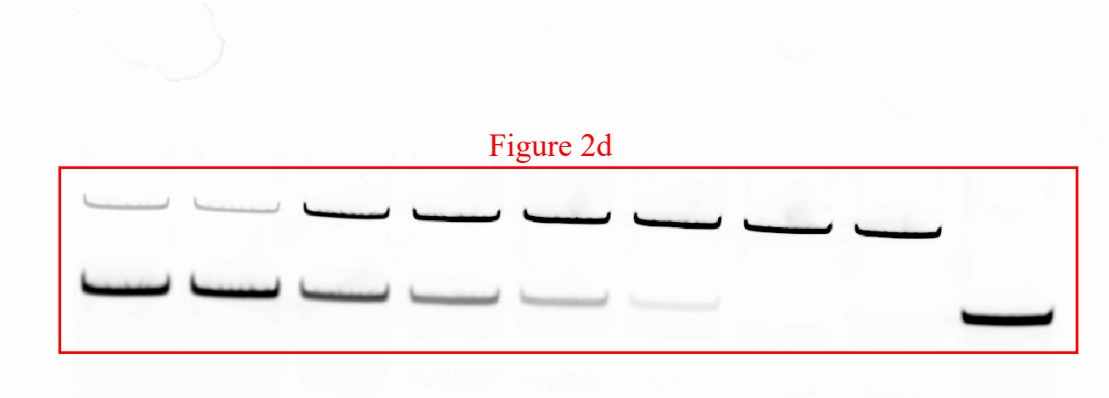


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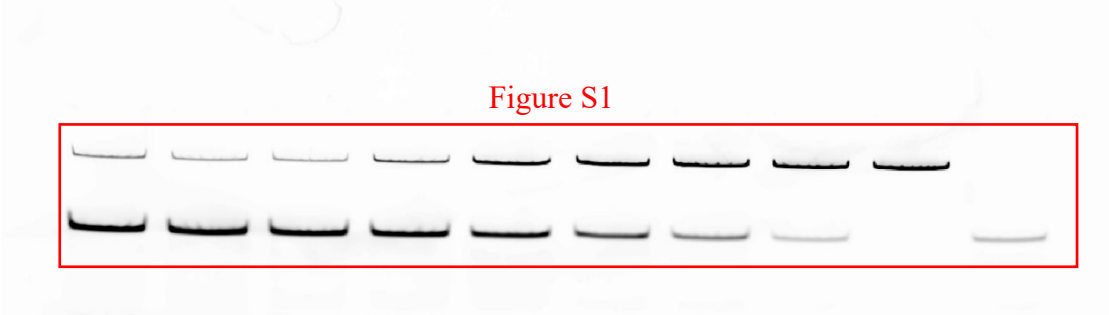
Figure S1



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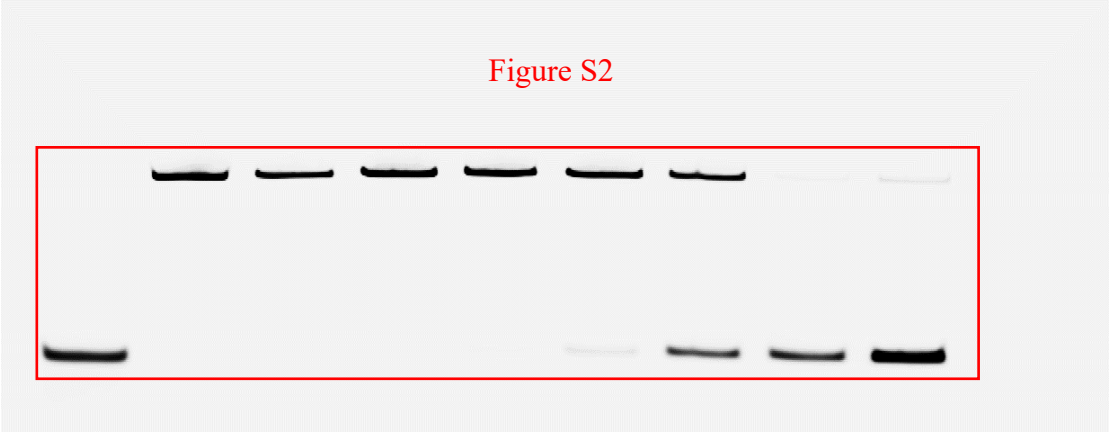
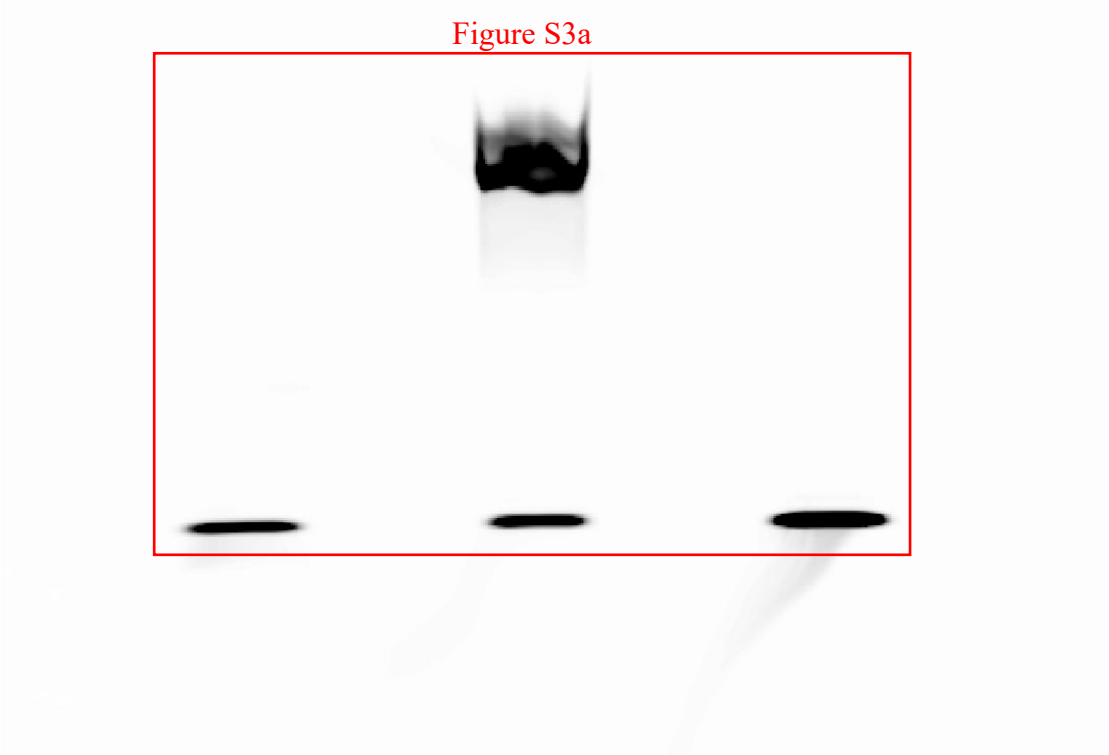
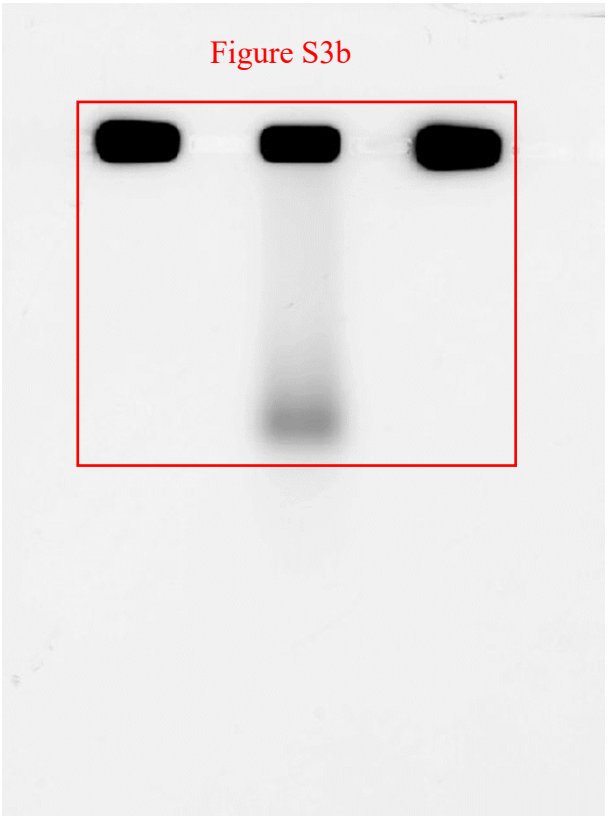


Uncropped dPAGE gel for Figure S2. The red box indicates the region presented in Figure S2.



Uncropped dPAGE gel for Figure S3a. The red box indicates the region presented in Figure S3a.



Uncropped dPAGE gel for Figure S3b. The red box indicates the region presented in Figure S3b.