Supplementary information

Directly single-cell antimicrobial susceptibility testing of *Escherichia coli* in urine using a ready-to-use 3D microwell array chip

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Chip fabrication

In brief, a SU-8 3010 (KAYAKU, Westborough, USA) layer (~20 μm) was firstly patterned to make channel structures. Next, the second layer of SU-8 3010 (~20 μm) was coated onto the channel layer and exposed to ultraviolet (UV) after mask alignment to form the permanent microwell array. The mold covered by 5:1 PDMS (RTV-615, Momentive) was vacuumed for 15 min. Following that, the wafer was spun at 1500 rpm for 20 s and put in an 80 °C oven for 3 min. 44 g degassed 10:1 PDMS was cast on the first PDMS layer and polymerized at 80 °C for 30 min. Then, the PDMS replicas were peeled off and punched by a 1 mm puncher for the inlets and outlets. Subsequently, these two layers were put face to face and aligned manually under a stereomicroscope (Olympus, 4 × lens, Japan), during which DI water could be optionally sprayed on the surface as lubricant to facilitate the alignment. The combinations were dried at 55°C overnight followed by UV exposure for 2 h after the removal of the top glass. Afterward, the assembled devices were baked at 80 °C for over 10 h to enhance the bonding strength. The fabrication procedure of ultra-thin chip was described in supplementary information.

For fabricating the ultra-thin PDMS chip, 5g PDMS precursor with 1g cross-linker (RTV-615, Momentive) was fully mixed, poured onto the mold and vacuumed for 15 min to remove air bubbles. The wafer was spun at 1500 rpm for 20 s and put in an 80 °C oven for 3 min to make PDMS harden. Then, 10:1 PDMS was coated on the first PDMS layer at 300 rpm and polymerized at 80 °C for 30 min. Oxygen plasma was used for bonding the PDMS with glass slides (~500 μm) with/without holes (1.0 mm in diameter). To facilitate the releasing of glass bonded PDMS sheets, the PDMS film was firstly cut into pieces along the glass edge and then covered by methanol for 30 min. PDMS film was punched for inlet and outlets after detaching from the mold. Subsequently, these two layers were put face to face and aligned manually under a stereomicroscope (Olympus,4 × lens), during which DI water could be optionally sprayed on the patterns as lubricant to facilitate the alignment. The combinations were dried at 55°C overnight and exposed to UV for 2 h. Afterward, the assembled devices were baked at 80 °C for over 10 h to enhance the bonding strength.

Chip operation

The chip was firstly degassed in a vacuum microwell for 50 min to produce negative pressure in PDMS. Before degassing, it was assembled with two pieces of PDMS (PDMS batteries) and covered by optic tape to keep the negative pressure. For the experiments not involving lyophilized reagents, 10 μL test solution composed of *E. coli*, 0.07 mM resazurin, 1× LB Broth and antibiotics was pipetted into a tip and covered by thermosetting oil. Following with sticking the cover tap with a needle, the tip was inserted into the inlet. When the oil phase entered into the outlets, the PDMS batteries and cover tape were removed. The inlet and outlets were sealed with 10:1 PDMS. An optional procedure, keeping the chip in air for ~15 min, helps to release the negative pressure in PDMS and reduce the water evaporation of peripheral microwells. Finally, the chip was enclosed by a coverslip incubated at 42 °C in a miniature incubator.

Bacteria culture and sample preparation

Escherichia coli O114 (ATCC 25922), *Escherichia coli* K-12 (ATCC 19020) and *Escherichia coli* EK-19 (isolated from soil) were inoculated in sterile Luria Broth (LB, L3522, Sigma) and incubated at 37 °C overnight with a shaking speed of 200 rpm. If not mentioned, all the bacterial samples used in this work were harvested after overnight cultivation. The logarithmic phase *E. coli* EK-19 was obtained at the OD value of 0.15. To prepare the test sample, 1 mL *E. coli* suspension was centrifuged at 1540 RCF for 15

min and resuspended in 1 mL PBS. After diluting the stock sample with PBS, the *E. coli* samples were ready for usage.

Test solutions and optimizing test conditions in 96-well plate

The concentration of mediums, LB, Tryptic Soy Broth (T8907, Sigma), Tryptic Soy Broth without dextrose (T3938, Sigma) and Hi-Def Azure Media with 1% Glucose (3H5000, Teknova) were optimized. The optimization of resazurin (0.01-0.2 mM) in the test solution and the incubation temperature (35-42°C) were also conducted. For the optimization in 96-well plates, each 320 μL test solution consisted of 6.4 μL *E. coli* sample (~8×10^5 CFU/mL), 11.2 μL resazurin and 302.4 μL medium and were aliquoted into 3 parallel wells with 100 μL per well. To confirm the AST, 32 μL ampicillin (Roche) or PMB (P1004, Sigma) with different concentrations were added to the above solutions.

The determination of *E. coli* concentration according to Poisson distribution

Similar to digital PCR, the quantification of *E. coli* depends on the random distribution of single bacterium in the microwell array. It has been demonstrated that the bacteria were randomly encapsuled in microwells. The number of cells in each microwells fitted Poisson distribution. Therefore, the number of targets can be calculated by Poisson distribution:

$$P(n,\lambda) = (\lambda^n \cdot e^{-\lambda})/n! \quad (1)$$

Here, n denotes the bacteria number in a microwell (0, 1, 2, 3…) and λ is the average number of bacteria per microwell (total number of *E. coli* in chip / the number of microwells). *P* is the probability that the microwell contains n cells. The stochastic distribution of targets gives the microwell two conditions, with cells and without cells. When a microwell shows bright fluorescence after incubation, there is at least 1 cell in it and the probability is

$$P(n>0) = 1 - P(n=0) = 1 - e^{-\lambda} \quad (2)$$

And, the probability also equals the proportion of positive microwells in the chip, then

$$P(n>0) = 1 - e^{-\lambda} = a/b \quad (3)$$

In which *a* and *b* are the number of positive microwells and the total amount of microwells, respectively. After incubation, the counting of positive is a known parameter. Therefore, the λ can be calculated as follow:

$$\lambda = -ln(1 - a/b) \quad (4)$$

As indicated above, λ equals the number of *E. coli* in chip divided by the number of microwells. The cell number in chip is unknown and defined as *X*. The formula 4 can be rewritten as

$$X/b = -ln(1 - a/b) \quad (5)$$

Or

$$X = ln(1 - a/b) \cdot (-b) \quad (6)$$

In this work, the total number of microwells per chip is 22660.

So, the relation between cell number (X) and the number of positive microwell (a) is as followed:

$$X = ln(1 - a/22660) \cdot (-22660) \quad (7)$$

The volume of the microwell array is 2.55 μL. Eventually, the concentration of the *E. coli* in test solution can be calculated.

The calculation of threshold for MIC determination

Due to the random distribution of single cells in 3D-MAC, there are some microwells containing multiple cells. Our result (Figure S14) and the previous study indicate that the inhibition efficiency of antibiotic is determined by the amount of antibiotic used per cell rather than the antibiotic concentration.[1] Therefore, the data of microwells with multiple cells may result in incorrected MIC. In this work, we use

cell viability, which is defined by the number of positive microwells (containing 1 or more than 1 *E. coli*) on the chip with antibiotics divided by the number of positive microwells on control chip, to indicate cell response to antibiotics. A threshold is set as the ratio of the number of microwells with more than 1 cell to the number of cell-containing microwells to exclude the effect of the microwells with multiple cells on MIC determination. When the cell viability is below than the threshold, all single *E. coli* are inhibited by antibiotics. Prior to calculating the threshold, the number of cell-containing microwells and the number of microwells with multiple cells must be figured out. The number of cell-containing microwells on the chip with antibiotics should be same as that of the control chip and equals to the number of positive microwells on the control chip. The number of microwells with multiple cells depends on the number of cells loaded and can be calculated via Poission distribution.

Firstly, the amount of loaded cells (X) is calculated by Equation (7) with the input of the number of positive microwells on control chip (a). The average number of bacteria per microwell (λ) is:

$$\lambda = X/22660 \tag{8}$$

According to Equation (1), the probabilities that the microwell contains 0 and 1 cells are:

$$P(n=0) = e^{-\lambda} \text{ and } P(n=1) = \lambda \cdot e^{-\lambda} \tag{9}$$

respectively.

Therefore, the probability that the microwell contains more than 1 cell is:

$$P(n>1) = 1 - P(n=0) - P(n=1) = 1 - e^{-\lambda} - \lambda \cdot e^{-\lambda} \tag{10}$$

The numbers of microwells with multiple cells (M) can be calculated as:

$$M = 22660 \cdot (P\ (n>1)) = 22660 \cdot (1 - e^{-\lambda} - \lambda \cdot e^{-\lambda}) \tag{11}$$

Finally, the threshold (T) is defined as follows:

$$T = 100\% \times M/a \tag{12}$$

The lowest antibiotic concentrations at which the cell viability is less than the threshold is defined as MICs. The susceptibility of bacteria is then categorized with measured MICs according to CLSI guideline.

Supplementary results

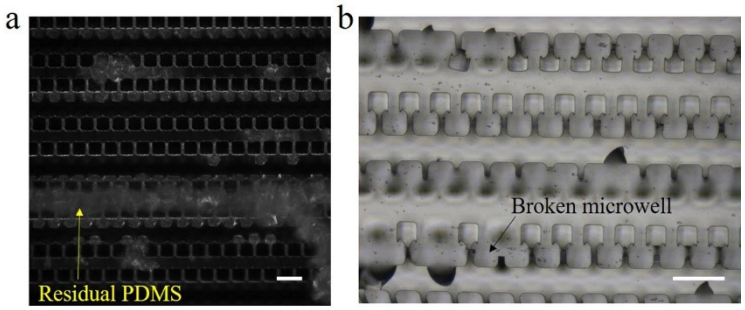

Figure S1. The broken PDMS chip of 2D microwell array with 20 μm interval. a) The SU-8 mold with residual PDMS. b) The broken microwells in the PDMS chip. When the interval between microwells decreases to 20 μm, it is a challenge to totally remove the unsolidified photoresist. More importantly, the 20 μm PDMS wall cannot resist the frictional drag during peeling off the PDMS elastomer, leading to microwell crushing. The scale bar denotes 100 μm.

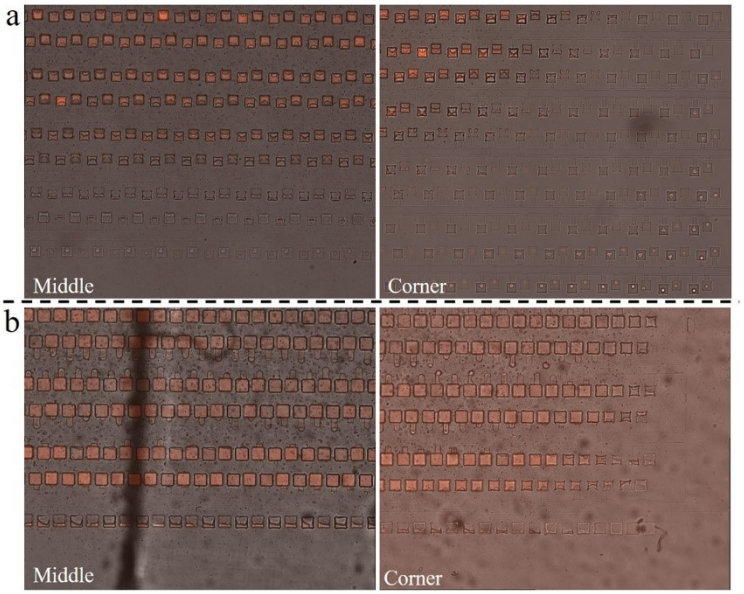

Figure S2. The anti-evaporation performance of the ultra-thin chip. a) Water loss of the peripheric microwells in normal chip after 3 h incubation at 42℃. Left: the middle of the microwell array; Right: the corner of the microwell array. b) Water loss of the peripheric microwells in ultra-thin chip after 3 h incubation at 42℃. Left: the middle of the microwell array; Right: the corner of the microwell array.

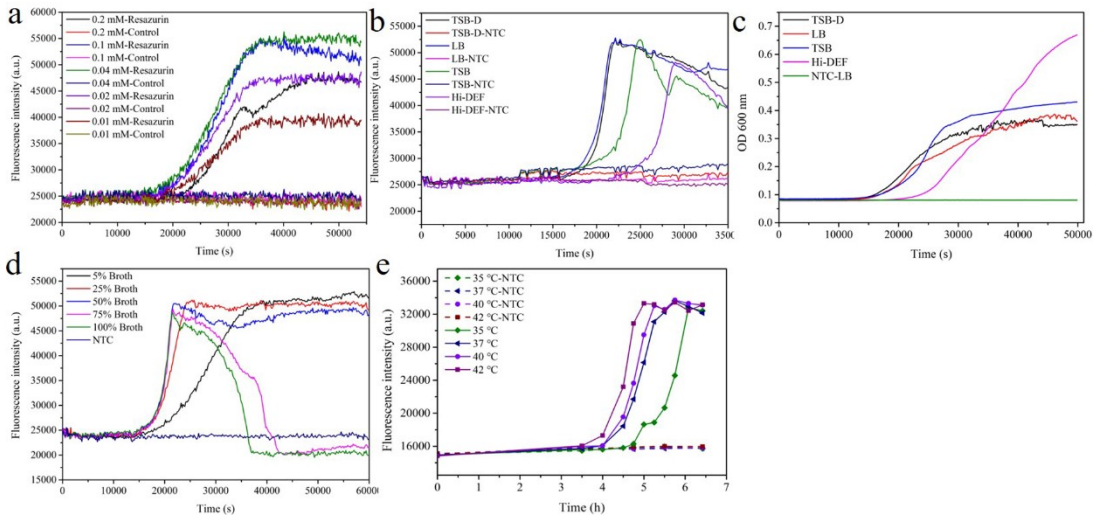

Figure S3. The optimization of resazurin (a), medium (b, c and d) and reaction temperature (e) for fast resazurin reaction in 96 well plate. The curves indicate the average of triplicate wells.

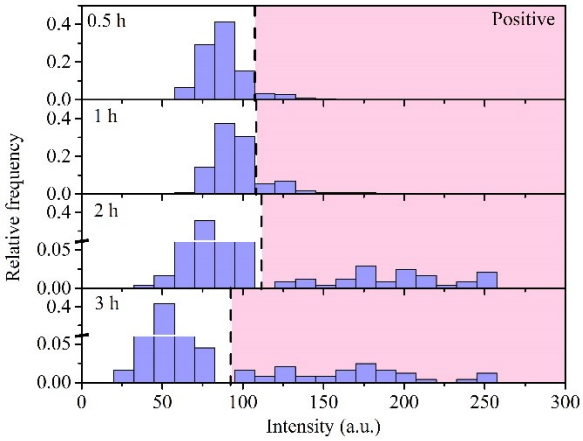

Figure S4. Distribution of the intensity of microwells over time.

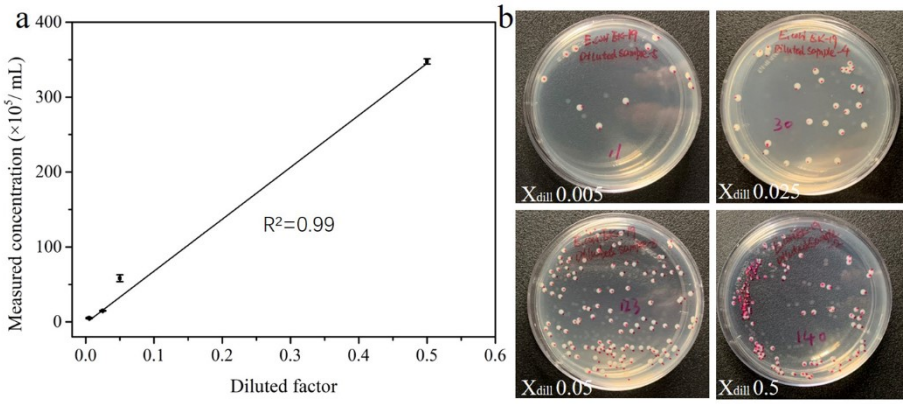

Figure S5. Enumerating the *E. coli* on agar plates. a) The correlation between the measured concentrations and diluted factors. b) The pictures of agar plates after overnight incubation. Before spreading, the samples with diluted factors of 0.05, 0.025 and 0.005 were 1000-fold diluted. The sample with diluted factors of 0.5 was 1000-fold diluted. Then, 20 μL diluted solutions were pipetted on an agar plate for spreading. Error bars represent standard deviation of three replications.

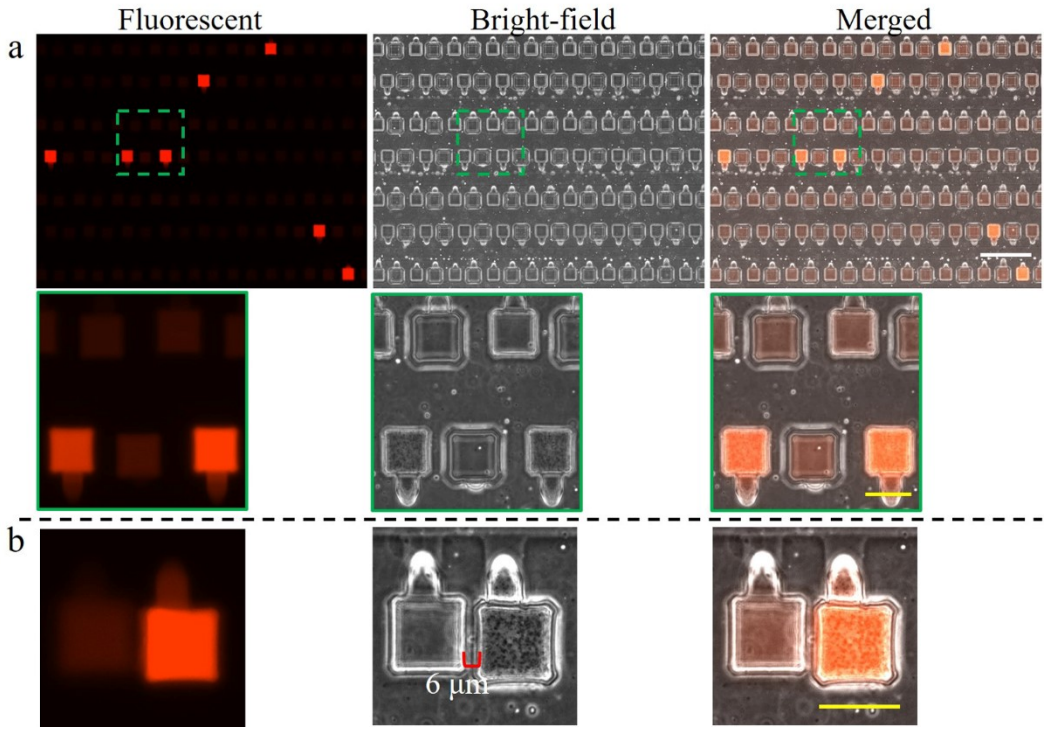

Figure S6. The confirmation of positive microwells. a) The correlation between bright fluorescence and *E. coli* colonies proliferated from single cells in microwells. b) The reliable chip bonding and microwell isolation. Scale bar: 200 μm (white) and 50 μm (yellow).

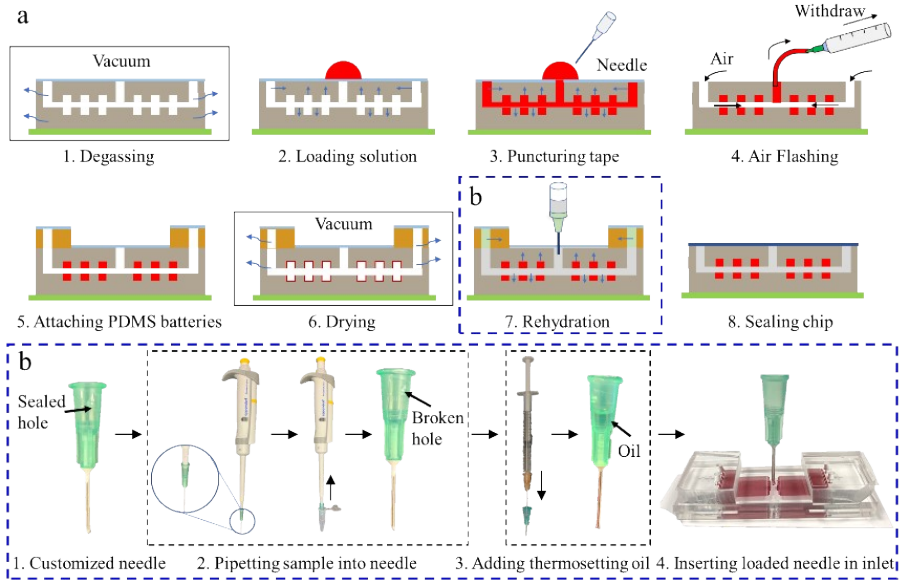

Figure S7. Flow chat of chip operation (a) and liquid transferring (b). A 20 G needle (OD 0.8 mm, ID 0.3 mm) was cut and drilled to make a hole on its adaptor. The hole was used for balancing the pressure when taking off the needle from the pipette. Before liquid transferring, the hole was sealed with a piece of tape. A 20 μL pipette was used for transferring 8 μL sample solution into the needle, which was stored in the metal capillary. Then, the tape was sticked and the needle was detached from the pipette. The oil was added in the needle adaptor. With the sharp tip, the needle can puncture the seal tape on the inlet and be inserted into the inlet directly.

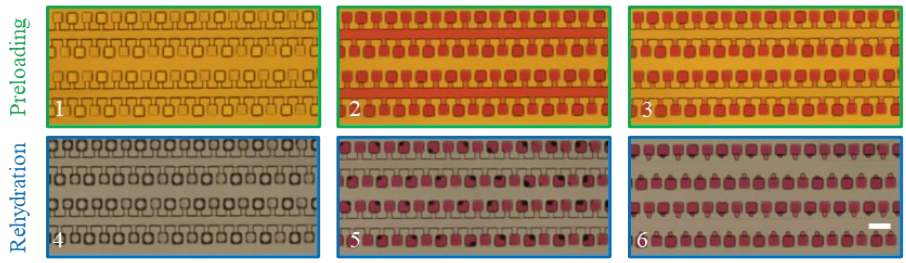

Figure S8. The process of reagent pre-incorporation and rehydration in a 3D-MAC. 1. Degassed chip; 2. Reagent preloading; 3. Air flushing; 4. Drying; 5. Rehydration; 6. Sample digitalization. Scale bar denotes 100 μm.

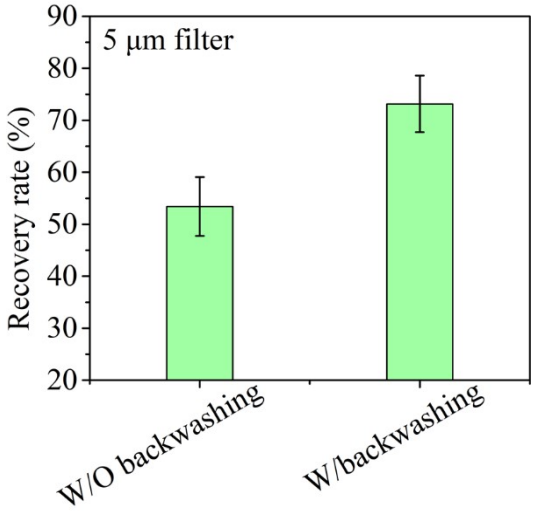

Figure S9. The increased recovery rate of 5 μm filter after backwashing by 2mL PBS.

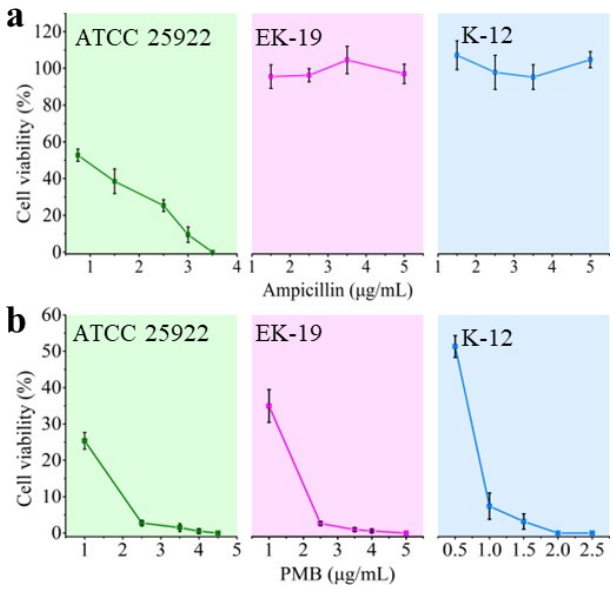

Figure S10. MIC of AMP (a) and PMB (b) for *E. coli* ATCC 25922, EK-19 and K-12. MICs were determined with fresh test solution at 42℃.

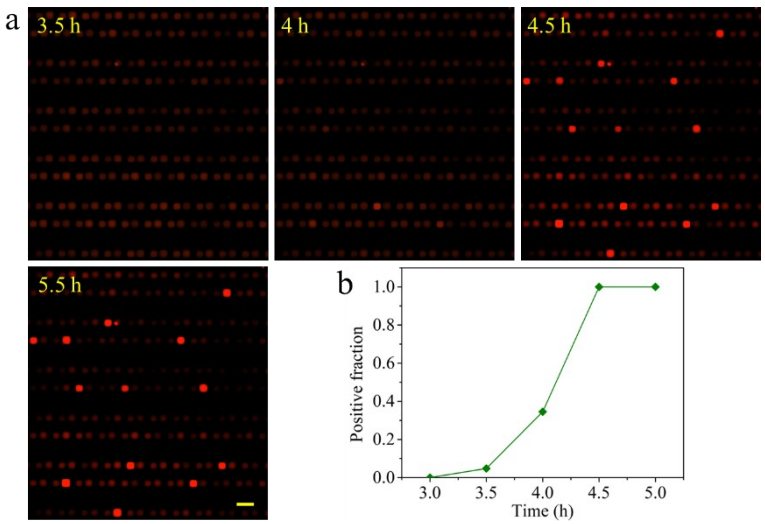

Figure S11: a) Time-lapse imaging of the microwells with EK-19 and 0.5 μg/mL PMB. b) Changes of positive fraction of EK-19 with 0.5 μg/mL PMB. The chip was incubated at 37°C. Scale bar denotes 100 μm.

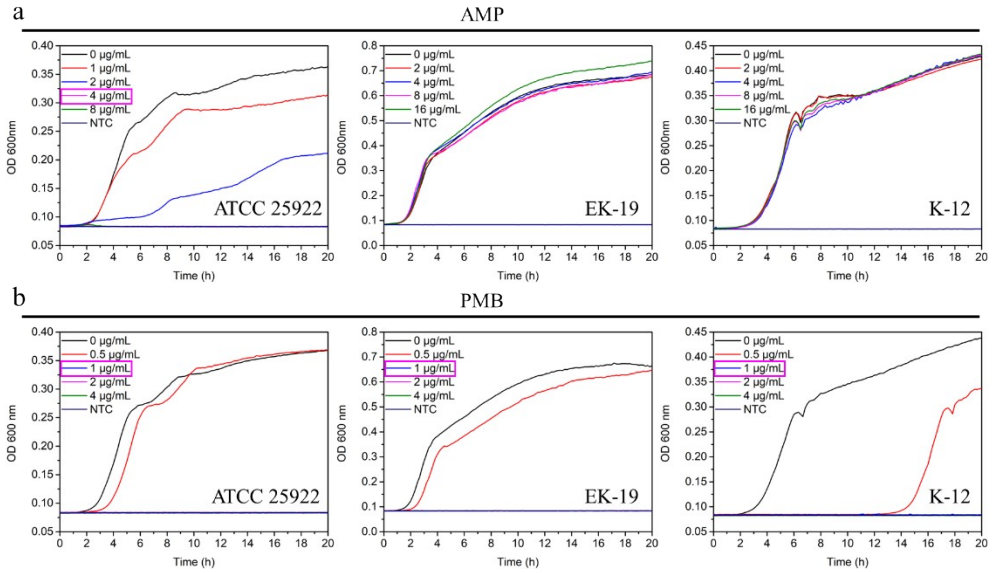

Figure S12. MIC determination of *E. coli* ATCC 25922, EK-19 and K-12 to AMP (a) and PMB (b) via broth dilution. MICs were determined at 37°C. The rose rectangles indicate the measured MICs.

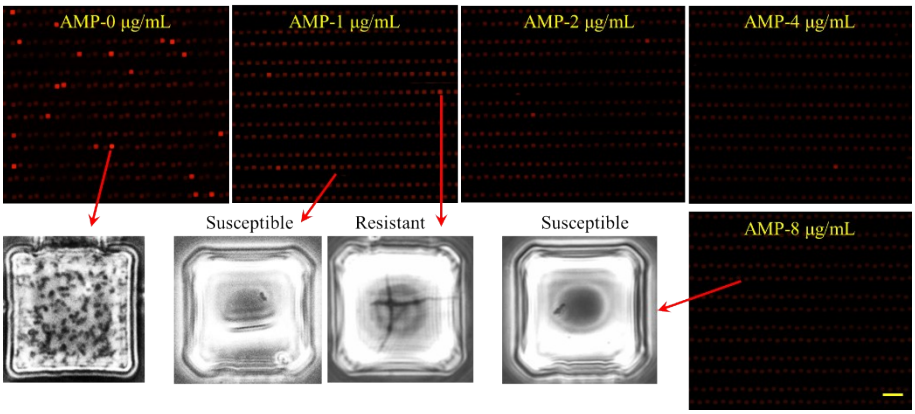

Figure S13. Micrographs of the microwells with ATCC 25922 and varying concentrations of AMP.

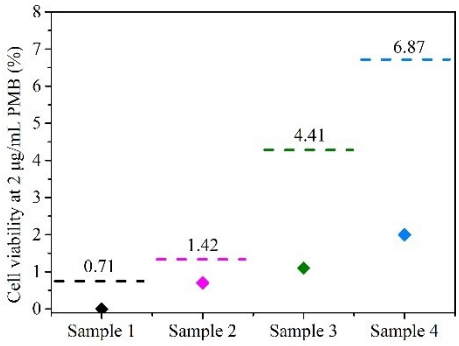

Figure S14. Alleviation of inoculum effect on susceptibility categorization. The cell viability of EK-19 with different concentrations to 2 μg/mL PMB was measured to indicated MICs. Sample 1: 6.35×10^4 CFU/mL, Sample 2: 1.27×10^5 CFU/mL, Sample 3: 6.35×10^5 CFU/mL, Sample 4: 1.27×10^6 CFU/mL.

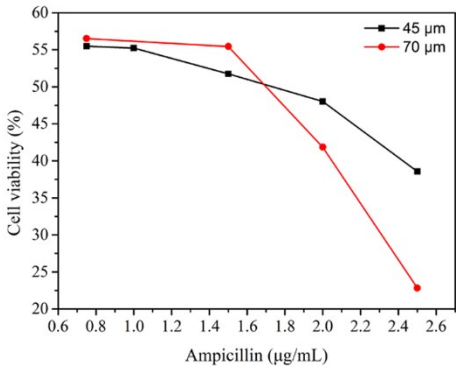

Figure S15. The inhibition efficiency of ampicillin to *E. coli* ATCC 25922 in 45 and 70 μm microwells. 100% LB was used in this experiment.

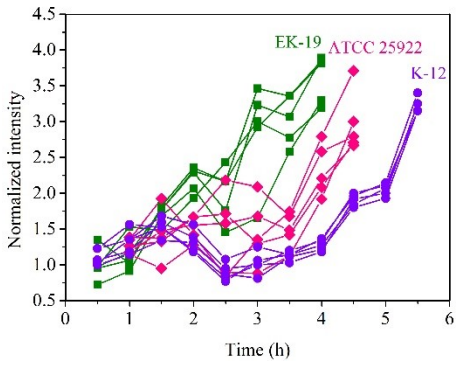

Figure S16. Fluorescence evolution of the microwells with different strains. 5 microwells were randomly selected from the chip with pure bacteria. The fluorescence evolution of the microwells containing different *E. coli* strains exhibited significant difference. The normalized intensity indicated the ratio of the intensity of positive microwells to that of negative microwells.

Table S1. The performance of Dsc-AST and the portable platforms reported previously.

Method	Samples	AST with MICs	Dynamic range (CFU/mL)	Sample-to-Answer Time (h)	Measurement of Heteroresistance	Sample preparation	Inoculum effect	Ref.
Dsc-AST	Urine	Y	10^2 to 10^7	~3	Y	NR	N	This work
Nanoliter array	Urine	Only AST	≥5 × 10^5	6	N	NR	Y	25
Gravity-driven droplet assay	Isolates	Y	10^2 to 10^7	>5	Y	R	N	26
Resazurin assay	Isolates	Y	-	>5	N	-	Y	21
Phenol red assay	Isolates	Y	5 × 10^5	>18	N	NR	Y	27

Y: Yes; N: No; R: Required; NR: No required.

Reference

1. O. Scheler, K. Makuch, P. R. Debski, M. Horka, A. Ruszczak, N. Pacocha, K. Sozanski, O. P. Smolander, W. Postek and P. Garstecki, *Sci. Rep.*, 2020, **10**, 3282.