Electronic Supplementary Material (ESI) for Analyst.

Supporting Information:

Rapid Genotypic Antibiotic Susceptibility Test Using CRISPR-Cas12a for Urinary Tract Infection

Juhong Chen,*[ab] Fuguo Jiang,[c] Chao-Wei Huang,[a] and Liwei Lin*,[a]

[a] Department of Mechanical Engineering, University of California, Berkeley, Berkeley, CA 94760, USA

[b] Department of Biological Systems Engineering, Virginia Tech, Blacksburg, VA 24061, USA

[c] Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX 77054, USA

Corresponding Author: J. Chen (email: jhchen@vt.edu)

L. Lin (email: lwlin@berkeley.edu)

Experimental Procedures

Protein Expression and Purification. LbCas12a expression and purification were carried out following the procedures with minor modification. In brief, bacterial expression vectors containing an N-terminal 10×His-tag, maltose-binding protein, and TEV protease cleavage site were transformed into *E. coli* BL21 (DE3). The *E. coli* cells were then cultured in Terrific Broth at 16 °C for 14 hours to express LbCas12a. To collect the LbCas12a protein, cells were lysed using lysozyme and purified using Ni-NTA resin. After TEV cleavage overnight, proteins were further purified over MBPTrap HP column connected to a HiTrap Heparin HP column for cation exchange chromatography. The LbCas12a proteins were finally purified using gel filtration purification and stored in the buffer consisting of 20 mM Tris-HCl (pH 7.5) 200 mM NaCl, 5% (v/v) glycerol, and 1mM TCEP.

Characterization and Optimization of pUC19 Plasmid Cleavage Assay. In this study, all nucleic acids were synthesized commercially by IDT, Inc. (listed in Supplementary Table S1). LbCas12-medicated cleavage assays were performed in the buffer solution (200 μL) consisting of 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl2, and 10% bovine serum albumin (BSA). In detail, LbCas12a, crRNA-1 or crRNA-2, ssDNA-FQ probe, and pUC19 plasmid were mixed in a 1.5 mL centrifuge tube and shake at 37 °C under 1000 rpm. After incubation of 30 min, 150 μL reaction solutions were immediately added into a 96-well fluorescence plate to measure the fluorescence intensity using spectrometer (fluorescence spectra from 515 to 600 nm under the excitation wavelength of 485 nm). For the optimization, the LbCas12a concentration at 50 nM was firstly decided, and the rest of the reagents were then optimized based on the fluorescence readout.

Bacteria Culture and Cell Harvest. To prepare bacteria culture, a colony of AmpR *E. coli* BL21 with pUC19 plasmid was inoculated into LB broth with ampicillin (10 μg/mL). After overnight growth at 37 °C under 200 rpm,

the bacteria cells were harvested by centrifuging at 6,000x g for 2 min and resuspending in PBS. The harvested bacteria stock was serially diluted into desired concentrations for further experiment. All other bacteria strains (AmpR *E. coli* DHα5, *E. coli* BL21, *B. subtilis*, and *S. epidermidis*) were cultured and harvested in the same way. The colony-forming unit (CFU) of each bacteria concentration was determined using standard LB agar plates.

Detection of AmpR Bacteria Using CRISPR-Cas12a Cleavage Assay. To determine AmpR bacteria, the harvested bacteria cells were lysed using lysozyme. In detail, 10 mg/mL lysozyme (50 μL) was added into bacteria solution (450 μL) and the mixtures were incubated at 33 °C for 30 min. The bacteria lysis (20 μL) were then added into a premix reagent (180 μL) consisting of LbCas12a, crRNA-1, and ssDNA-FQ probe in the buffer solution. The final concentrations were 50 nM for LbCas12a, 60 nM for crRNA-1, and 700 nM for ssDNA-FQ probe. After incubation for 30 min at 33 °C, the fluorescence intensities of different AmpR bacteria concentrations were measured (λex: 485 nm and λem: 525 nm). For the one step assay, the bacteria solutions with lysozyme were added into a premix reagent before bacteria lysis and incubated at 33 °C for 60 min, which allowed the bacteria lysis and plasmid cleavage assay to happed in parallel. The specificity of this proposed method was investigated using other bacteria strains and bacteria mixtures (AmpR *E. coli* DHα5, *E. coli* BL21, *B. subtilis*, *S. epidermidis*, AmpR *E. coli* BL21 + *E. coli* BL21, AmpR *E. coli* BL21 + *B. subtilis*, and AmpR *E. coli* BL21 + *S. epidermidis*).

Determination of AmpR Bacteria in Urine Samples. To determine the bacteria AmpR gene from the urine samples, the AmpR bacteria at different concentrations (10^3, 10^4, and 10^5 CFU/mL) were inoculated into synthetic urine. Synthetic urine without the addition of any bacteria was used as a negative control. In the workflow of AST-1, bacteria solutions (0, 10^3, 10^4, and 10^5 CFU/mL) were added into growth media containing ampicillin (10 μg/mL) and incubated at 33 °C. After pre-enrichment of

2 – 8 hours, the AmpR bacteria were measured using the CRISPR-Cas12a cleavage assay. In the workflow of AST-2, the bacteria solutions were lysed and the target genes were amplified using RPA in parallel for 20 min. The RPA amplifications were performed using TwistAmp® Basic Kit with the primers listed in Supplementary Table S1. The amplified target DNA were then measured using cleavage assay.

Results and Discussion

Control experiments to characterize CRISPR-Cas12a system. In order to further characterize our design, control experiments including LbCas12a + ssDNA-FQ probe + pUC19, LbCas12a + crRNA + ssDNA-FQ probe, crRNA + ssDNA-FQ probe + pUC19, and LbCas12a + crRNA + ssDNA-FQ probe + pUC19 were studied, respectively (Figure S1). Based on the combination in Figure S1a, these mixed solutions (No.1 – No.9) were prepared and incubated at 37 °C. After incubation of 30 min, the fluorescence spectra from 515 nm to 600 nm were recorded at the excitation wavelength of 485 nm (Figure S1b). The peak at the wavelength of 525 nm represented the fluorescence intensity of the cleaved fluorophore (FAM). There were no peaks observed except the control experiments (No. 8 and No.9), indicating that ssDNA-FQ probes were not cleaved by CRISPR-Cas12a system (No.1 – No.5) or there were no ssDNA-FQ probes (No.6 and No.7). On the contrary, control experiments (No.8 and No.9) showed a strong fluorescence intensity. In order to quantify the cleavage of the ssDNA-FQ probe using CRISPR-Cas12a system, the fluorescence intensities of all control experiments at the peak wavelength of 525 nm were plotted in Figure S1c. Strong fluorescence intensities were observed for control experiments (both No.8 and No.9) that included four reagents of LbCas12a + crRNA + ssDNA-FQ probe + pUC19. The reason was that Cas12a-crRNA system was activated by target pUC19 plasmid, and then cleave ssDNA-FQ probes to generate fluorescence readouts. Compared to the control experiment (No.9),

control experiment (No.8) had higher fluorescence intensity, indicating that crRNA-1 had higher cleavage efficiency than crRNA-2. This phenomenon was consistent with the result in Figure 1.

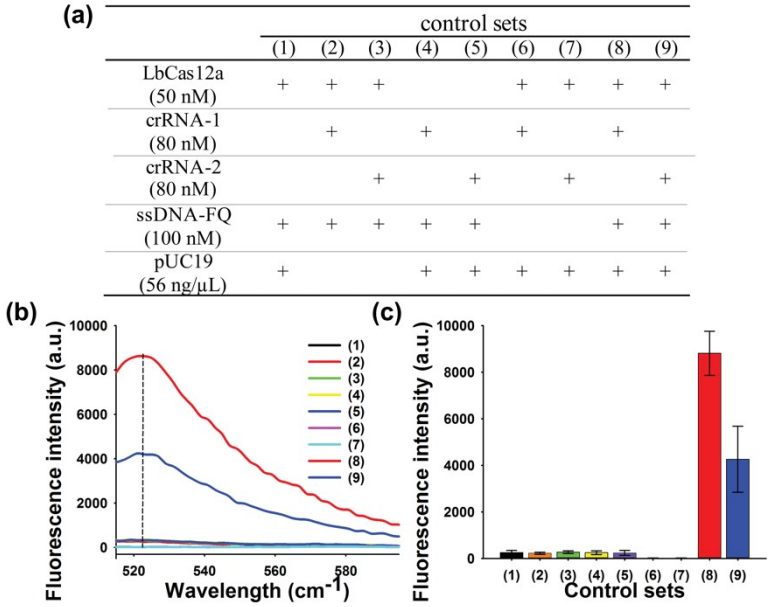

Figure S1. Control experiments (No.1 – No.9) to characterize the efficiency of CRISPR-Cas12a system. (a) Nine different combinations of the four reagents, including LbCas12a, crRNA (crRNA-1 or crRNA-2), ssDNA-FQ probe, and pUC19 plasmid. (b) Fluorescence spectra (515 – 600 nm) and (c) the fluorescence intensity at the peak wavelength of 525 nm of these nine combinations. Error bar represents the standard deviation (n = 3).

Optimization of experimental conditions to determine bacteria antibiotic-resistant type using CRISPR/Cas12a. *Escherichia coli* (*E. coli*) BL21 with pUC19 plasmids were cultured in LB broth (containing 100 μg/mL ampicillin). After incubation overnight, *E. coli* cells were centrifuged and washed using PBS buffer, and then resuspended in PBS buffer. The bacteria concentration was enumerated using standard ten-times serial dilution. Based on suggestion in the previous publication, the final Cas12a concentration of 50 nM was used in this study.[1] Under this condition, other experimental parameters including crRNA concentration, ssDNA-FQ probe concentration, and temperature were investigated using pUC19 at the concentration of 56 ng/μL in detail below.

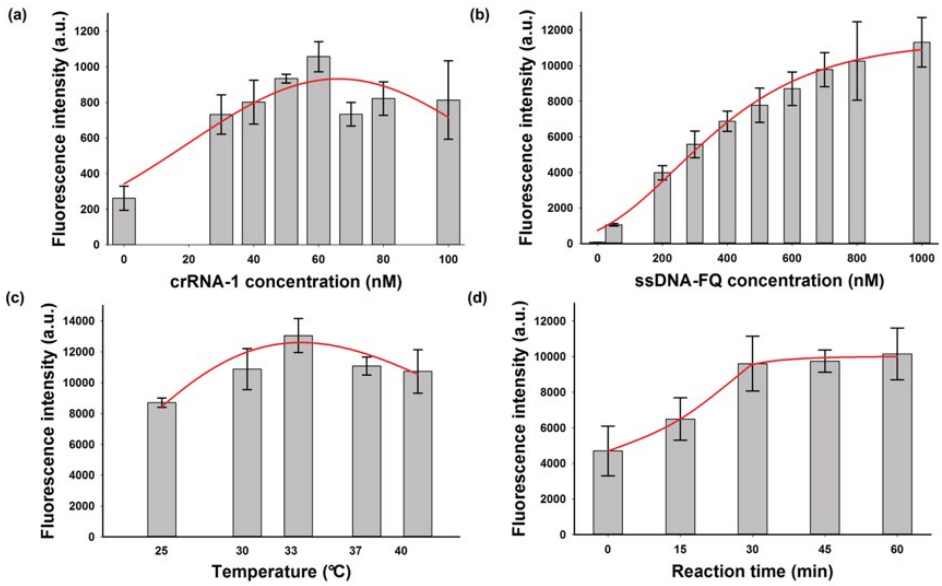

Figure S2. Optimization of AmpR detection. Effects of (a) crRNA-1 concentration, (b) ssDNA-FQ concentration, (c) temperature, and (d) reaction time for the fluorescence intensity. The pUC19 concentration: 56 ng/μL. Error bar represents the standard deviation (n = 3).

Specifically, crRNA from the CRISPR/Cas12a-crRNA complex can recognize target DNA and cut it. The amount of crRNA can affect the cleavage efficiency. As concluded from Figure S1, crRNA-1 had higher cleavage efficiency than crRNA-2. Thus, only crRNA-1 concentration was investigated. As indicated from Figure S2a, the fluorescence intensity at the wavelength of 525 nm initially increased with the increment of crRNA-1 concentration from 0 to 60 nM, and then decreased. The reason could be that excess crRNA-1 concentration would bind to target DNA, decreasing the recognition of Cas12a-crRNA-1 complex to find the target DNA. The maximum fluorescence intensity was observed at the crRNA-1 concentration of 60 nM, which was employed for the development of CRISPR-Cas12a system. In this assay, fluorescence signal was from the cleaved ssDNA-FQ probes. The ssDNA-FQ probe concentration was expected to affect the fluorescence readout. Thus, the ssDNA-FQ probe concentration was next investigated. Using pUC19 at the concentration of 56 ng/μL and crRNA-1 at the concentration of 60 nM, as seen from Figure S2b, the

fluorescence intensity increased with the increase of ssDNA-FQ probe concentration and tended to level off after the concentration of 700 nM. Thus, ssDNA-FQ concentration of 700 nM was used for the further CRISPR-Cas12a system. Using pUC19 at the concentration of 56 ng/μL and crRNA-1 at the concentration of 60 nM, the reaction temperature end reaction time were next optimized. Briefly, the reaction temperature for the CRISPR/Cas12a cleavage of ssDNA-FQ probes affected the fluorescence signal readout. As indicated in Figure S2c, the fluorescence intensity initially increased with the increase of temperature, then decreased after 33 °C. Higher temperature would decrease the Cas12 activity, resulting in the reduction of fluorescence intensity. There, a reaction temperature of 33 °C was used for the reminder of the study. Finally, the reaction time was investigated and reaction of 30 min was selected.

Comparison of the AmpR bacteria determination using crRNA-1 and crRNA-2. As shown in Figure 1 and S1, the cleavage efficiency using crRNA-1 is higher than crRNA-2. For comparison, we also investigated the analytical performance of AST towards AmpR bacteria concentration using crRNA-1 and crRNA-2. Bacteria concentrations (10^4 – 10^8 CFU/mL) were tested, and PBS buffer without any bacteria cells was used as a negative control. The fluorescence intensity towards bacteria concentration was measured at 0, 30, and 60 min (Figure S3). At 0 min, all fluorescence intensities were around 4000 a.u., and there weren't significant differences observed between crRNA-1 and crRNA-2. After reaction for 30 min, significant difference (p value < 0.05) between crRNA-1 and crRNA-2 at the concentration of 10^8 CFU/mL was observed. After incubation for 60 min, significant difference (p value < 0.01) between crRNA-1 and crRNA-2 was observed at the concentration of 10^7 CFU/mL. Compared with the negative control, bacteria at the concentration of 10^8 CFU/mL can be detected after reaction of 30 min using crRNA-1. And, bacteria at the concentration of 10^7 CUF/mL and 10^8 CFU/mL can be measured after incubation of 60 min using crRNA-1 and

crRNA-2, respectively. All these results indicated that the cleavage efficiency of crRNA-1 was higher than that of crRNA-2, which was consistent with our early findings in this study.

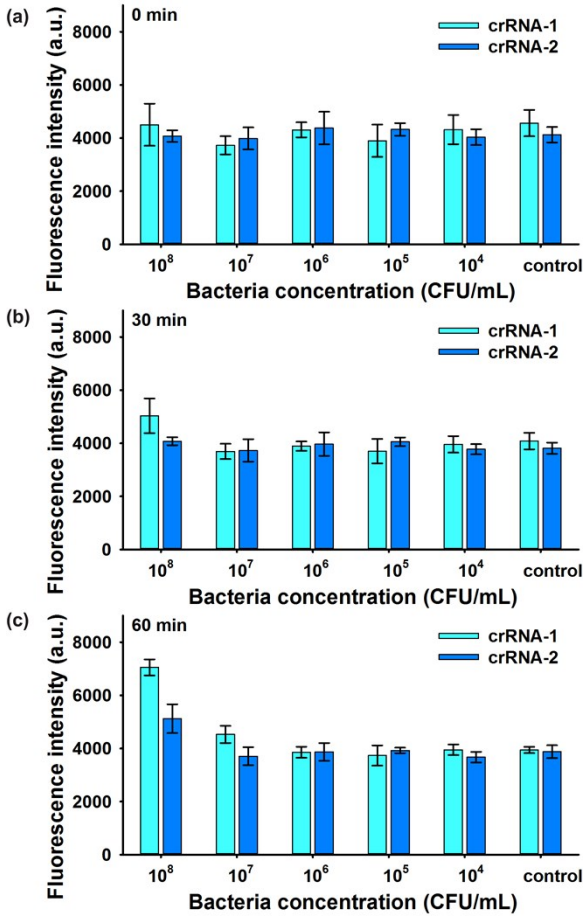

Figure S3. Comparison of the AmpR bacteria detection using crRNA-1 and crRNA-2. Plots of fluorescence intensity towards bacteria concentration after the incubation of (a) 0, (b) 30, and (c) 60 min.

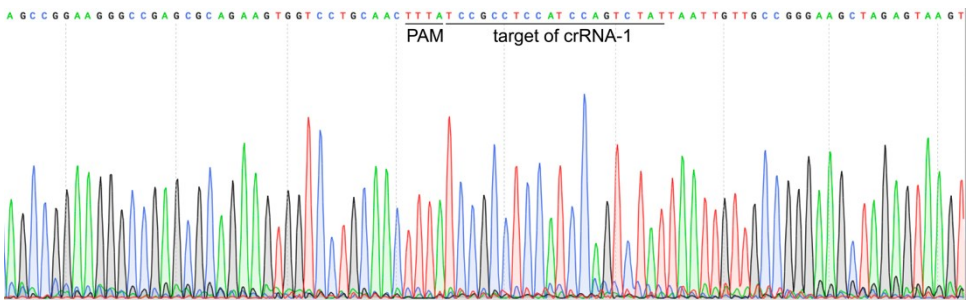

Figure S4. DNA sequencing of RPA product (band-2 in Figure 4c).

Table S1. All nucleic acids used in this study.

Name	Sequence
Target DNA-1	5'-TCCGCCTCCATCCAGTCTAT-3'
Target DNA-2	5'-TGTGACTGGTGAGTACTCAA-3'
crRNA-1	5'-ggUAAUUUCUACUAAGUGUAGAUUCCGCCUCCAUCCAGUCUAU-3'
crRNA-2	5'-ggUAAUUUCUACUAAGUGUAGAUUGUGACUGGUGAGUACUCAA-3'
ssDNA-FQ Probe	/56-FAM/TTATT/3IABkFQ/
RPA Forward Primers	CACAACATGGGGGATCATGTAACTCGCCTTG
RPA Reverse Primers	CCAATGCTTAATCAGTGAGGCACCTATCTCAG

References

(1) Chen, J. S.; Ma, E. B.; Harrington, L. B.; Da Costa, M.; Tian, X. R.; Palefsky, J. M.; Doudna, J. A. *Science* **2018**, *360*, 436-439.