Supporting Information

Combinatorial Screening SlipChip for Rapid Phenotypic Antimicrobial Susceptibility Testing

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Supplementary Materials and Methods

1. Materials

Sodium hydroxide, perchloric acid, ethanol, chloroform, acetone, hydrogen peroxide, and sulfuric acid were purchased from SinoPharm Chemical Reagent Co., Ltd. (Beijing, China). Hydrofluoric acid, dichlorodimethylsilane and PMX-200 silicone fluid were purchased from Aladdin Co., Ltd. (Shanghai, China). Mineral oil was purchased from Innochem Technology Co., Ltd. (Beijing, China). Spectrum food colors were ordered from August Thomsen Corp (Glen Cove, NY). LB broth was purchased from Sangon Biotech (Shanghai, China). Wild type *Escherichia coli* (*E. coli* ATCC 25922) and all clinical isolates were provided by Shanghai Public Health Clinical Center.

2. Fabrication of the cs-SlipChip for phenotypic AST

The fabrication of cs-SlipChip were conducted as described in the previous study[1]. First, we designed the photomask using CAD software (Autodesk, San Rafael, CA, USA) and printed it onto a film (Shenzhen Microcad Photo-mask Ltd, China). The cs-SlipChip has a width of 5.5 cm and a height of 3.5 cm. The cs-SlipChip was composed of two glass plate layers. The diameter of top-layer microwell is 500 μm, and the microwells are connected with the channel that were 100 μm in

width. In the bottom layer, the microwells are 400 μm in diameter (Figure S1). For the bottom plate, the soda-lime glass plate coated with chromium and photoresist was aligned with the photomask with straight expansion channels and then exposed to UV light in a UV flood curing system for 15 s. Then, the exposed glass plate was immersed in 1 M NaOH solution for 2 minutes to remove the reacted photoresist. Next, the glass plate was submerged in a chromium etchant solution to erase the chromium layer. Glass etching was conducted at 45 °C in a shaking water bath (50 rpm). The exposed glass was etched to 30 μm in depth. A two-step etching method was used to fabricate the top plate. First, the soda-lime glass plate was aligned to the photomask with the "pearls" shape and exposed to UV light for 15 s. Then, the NaOH solution and chromium etchant solution were used sequentially to remove the reacted photoresist and exposed chromium. After thoroughly rinsing the glass with water and drying in air, the glass was aligned to the second film photomask with a "chain" shape and exposed to UV light for another 15 s. Then, the NaOH solution was used to remove the reacted photoresist. The glass plate was then immersed in the etchant solution. The exposed glass with a "pearl" shape was etched to a depth of 20 μm. Then, the chromium etchant solution was used to remove the exposed chromium on the glass with a "chain" shape. Finally, the "chain-of-pearls" was etched to a 50-μm depth. A profilometer (Bruker, Billerica, MA) was used to measure the microwell depths during the etching process. After etching, the two glass plates were rinsed with water and placed in EtOH for 1 min to remove the remaining photoresist. Then, the glass plates were rinsed with water and submerged in chromium etchant solution to remove the remaining chromium layer. Prior to use, the surface of cs-SlipChips was silanized with dimethyldichlorosilane by gas phase salinization for 1 h and then thoroughly cleaned with chloroform, acetone, and ethanol.

3. Design and operation of cs-SlipChip

This cs-SlipChip utilizes a slip-induced self-partitioning mechanism to generate arrays of nanoliter droplets which do not require accurate alignment of the top and bottom microfeatures compared to the traditional SlipChip. The microwell on the top plate was 500 μm in diameter and 50 μm in depth, and was connected by narrow fluidic loading channels. The channel was 100 μm in width and 20 μm in depth. On the bottom plate, we designed an array of 192 circular microwells that were 400 μm in diameter and 30 μm in depth (Figure S1A). A 200 nL solution with a defined concentration of antibiotics was loaded into the microwells on the bottom plate through a liquid dispensing

apparatus controlled by a programmable syringe pump (Harvard Apparatus, Holliston, MA). After the antibiotic droplets dried, the two plates were assembled with a thin layer of mineral oil in between. This organic phase between the two plates could reduce the physical resistance during the slipping operation and avoid the evaporation of the aqueous solution during incubation. The aqueous solution containing bacteria was loaded into the "chain-of-pearl" micro-channels by pipetting through the inlet. Then, by a manual slipping operation, the "chain-of-pearl" micro-channels on the top plate were in contact with the expansion microwells on the bottom plate, and the aqueous solution self-partitioned into droplets. The preloaded antibiotic in the expansion microwells was dissolved into the droplets.

4. Bacteria culture

Escherichia coli reference strain (ATCC 25922) and five clinical strains with AST reports were provided by Shanghai Public Health Clinical Center. To prepare the bacterial samples, a small number of cells were picked up from individual colonies on an agar plate using an inoculation loop and then suspended in a culture tube with 5 mL LB Broth. The tube was then placed in a shaking incubator at 200 rpm and cultured overnight (~12–18 h) at 37 °C. Next, a 10 μL sample from the overnight culture was added to 1 mL fresh medium and sub-cultured for 3 h. The sub-cultured sample was diluted to 5-10 × 10^5 CFU/mL.

5. Antimicrobial susceptibility testing on the cs-SlipChip

Ceftazidime was purchased from Ark Pharm, Inc. (Arlington Heights, USA). Ampicillin, ciprofloxacin and nitrofurantoin were purchased from Macklin (Shanghai, China). Stock solutions (10 mg/mL) of ampicillin (Amp), ciprofloxacin (Cip) and ceftazidime (Cef) were freshly prepared in sterile deionized water for the AST experiments. The stock solution (10 mg/mL) of nitrofurantoin (Nit) was freshly prepared in DMF (N, N-Dimethylformamide). A series of 2-fold antibiotic dilutions were prepared according to the MIC, and 200 nL of each dilution series was preloaded into the microwells and dried. After loading the bacterial solution, the device was slipped to overlay the microwells containing the captured bacteria on the top plate with the microwells on the bottom plate preloaded with antibiotics. The bacteria were cultivated at 37 °C and monitored every hour. Bright-field images were captured by an inverted fluorescence microscope (Eclipse Ti2, Nikon) equipped

with 4×, 10×, and 20× objectives. The microscope was focused near the bottom of the microwells to ensure most of the bacterial cells stay in focus during the analysis. The field of view of the microscope with 20 × objective is approximately 720 μm × 480 μm in actual imaging area, and it was selected around the middle of each compartment. The whole image was applied for AST analysis.

6. Assessing the effect of antibiotic combinations by broth microdilution in the 96-well plate

For the antibiotic combination experiments, antibiotics were diluted in lysogeny broth (LB) to the appropriate concentrations in 96-well plates, with each well containing 100 μl in total. Overnight cultures of clinical *E. coli* isolates were diluted to OD600 ~ 0.4 and mixed with the antibiotic combinations. The plates were incubated at 37 °C in the constant temperature rocker rotator (150 rpm). OD600 was measured by a SpectraMax i3x multimode microplate reader (Molecular Devices, San Jose, CA) at 1 h, 2 h, 3 h and 16 h. The combinations were defined as susceptible(S) when the OD600 dropped to 0.6 at 16 h. Each antibiotic combination contained three concentrations, and each concentration had three replicate wells. ACiCe: ampicillin, ciprofloxacin and ceftazidime; ACiN: ampicillin, ciprofloxacin and nitrofurantoin; ACeN: ampicillin, ceftazidime and nitrofurantoin; CiCeN: ciprofloxacin, ceftazidime and nitrofurantoin; ACiCeN: ampicillin, ciprofloxacin, ceftazidime and nitrofurantoin. S: susceptible.

7. Statistical Analysis

ImageJ software was used to quantify the bacterial numbers in the microwells. Specifically, the images were converted to the 8-bit format, and the threshold was adjusted to capture all cells and analyze the particles. Python was used to analyze the bacterial length. The details of the image-analysis process are shown in Figure S4 of the supplementary information. All statistical analyses were performed using GraphPad Prism software V8.0 (La Jolla, CA, USA). Data are represented as mean ± SD.

Supplementary figures

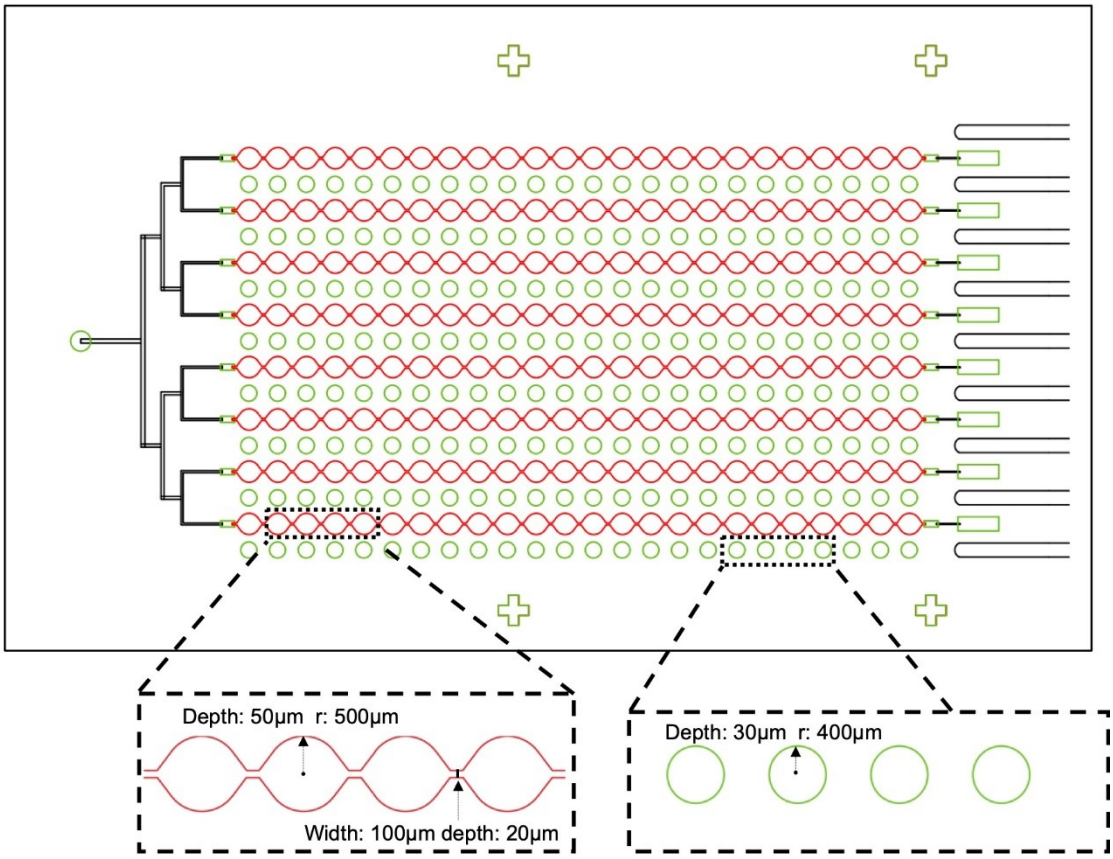

Figure S1. The design of cs-SlipChip. The dimensions of the cs-SlipChip are 55 mm × 35 mm. The microwell on the top plate was 500 μm in diameter and 50 μm in depth, and was connected by narrow fluidic loading channels. The channel was 100 μm in width and 20 μm in depth. On the bottom plate, we designed an array of 192 circular microwells that were 400 μm in diameter and 30 μm in depth.

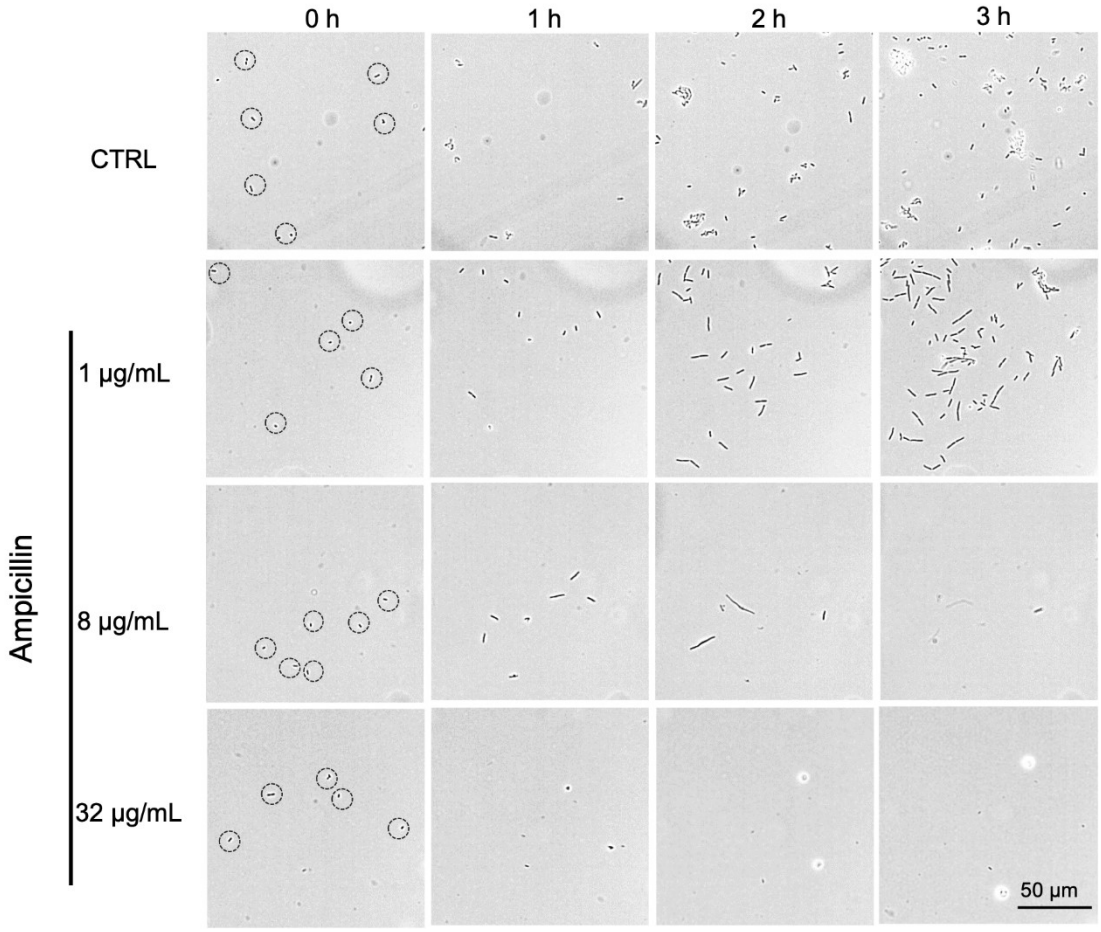

Figure S2. AST results of *E. coli* ATCC 25922 on cs-SlipChip with ampicillin**.** Representative 20 × magnification images of the *E. coli* treated with different concentration of ampicillin from the cs-SlipChip are shown (scale bar, 50 μm).

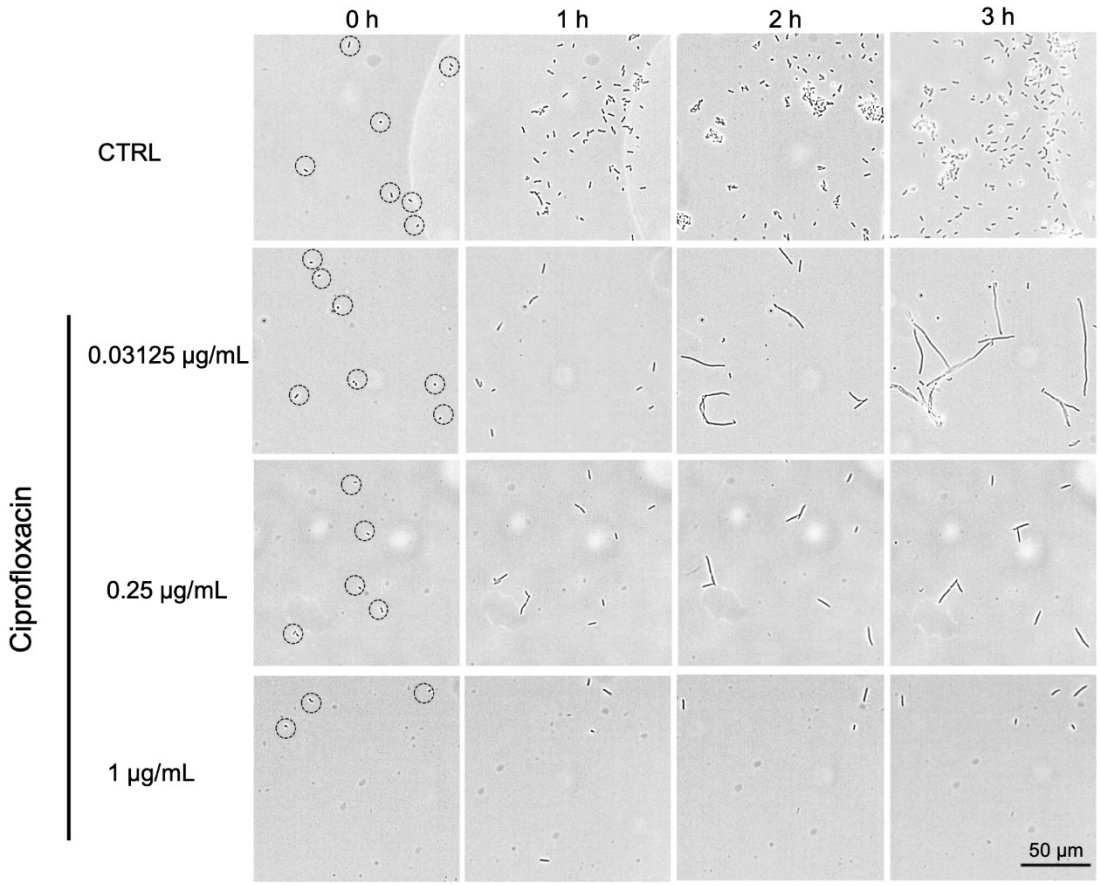

Figure S3. AST results of *E. coli* ATCC 25922 on cs-SlipChip with ciprofloxacin**.** Representative 20 × magnification images of the *E. coli* treated with different concentration of ciprofloxacin from the cs-SlipChip are shown (scale bar, 50 μm).

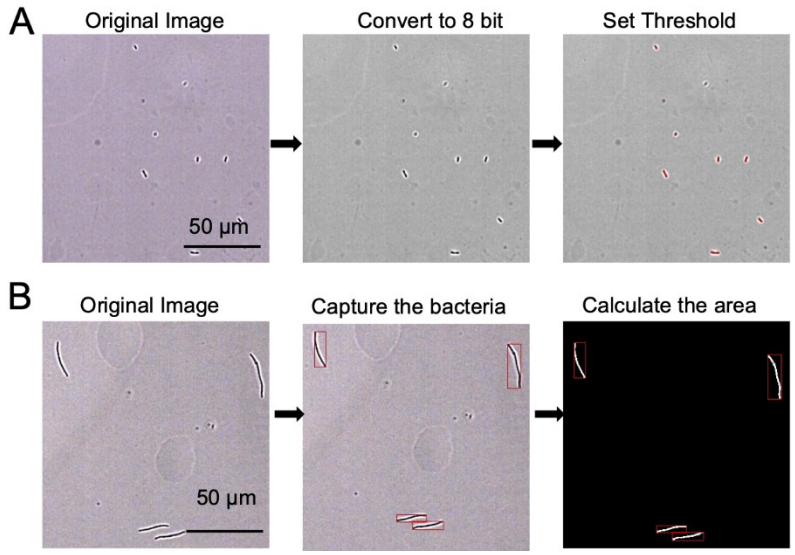

Figure S4. Automated image processing of bacteria. (A) Data were analyzed with the Fiji software (NIH). Images represent output at each step. Briefly, to obtain bacterial number from the raw CCD images, several steps of image processing were used. First, we convert the original image from RGB to 8-bit, then we set the threshold to exclude the material smaller than bacterial cells and capture all the bacteria. Next, we analyze particles captured in the previous step to get the absolute number of the bacteria. (B) The data were analyzed with python and OpenCV package. Images represent output at each step. First the image was read and convert it to binary image. Next, the bacteria were captured and labeled. By setting the filter, the total number of non-zero pixels occupied by a single bacterium was calculated.

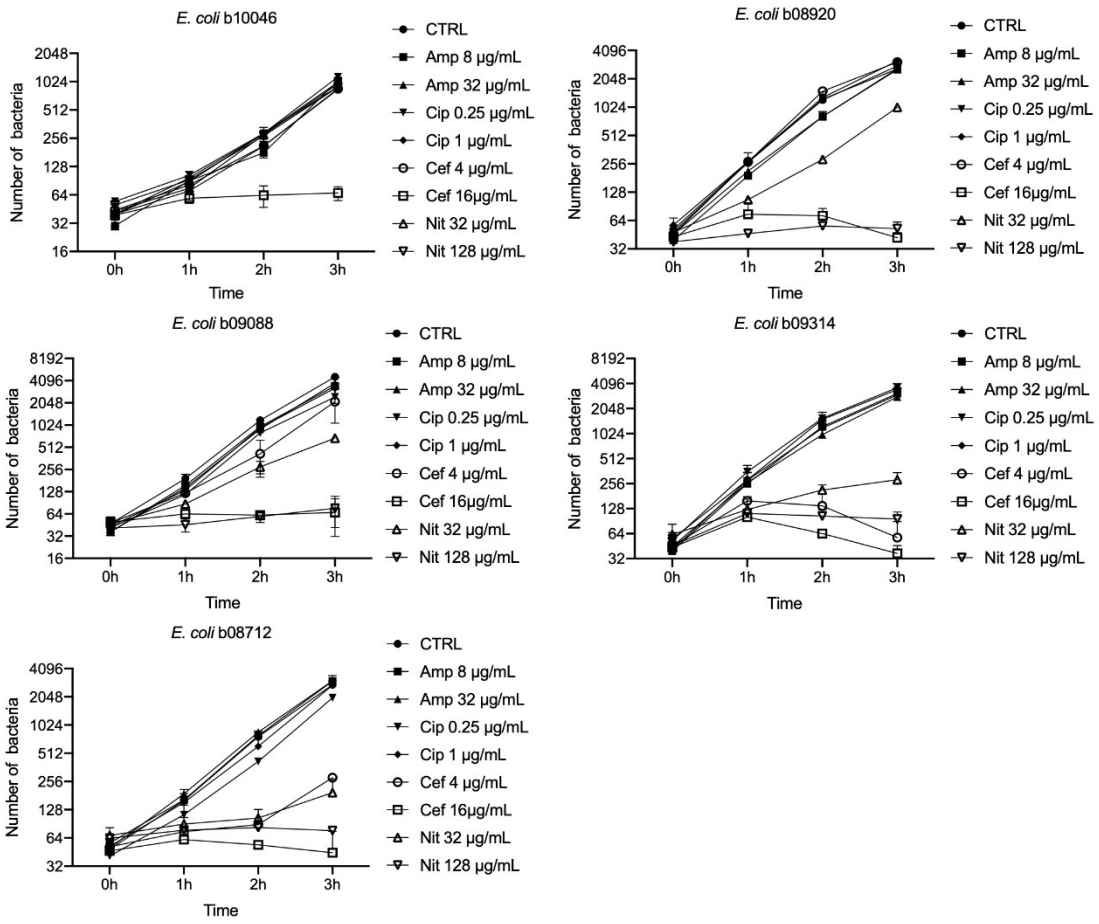

Figure S5. Analysis of the bacterial numbers of five clinical isolates treated with single antibiotic (in Figure 4), including ampicillin (Amp), ciprofloxacin (Cip), ceftazidime (Cef) and nitrofurantoin (Nit) in two concentrations, S (equal to MIC) and R (fourfold of MIC). Each dot represents the mean of the bacterial absolute number in four replicated microwells. Error bars represent standard deviation. (n=4)

Table S1. The AST results of *E. coli* ATCC 25922reported by the cs-SlipChip compared to the CLSI QC range.

Antibiotics	CLSI QC range (μg/mL)	cs-SlipChip (μg/mL)
Ampicillin	2 – 8	2
Ciprofloxacin	0.004 – 0.016	0.004
Ceftazidime	0.06 – 0.5	0.5
Nitrofurantoin	4 – 16	4

Table S2. The concentration of antibiotics in the panel used in Figure 4. (μg/mL)

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20	21 22	23 24
A B	CTRL	CTRL	CTRL	Nit16	Nit32	Nit128	Cip 0.125 Cef 2	Cip 0.25 Cef 4	Cip 1 Cef 16	Amp 4 Cip 0.125 Nit 16	Amp 8 Cip 0.25 Nit 32	Amp 32 Cip 1 Nit 128
C D	Amp 4	Amp 8	Amp 32	Amp 4 Cip 0.125	Amp 8 Cip 0.25	Amp 32 Cip 1	Cip 0.125 Nit 16	Cip 0.25 Nit 32	Cip 1 Nit 128	Amp 4 Cef 2 Nit 16	Amp 8 Cef 4 Nit 32	Amp 32 Cef 16 Nit 128
E F	Cip 0.125	Cip 0.25	Cip 1	Amp 4 Cef 2	Amp 8 Cef 4	Amp 32 Cef 16	Cef 2 Nit 16	Cef 4 Nit 32	Cef 16 Nit 128	Cip 0.125 Cef 2 Nit 16	Cip 0.25 Cef 4 Nit 32	Cip 1 Cef 16 Nit 128
G H	Cef 2	Cef 4	Cef 16	Amp 4 Nit 16	Amp 8 Nit 32	Amp 32 Nit 128	Amp 4 Cip 0.125 Cef 2	Amp 8 Cip 0.25 Cef 4	Amp 32 Cip 1 Cef 16	Amp 4 Cip 0.125 Cef 2 Nit 16	Amp 8 Cip 0.25 Cef 4 Nit 32	Amp 32 Cip 1 Cef 16 Nit 128

Table S3. The effect of antibiotics combinations on *E. coli* b09088 and b08712 assessed by broth microdilution in 96-well plate

Bacteria	ACiCe	ACiN	ACeN	CiCeN	ACiCeN
E. coli b09088	S	S	S	S	S
E. coli b08712	S	S	S	S	S

Reference:

1. Z. Yu, W. Lyu, M. Yu, Q. Wang, H. Qu, R. F. Ismagilov, X. Han, D. Lai and F. Shen, *Biosens Bioelectron*, 2020, **155**, 112107.