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Supplementary Materials for

Rapid pathogen-specific phenotypic antibiotic susceptibility testing using digital LAMP quantification in clinical samples

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Materials and Methods

Supplementary materials and sample handling

Materials and reagents

All reagents purchased from commercial sources were used as received unless otherwise stated. BBL trypticase soy agar (TSA) plates with 5% sheep blood and Bacto brain heart infusion (BHI) media were purchased from BD Biosciences. All antibiotic stock solutions and nucleic acid amplification reactions were prepared using sterile, nuclease-free water (NF-H_2O) purchased from Thermo Fisher. Ciprofloxacin was purchased from Sigma-Aldrich and prepared as a 1 mg/mL stock solution in NF-H_2O. Nitrofurantoin was purchased from Sigma-Aldrich and prepared as a 10 mg/mL stock solution in NF-H_2O. QuickExtract DNA Extraction was purchased from Epicentre. QX200 ddPCR EvaGreen Supermix was purchased from Bio-Rad Laboratories. Bst 3.0 and 10 mM dNTPs were purchased from NEB. Pooled healthy human urine was obtained from Lee Biosolutions. Primer sequences were ordered as dried stocks from IDT.

Digital quantification with dPCR

Droplet digital PCR reactions were carried out as described previously (*26*).

Design, fabrication and preparation of SlipChips

Details of the design, fabrication, preparation, and assembling of the single-volume 1,280-well SlipChip glass devices are described in previous work (*50*). For this manuscript, the workflow in Fig. 5 was performed with lab-made reusable glass microfluidic chips (Samples 28-29,48-51). To run the rest of the 54 samples with the rapid dLAMP assay, we obtained a set of disposable injection molded chips (5,376 2.4-nL compartments) from SlipChip Corp, which enabled shorter turnaround times between experiments (Samples 1-27,30-47).

Clinical sample handling and gold-standard broth microdilution AST

Urine from patients suspected of having urinary tract infections (UTIs) was collected and transported in a BD Vacutainer Urine Collection Tube containing formate and borate as preservatives. Next, pathogens from the urine samples were isolated and identified using mass spectrometry. Broth microdilution AST was performed on samples positive for *E. coli*.

dAST with clinical samples

One modification to our original dAST protocol (*26*) is the addition of DNase to digest extracellular DNA. We did this to eliminate the confounding effect that extracellular DNA could have on the CT ratio. Consider an antibiotic-susceptible sample with 500 cop/μL of cell-free DNA and 300 cop/μL DNA inside cells. If the genomes replicate 1.5X over a 15 min exposure time, then the CT ratio in the case where cell-free DNA is also detected would be 950 cop/uL ÷ 800 cop/uL = 1.19; in the case where cell-free DNA is digested by DNase and not detected, the CT ratio would be 450 cop/uL ÷ 300 cop/uL = 1.50.

If discordant AST calls (compared to the gold-standard) were noticed on the same day, we re-ran that sample to resolve the discordancy. Some reruns are accepted even in the FDA submissions of diagnostic AST devices, so rerunning samples in itself is not a problem. It would have been better to rerun the samples twice, to get a third measurement as a tie-breaker. Unfortunately, we could not do so due to the limitations of our protocols and the concern for aging of clinical samples over time (and we were not able to rerun all of the samples).

Because our data provide a quantitative measurement (CT ratio), we averaged the two runs to obtain a consensus value of the CT ratio. When we do this (using dPCR values as an example), we find that three samples (#28, #29, #43) returned average CT ratios (1.48, 1.07, 1.48) that were in agreement with the gold

standard (S, R, S). For a fourth sample (#36), the average CT ratio (1.09) was also discordant with the gold standard (S) and we recorded it as an error in our analysis for both dPCR and dLAMP (see Table S1).

Isolate maintenance and exposure experiments.

For all experiments involving isolates (Fig. 2), isolates were maintained and antibiotic exposure carried out as described in previous work (*26*). All *E. coli* isolates were maintained on solid or liquid BHI media (BD), all *Lactobacillus jensenii* isolates were maintained on solid or liquid MRS media (BD).

Intermediate samples

In this manuscript, we focus on categorical agreement of our binary susceptibility determination (susceptible or resistant). We chose to design our study this way and to exclude intermediate samples for the following reasons:

The current gold-standard antibiotic susceptibility testing method is broth dilution. This method, used every day in central clinical laboratories, is only accurate to +/- one dilution step. For example, *E. coli* with an initially determined ciprofloxacin MIC of 2.0 μg/mL might have an MIC of 1, 2, or 4 μg/mL if tested again using the same gold-standard method. According to the CLSI standards used in the US, 1.0 μg/mL is considered "susceptible", while 2 μg/mL is considered "intermediate" and 4.0 μg/mL is considered resistant. This is well-known in the clinical microbiology community. In fact, the CLSI manual (*59*) states that one of the roles of the intermediate category is to include a buffer zone which should prevent small, uncontrollable, technical factors from causing major discrepancies in interpretation. Furthermore, when gold standard broth dilution vs gold standard inhibition zone diameter is compared, intermediate samples do not show consistent results (see Fig. 4 of (*60*); of the five samples tested with intermediate MICs (as determined by the gold-standard), the inhibition zone method called one of them resistant, two intermediate, and two susceptible (*60*)).

A further issue is the discrepancy of the meaning "resistant and susceptible" around these concentrations. For example, using EUCAST standards, susceptible isolates are those with ciprofloxacin MIC of 0.25 μg/mL and below, while intermediate isolates have MIC of 0.5 μg/mL and resistant isolates are 1.0 μg/mL and above.

We chose to exclude samples with MICs of 0.5, 1.0, and 2.0 μg/mL to ensure that the gold standard method would not frequently switch between a susceptible and resistant call if repeated.

Importantly, this approach is still applicable to "real world" samples and does not correspond to only looking at extremes of MIC. Excluding these samples only eliminates a small percentage of *E. coli* samples based on epidemiological data [see "Ciproflaxin / *Escherichia coli* international MIC distribution" reference database (62)], with the caveat that these distributions may change at different times in different locations. For example: a broader range of antibiotic concentrations is tested when generating epidemiological data than is tested in clinical microbiology laboratories. The cut-off MIC for defining resistant and susceptible organisms is different between the epidemiological and clinical microbiological data. Epidemiological cut off is defined relative to the wild-type susceptibility whereas the clinical cut off is defined relative to clinically relevant susceptibility. These data should not be used to infer the rates of resistance in a particular geographical location at a particular time (62).

For nitrofurantoin (nit), MIC of >=128 is considered resistant and MIC of <= 32 is considered susceptible. Similarly, we chose to exclude the minimal possible number of samples with MICs that might switch between a susceptible or resistant call when repeated. For this reason, we excluded samples with MICs of 32 and 64 μg/mL.

Therefore, it should not be surprising that when validating a new AST method with clinical samples, it is common to challenge the method only against susceptible and resistant samples that are above or below the MIC breakpoints, while avoiding intermediate samples (*34, 35, 61*).

To test whether intermediate or near-intermediate samples provide any unexpected results, we did run a small separate study of 8 clinical isolates (2 operators with 4 isolates each) with intermediate and near-intermediate MICs using dPCR readout. We exposed these isolates with (1.0 µg/mL ciprofloxacin) and without antibiotics for 15 min and measured the nucleic acid concentrations with dPCR. Isolates with MIC of 1.0 µg/mL are clustering very close to the threshold and slightly below, while isolates with MIC of 0.5 µg/mL are comfortably above the threshold and would be read as susceptible (fig. S3).

Theoretical analysis of phenotypic AST

To explore the tradeoffs among antibiotic exposure time, the growth rate of the bacteria in question, and the required resolution of the measurement method, we developed a simple model to inform optimal AST methods when DNA replication is used as the differentiating marker between susceptible and resistant bacteria. We assumed that i) a sample containing bacteria with an initial concentration of a specific NA sequence, C_0 [mol/L], has a DNA doubling time of t_{double} [min] when incubated in media for t_{inc} [min], ii) an antibiotic-susceptible bacteria sample incubated in media with antibiotics does not grow at all, and iii) antibiotic-resistant bacteria grow at the same rate with and without antibiotics.

Under these assumptions, the ratio of the NA concentrations of a control sample ($C_{control}$) compared to an antibiotic-treated sample (C_{ABX})—the control–treated ratio (*CT ratio*)—after a certain time of antibiotic exposure (t_{inc}) would be:

$$CT\ ratio = \frac{C_{control}(t_{inc})}{C_{ABX}(t_{inc})} = \frac{C_0 \cdot 2^{t_{inc}/t_{double}}}{C_0} = 2^{t_{inc}/t_{double}}$$

Plotting CT ratio as a function of t_{inc} and t_{double} yields Fig. 1B. Typically, qPCR is capable of resolving 2-fold differences in concentration, whereas digital PCR (dPCR) can resolve as low as 1.2-fold differences in concentration (*41*). Due to the higher resolving power of dPCR, phenotypic AST can be performed with shorter antibiotic exposure times than if qPCR was used as the measurement method.

Experimental details for LAMP primer design, optimization, and specificity.

LAMP primer optimization experiments (Fig. 3A, steps 1–2) were performed on a Roche LightCyler 96 using the SYBR Green I channel for readout, 6 µL reaction volumes, and the following concentrations of reagents: 20 mM Tris-HCl pH 8.8, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 1.4 mM dNTPs, 2 µM Syto-9, 400 U/mL Bst 2.0 (New England Biolabs), ~700 copies/µL *E. coli* gDNA, and 8 mM $MgSO_4$. All samples were run across a temperature gradient spanning 60 – 72 ˚C.

The experiments optimizing magnesium concentration (Fig. 3A, step 3) were performed using the same protocol as above with the following concentrations of reagents: 20 mM Tris-HCl pH 8.8, 150 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 1.4 mM dNTPs, 2 µM Syto-9, 360 U/mL Bst 3.0 (New England Biolabs), ~700 copies/µL *E. coli* gDNA, and variable concentrations of $MgSO_4$ (Fig. 3A). All samples were run across a temperature gradient spanning 60–74 ˚C.

Primer concentrations were kept constant in all experiments: 1.6 µM FIP/BIP, 0.2 µM FOP/BOP, and 0.4 µM loopF/loopB (when included). The final selected primer set was as follows: GGCGTTAAGTTGCAGGGTAT (FOP), TCACGAGGCGCTACCTAA (BOP), CGGTTCGGTCCTCCAGTTAGTGTTTTCCCGAAACCCGGTGATCT (FIP),

TAGCGGATGACTTGTGGCTGGTTTTTCGGGGAGAACCAGCTATC (BIP), ACCTTCAACCTGCCCATG (LoopF), GTGAAAGGCCAATCAAACC (LoopB).

Identification and specificity experiments were performed using the same concentration of reagents as the experiments to optimize $MgSO_4$ concentration, but were run with 5 mM $MgSO_4$. Although 6 mM $MgSO_4$ yielded the fastest TTP, 5 mM $MgSO_4$ was used in subsequent experiments in order to minimize the risk of background amplification. We have not observed background amplification with the primers described here, but other primer sets are sensitive to $MgSO_4$ concentration. The optimal TTP using 5 mM $MgSO_4$ was only 12 s slower than when using 6 mM $MgSO_4$.

BLAST was used to evaluate primer specificity against the families *Enterobacteriaciae*, *Staphylococcaceae*, and *Enterococcaceae*. The specificity of the LAMP primers targeting the *E. coli* 23S rDNA gene was tested against human genomic DNA (*Hs* gDNA), *Lj* gDNA, urine from healthy donors, and water (Fig. 3A,B). *Hs* gDNA was tested at 0.002, 0.02, and 0.2 ng/µL final reaction concentration as measured using a NanoDrop 2000c (Thermo Fisher Scientific). *Lj* gDNA was tested at final reaction concentrations of 0.16, 0.8, and 1.6 ng/µL, as measured using a NanoDrop 2000c. Urine from healthy donors was run at 10% final reaction volume. Real-time LAMP amplification was performed using a range of concentrations of *E. coli* gDNA (*Ec* gDNA) prepared from clinical UTI urine samples and quantified using droplet digital PCR (Fig. 3C).

Rapid digital LAMP (dLAMP)

Clinical urine samples were treated with and without 1 µg/mL cip or 16 µg/mL nit for 15 min and nucleic acids extracted as described above. The dLAMP mix consisted of 20 mM Tris-HCl pH 8.8, 150 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 1.4 mM dNTPs, 1X EvaGreen (Biotium), 360 U/mL Bst 3.0, 1X RNase Cocktail (ThermoFisher), 5 mM $MgSO_4$, and 1 mg/mL BSA prepared in NF-H_2O. Aliquots of NA extractions composed 10% or 20% of the final volume in the dLAMP mix. Two aliquots of dLAMP mix containing equal volumes of NA extractions from the control and treated samples were simultaneously loaded into two separate SlipChip devices. The top piece of each SlipChip was moved relative to the bottom piece, which partitioned the solution into 1,280 3-nL compartments (lab made glass SlipChips) or 5,376 2.4-nL compartments (injection-molded plastic SlipChips) (see Supplementary Materials). When using the injection-molded plastic SlipChips, the treated chip was loaded 30 s after the control chip. The SlipChips were then placed onto the thermal cycler of a digital real-time imaging instrument and incubated at 72 °C for 20 min (*56*). Amplification time was recorded starting from when the thermal cycler reached 72 °C.

Images were taken every 20 s and the fluorescent intensity was measured for each compartment (Fig. 4A/F) with LabView software as described in (*56*). Wells that showed liquid movement or bubbles were excluded from analysis. If there was a spatial amplification gradient (i.e., positives in one area of the chip appeared before other areas), then the experiment was excluded. The concentration of the target was calculated using Poisson statistics and was based on the number of "positive" compartments that exceeded the fluorescence intensity threshold, for time points where 13 or more compartments were positive. The concentration of the control and treated samples was calculated in real-time, along with a *P* value representing the probability that the ratio of concentrations being greater than 1.10 was a result of random chance (Fig. 4C/H). If $P < 0.05$, we can be reasonably certain that the bacteria are susceptible to the antibiotic. If the P-value remains > 0.05, we can be reasonably certain that the bacteria are resistant to the antibiotic. The CT ratio was calculated and plotted for each 20 s interval in Fig. 4 D/I for one susceptible sample and one resistant sample. For Fig. 6C, the CT ratio at 6.7 min was calculated and plotted for all 54 dAST experiments.

In some cases, the TTP distribution (Fig. 4 B/G) of one chip was delayed relative to the other chip. If this happened, the TTP maximums were aligned to normalize the data before concentrations and CT ratios calculated.

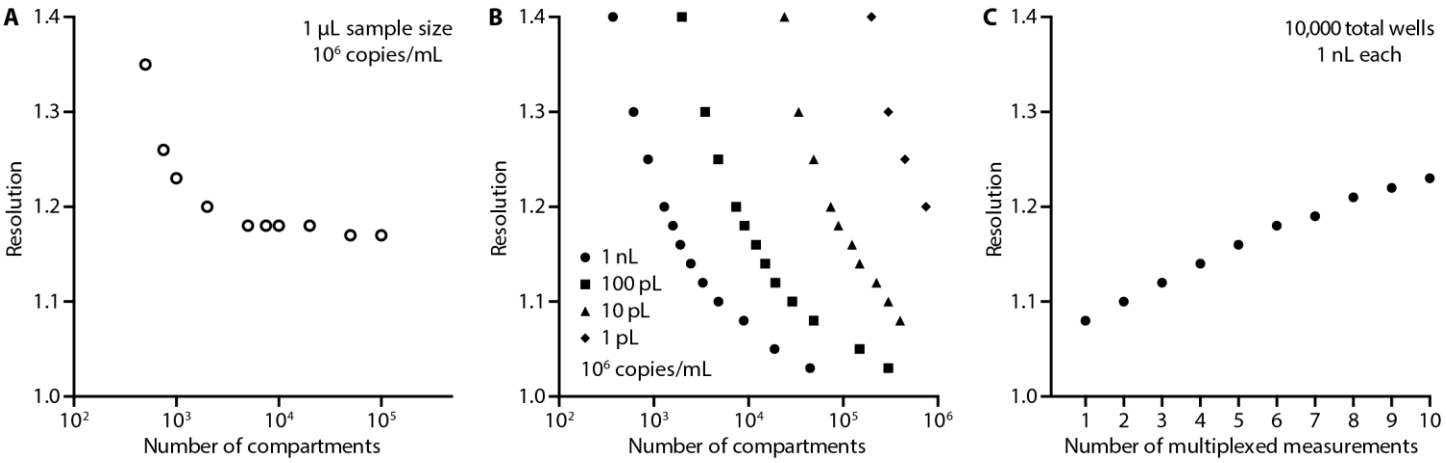

Fig. S1. **Resolution of digital devices.** The resolution of digital quantification depends on the number and volume of compartments. Simulations were performed with the methods described in (*45*). **A)** For a fixed sample size, and fixed input concentration of 10^6 cop/mL relevant to UTIs, increasing the number of compartments (and reducing the volume of each compartment accordingly) beyond 1,000 does not improve resolution in a useful way. **B)** For fixed compartment volume, and fixed input concentration of 10^6 copy/mL relevant to UTIs, the resolution improves with increasing number of compartments, although this increase requires a larger input of sample and amplification reagents. **C)** Dependence of resolution on the number of multiplexed measurements made for a constant number of total wells. For example, while 10,000 of 1 nL compartments provide 1.08 resolution, 2,000 of 1 nL compartments provide 1.16 resolution each, enabling a 4-plex dAST (1 control and 4 ABX treated samples) to be performed.

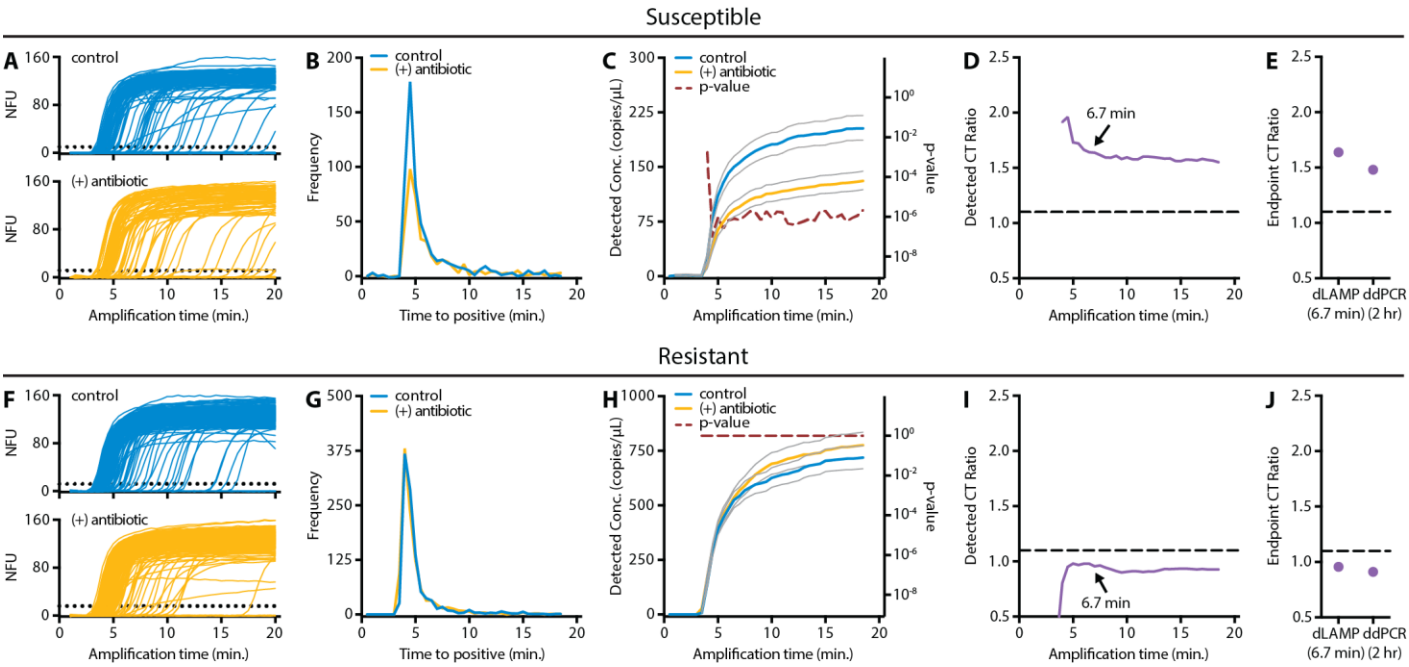

Fig. S2. Real-time dLAMP DNA quantification of a UTI sample with nit treatment. High-resolution single-molecule nucleic acid amplification was performed using ultrafast digital LAMP (dLAMP) for digital antimicrobial susceptibility test (dAST) of clinical UTI urine samples with antibiotic-susceptible (A–E) and antibiotic-resistant (F–J) *E. coli*. Aliquots of a clinical UTI sample were treated with and without 16 µg/mL nitrofurantoin. After 15 min, DNA was extracted and quantified with digital LAMP on SlipChips. The protocols followed and materials used are described in Materials and Methods, "Digital AST (dAST) using clinical UTI samples" and Supplementary Materials Section 5. (**A,F**) Real-time fluorescence amplification traces (only 200 of 1,280 traces shown for clarity). NFU = normalized fluorescence units; dotted line = positive threshold; when the normalized fluorescence intensity of a compartment crosses the threshold, that compartment is counted as positive. (**B,G**) Time-to-positive (TTP) distribution was determined by counting the number of compartments that crossed the positive threshold at each time point. (**C,H**) Detected concentrations of the target dAST marker in control and antibiotic-treated samples for successive image cycles. Grey lines represent 95% confidence intervals. Note these curves are not the amplification curves shown in A and F. (**D,I**) Detected control–treated (CT) ratios over time. Dashed line indicates susceptibility threshold. (**E,J**) Comparison of CT ratios for droplet digital PCR (dPCR) after 2 h and dLAMP (after 6.7 min of amplification).

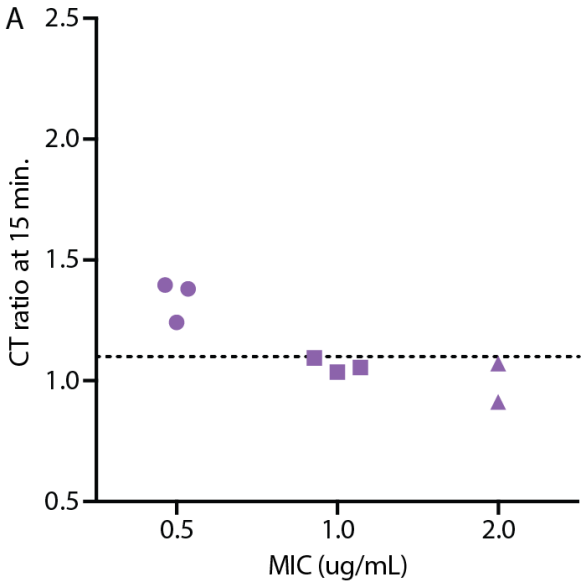

Fig. S3. **The dAST method tested with isolates with near-intermediate MICs.** The digital AST (dAST) method was tested with clinical isolates from urinary tract infections(UTIs) using a 15 min treatment of 1 µg/mL ciprofloxacin. Eight isolates with three near-intermediate MICs were analyzed with the dAST method (two operators with four isolates each). Control–treated (CT) ratios were calculated from dPCR 23S DNA concentration measurements.

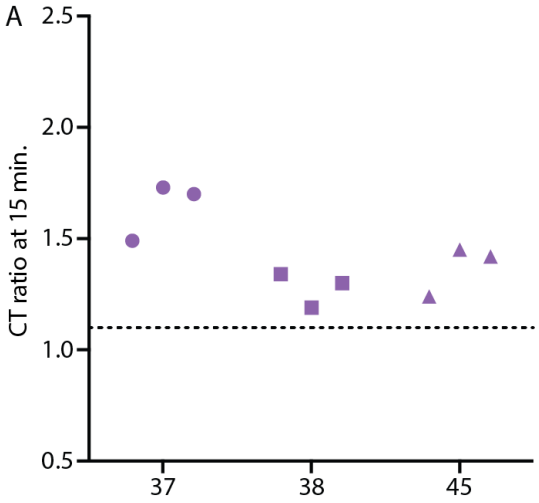

Fig. S4. **Reproducibility of the dAST method with clinical urine samples.** Three ciprofloxacin-susceptible samples (#37, #38, #45 from table S1) were analyzed with the dAST method in triplicate and control–treated (CT) ratios were calculated from DNA concentration measurements using digital droplet PCR.

Table S1. Concentration of clinical urine samples. Pathogen-specific 23S DNA concentration as determined by digital LAMP after 6.7 min of amplification time (Fig. 4C/H). Taking into account the number of rDNA copies per *E. coli* chromosome, and the efficiency of dLAMP in counting DNA in 6.7 min, the concentration of full genomes is ~6 times lower than the number reported in this table. CFU/mL was determined by plate counting at the UCLA Clinical Microbiology Laboratory.

Caltech Sample #	23S Conc. (cop/mL)	CFU/mL	Caltech Sample #	23S Conc. (cop/mL)	CFU/mL
1	1.59E+07	>100,000	**27**	4.63E+07	>100,000
2	2.52E+07	>100,000	**28**	3.62E+07	>100,000
3	3.94E+07	>100,000	**29**	6.21E+06	>100,000
4	5.63E+07	>100,000	**30**	2.38E+07	>100,000
5	3.14E+07	>100,000	**31**	2.98E+07	>100,000
6	7.86E+06	>100,000	**32**	9.57E+07	>100,000
7	7.07E+06	>100,000	**33**	1.08E+08	>100,000
8	5.08E+07	>100,000	**34**	1.13E+08	>100,000
9	1.72E+07	>100,000	**35**	4.84E+07	>100,000
10	2.64E+07	>100,000	**36**	5.73E+07	>100,000
11	7.44E+06	>100,000	**37**	1.59E+07	>100,000
12	2.75E+07	>100,000	**38**	8.49E+07	>100,000
13	2.07E+07	>100,000	**39**	3.18E+06	50,000
14	1.55E+07	>100,000	**40**	2.45E+07	>100,000
15	2.12E+08	>100,000	**41**	1.02E+08	>100,000
16	1.59E+07	>100,000	**42**	1.26E+07	>100,000
17	5.12E+07	>100,000	**43**	4.97E+06	>100,000
18	1.44E+07	>100,000	**44**	1.69E+08	>100,000
19	2.62E+07	>100,000	**45**	2.46E+08	>100,000
20	4.52E+06	>100,000	**46**	8.78E+06	>100,000
21	4.25E+07	>100,000	**47**	8.58E+06	>100,000
22	1.30E+08	>100,000	**48**	1.21E+07	>100,000
23	3.04E+07	>100,000	**49**	1.41E+07	>100,000
24	2.38E+07	>100,000	**50**	3.06E+06	>100,000
25	4.19E+07	>100,000	**51**	8.02E+06	>100,000
26	1.92E+07	>100,000			

Table S2. Clinical samples used in this study. Clinical urinary tract infection (UTI) urine samples tested for ciprofloxacin (cip) or nitrofurantoin (nit) susceptibility testing by gold-standard broth microdilution and by digital AST (dAST). Nucleic acids were quantified with both digital PCR (dPCR) and digital LAMP (dLAMP). Sample reruns (indicated by a "(2)") were performed several hours later on the same day when the control-treated ratio was discordant with the gold-standard AST call (CT ratio > 1.10 for a resistant sample or < 1.10 for a susceptible sample). S = antibiotic-susceptible; R = antibiotic-resistant; *major error; **very major error.

Caltech Sample #	UCLA ID #	Description (Color, Turbidity)	ABX	MIC (µg/mL)	Gold-standard AST call	CT Ratio (dPCR)	dAST call (dPCR)	CT Ratio (dLAMP, 6.7 min)	dAST call (dLAMP)
1	15-31A-020	red, clear	nit	<16	S	1.48	S	1.64	S
2	15-31A-022	light yellow, clear	cip	<=0.25	S	1.44	S	1.34	S
3	15-31A-025	light yellow, clear	nit	<16	S	1.33	S	1.33	S
4	15-31A-026	light yellow, clear	nit	<16	S	1.36	S	1.35	S
5	15-31A-027	light yellow, clear	nit	<16	S	1.25	S	1.24	S
6	15-31A-031	colorless, clear	cip	>=4	R	1.09	R	0.95	R
6	15-31A-031	colorless, clear	nit	256	R	0.95	R	0.77	R
7	15-31A-039	light yellow, clear	cip	>=4	R	0.99	R	0.84	R
8	15-31A-040	light yellow, clear	nit	128	R	1.06	R	1.09	R
9	15-31A-042	dark yellow, clear	cip	<=0.25	S	1.92	S	1.83	S
10	15-31A-043	light yellow, clear	cip	<=0.25	S	1.66	S	1.85	S
10	15-31A-043	light yellow, clear	nit	128	R	0.91	R	0.92	R
11	15-31A-049	light yellow, clear	cip	>=4	R	0.96	R	1.04	R
12	15-31A-050	dark yellow, cloudy	cip	>=4	R	0.88	R	0.96	R
13	15-31A-051	light yellow, cloudy	cip	>=4	R	0.98	R	0.97	R
14	15-31A-054	light yellow, cloudy	cip	<=0.25	S	1.42	S	1.48	S
15	15-31A-056	light yellow, cloudy	nit	256	R	1.09	R	1.106	S**
16	15-31A-060	light yellow, cloudy	cip	<=0.25	S	1.83	S	1.31	S
17	15-31A-063	yellow, cloudy	cip	<=0.25	S	1.28	S	1.111	S
18	15-31A-066	yellow, cloudy	cip	>=4	R	0.85	R	0.80	R
19	15-31A-067	light yellow, cloudy	cip	>=4	R	0.82	R	0.59	R
20	15-31A-068	light yellow, cloudy	cip	>=4	R	0.84	R	0.57	R
21	15-31A-071	light yellow, cloudy	cip	>=4	R	1.04	R	0.92	R
22	15-31A-079	light yellow, cloudy	nit	128	R	1.25	S**	1.43	S**
23	15-31A-084	yellow, clear	cip	>=4	R	1.01	R	0.96	R
24	15-31A-086	yellow, cloudy	cip	<=0.25	S	2.01	S	2.21	S
25	15-31A-088	yellow, cloudy	cip	<=0.25	S	1.25	S	1.22	S
26	15-31A-089	light yellow, clear	cip	>=4	R	0.94	R	0.91	R
27	15-31A-091	yellow, cloudy	cip	<=0.25	S	1.18	S	1.19	S
28	15-31A-093	orange/red, clear	cip	<=0.25	S	1.08	R	-	-
28(2)	15-31A-093	orange/red, clear	cip	<=0.25	S	1.88	S	1.59	S
28_avg	15-31A-093	orange/red, clear	cip	<=0.25	S	1.48	S	-	-
29	15-31A-096	light yellow, cloudy	cip	>=4	R	1.20	S	-	-
29(2)	15-31A-096	light yellow, cloudy	cip	>=4	R	0.93	R	0.98	R
29_avg	15-31A-096	light yellow, cloudy	cip	>=4	R	1.07	R	-	-
30	15-31A-097	light yellow, cloudy	cip	>=4	R	1.13	S**	0.98	R
31	15-31A-101	light yellow, clear	nit	<16	S	1.39	S	1.19	S
32	15-31A-102	dark yellow, clear	nit	<16	S	1.63	S	1.68	S
33	15-31A-103	light yellow, clear	nit	<16	S	1.38	S	1.28	S
34	15-31A-105	light pink, cloudy	nit	<16	S	1.47	S	1.44	S
35	15-31A-108	yellow, cloudy	nit	<16	S	1.29	S	1.37	S
36	15-31A-111	yellow, clear	nit	<16	S	1.02	R*	-	-
36(2)	15-31A-111	yellow, clear	nit	<16	S	1.16	S	0.95	R*
36_avg	15-31A-111	yellow, clear	nit	<16	S	1.09	R	-	-
37	15-31A-112	yellow, clear	nit	<16	S	1.49	S	1.12	S
38	15-31A-114	light yellow, clear	nit	<16	S	1.34	S	1.36	S
39	15-31A-115	yellow, clear	nit	<16	S	1.44	S	1.48	S
40	15-31A-116	dark yellow, cloudy	cip	>=4	R	1.05	R	0.75	R
40	15-31A-116	dark yellow, cloudy	nit	<16	S	1.96	S	2.33	S
41	15-31A-118	yellow, clear	nit	<16	S	1.25	S	1.15	S
42	15-31A-119	light yellow, clear	cip	<=0.25	S	2.21	S	1.95	S
43	15-31A-122	light yellow, clear	nit	<16	S	1.17	S	-	-
43(2)	15-31A-122	light yellow, clear	nit	<16	S	1.79	S	1.45	S
43_avg	15-31A-122	light yellow, clear	nit	<16	S	1.48	S	-	-

44	15-31A-123	yellow, cloudy	nit	<16	S	1.18	S	1.15	S
45	15-31A-126	light yellow, clear	nit	<16	S	1.24	S	1.19	S
46	15-31A-131	light yellow, clear	cip	<=0.25	S	1.61	S	1.28	S
47	15-31A-132	dark yellow, clear	cip	<=0.25	S	1.27	S	1.14	S
48	15-31A-133	dark yellow, cloudy	cip	<=0.25	S	1.30	S	1.29	S
49	15-31A-134	dark yellow, clear	cip	<=0.25	S	2.36	S	1.85	S
50	15-31A-136	light yellow, clear	cip	<=0.25	S	2.04	S	1.89	S
51	15-31A-137	dark yellow, clear	cip	<=0.25	S	1.43	S	1.28	S

Table S3. Rapid phenotypic AST literature summary showing the state of the art. Phenotypic antibiotic susceptibility tests using clinical samples, blood culture, contrived samples, clinical isolates, or reference strains with reported total assay time less than 3.5 hrs (210 min). References are sorted by sample type then by combined time of all steps. NR = not reported. Literature from 1997–2017.

Sample Type	*Method*	*Pre-assay Enrichment Time (min)*	*Minimum ABX Exposure Time (min)*	***Combined Time of All Steps (min)***	*Fastest Reported Sample-to-Answer Time (min)*	*Reference*
Clinical Samples	dAST (using dLAMP)	0	15	24	29	*This work*
Clinical Samples	ATP Bioluminescence	0	90	105	NR	(*61*)
Clinical Samples	Microscopy	120	30	155	NR	(*28*)
Clinical Samples	NA Quantification	0	120	204[a]	NR	(*35*)
Clinical Samples	Microscopy	0	206	206[b]	NR	(*88*)
Clinical Samples	Electrochemical	0	150	NR	210	(*34*)
Contrived Samples	Microfluidics	0	60	60	NR	(*89*)
Contrived Samples	Electrochemical	0	60	100	NR	(*90*)
Contrived Samples	Microfluidics	0	120	120	NR	(*91*)
Blood Culture	Microscopy	0	40	45[c]	NR	(*39*)
Clinical Isolates	Microscopy	0	10	<30[d]	NR	(*27*)
Clinical Isolates	Microscopy	0	40	40	NR	(*39*)
Clinical Isolates	Electrochemical	0	15	45	NR	(*33*)
Clinical Isolates	FACS	0	90	95	NR	(*92*)
Clinical Isolates	Magnetic Bead Rotation	90	15	120[e]	NR	(*93*)
Clinical Isolates	Microscopy	120	6	126	NR	(*28*)
Clinical Isolates	Raman Spectroscopy	0	120	130[f]	NR	(*94*)
Clinical Isolates	Raman Spectroscopy	0	120	130[f]	NR	(*95*)
Clinical Isolates	dAST (using dPCR)	0	15	140	NR	(*26*)
Clinical Isolates	FACS	0	120	150	NR	(*96*)
Clinical Isolates	FACS	0	60	180	NR	(*97*)
Clinical Isolates	Mass Spectrometry	0	60	180	NR	(*98*)
Clinical Isolates	Microscopy	0	180	200[g]	NR	(*29*)
Clinical Isolates	Electrochemical	0	90	NR	NR	(*34*)
Reference Strains	Microscopy	0	3	<30[d]	NR	(*27*)
Reference Strains	Electrochemical	0	10	25	NR	(*32*)
Reference Strains	Raman Spectroscopy	0	20	25[f]	NR	(*95*)
Reference Strains	Electrochemical	0	20	42	NR	(*99*)
Reference Strains	Microfluidics	0	60	60	NR	(*100*)
Reference Strains	FACS	0	120	120[h]	NR	(*101*)
Reference Strains	Raman Spectroscopy	0	120	130[f]	NR	(*94*)

[a]time does not include washing and centrifugation steps

[b]detailed times of each step not reported, listed time is median time reported for all samples

[c]does not include time of overnight blood culture growth
[d]total time of all steps reported as "less than 30 min."
[e]time does not include washing steps
[f]washing, imaging, and agarose embedding time not included
[g]reported as "clinical samples" in the abstract, but methods clearly state that all work was performed with clinical isolates: "We tested 189 clinical isolates…Before testing, each isolate was subcultured on cation-adjusted MHA for 20-24 hours"
[h]does not include time of FACS measurement