Electronic Supplementary Materials for

Rapid Detection of Uropathogens using an Integrated Multiplex Digital Nucleic Acid Detection Assay Powered by a Digital-to-Droplet Microfluidics Device

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Author Contributions

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Supplementary methods:

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Supplementary tables:

Supplementary movies:

References

Supplementary method S1

Routine bacterial culture and identification

Standard bacterial strains were grown overnight on standard agar plates. One colony was picked using an inoculation loop and subjected to 1 mL of saline, and the resulted bacterial suspension was ready for nucleic acid extraction.

Clinical urine samples were subjected to routine microbiological examination, including Gram staining, quantitative bacterial culture and antibiotic susceptibility testing.

Supplementary method S2

Fabrication of the droplet microfluidics module

The PDMS layer of the droplet microfluidics module was prepared by conventional soft lithography process. The SU8-3050 photoresist (Microchem) was spin-coated onto a 4-inch silicon wafer and molded using the standard lithography techniques. The PDMS precursor (Dow Corning, Sylgard 184) is supplemented with mineral oil following a mass ratio of 6.5:1. Afterward, the PDMS mixture and curing agent (Dow Corning, Sylgard 184) was prepared at a ratio 8:1. After degassing, the mixture was poured onto the SU-8 molds and baked at 120 °C for 10 min to replicate the features of the SU-8 mold into the cured PDMS layer. The PDMS layer was subsequently punched with a 1 mm-diameter puncher to form inlets/outlets and then bonded to the upper surface of the ITO-glass plate assisted by plasma treatment. Finally, the entire chip was baked at 80 °C for 10 h.

Supplementary method S3

DNA extraction on the droplet microfluidics module

Various reagents required for nucleic acid extraction were preloaded into the reservoir of the DMF module using a pipette, and the LAMP premix was injected into the chip using a syringe pump.

A total of 50 μL of the sample was mixed with an equivalent volume of lysis buffer, followed by the addition of 3.5 μL of protease K. The resulting mixture was subsequently incubated at 75 ℃ for 15 min. Subsequently, 50 μL of isopropanol and 2.5 μL of MBs suspension were introduced, and the mixture was allowed to stand at room temperature for 9 min.

The lysate along with MBs was introduced to the DMF module at a flow rate of 100 $\mu L \cdot min^{-1}$ with a syringe pump. At the same time, the waste liquid was discharged by another syringe pump at a flow rate of 120 $\mu L \cdot min^{-1}$, resulting in a total volume of 155 μL of lysate to be exhausted within 2 min. During this process, the magnet column is positioned close to the bottom plate to capture the MBs. Subsequently, washing buffer 1 and washing buffer 2 were dispensed from the respective reservoirs and the MBs were washed twice. The waste liquid was then directed to the waste

liquid area and is pumped out. After MBs washing, the magnet column was descended to facilitate the resuspension of MBs. Then, the magnet column moved horizontally to transfer the MBs into 20 μL of elution buffer. The elution process was performed in active motion droplets at 55℃ for 5 min.

Supplementary method S4

Digital PCR

The reagent for digital PCR (20 μL) contains: 10 μL of 2× PCR premix (Biorain, China), 2 μL of primer mixture (10 mM each, B3/F3 primers for LAMP), EvaGreen 1 μL, template DNA 5 μL, and 2 μL of nuclease-free water (Takala Bio, China). Digital PCR was conducted on a DropXpertS6 instrument (Biorain, Shenzhen, China). The LAMP primers are presented in Table S1, and the variation of their specificity were showed in Fig. S3. The thermal cycling was as follows: 95 °C 2 min, followed by 40 cycles at 95 °C for 10 s and 55 °C for 30 s.

Supplementary method S5

Digital LAMP

The LAMP premix droplets contained the following components: 2× isothermal amplification buffer (New England BioLabs), 8 mM $MgSO_4$ (2×, New England BioLabs), 640 $U \cdot mL^{-1}$ Bst 2.0 (2×, New England BioLabs), 2.8 mM dNTPs (2×, New England BioLabs), 1.6 M betaine (2×, Sigma), 2 $mg \cdot mL^{-1}$ BSA (2×, Sigma), 4 μM SYTO 82 (2×, ThermoFisher), 3.2 μM FIP/BIP, 0.4 μM F3/B3, and 1.6 μM LF/LB. After merging the template droplet with the LAMP premix droplet, the final concentration of each LAMP component would be 1×. To perform LAMP, the chip was heated and kept at a constant temperature of 65°C.

Supplementary figure S1

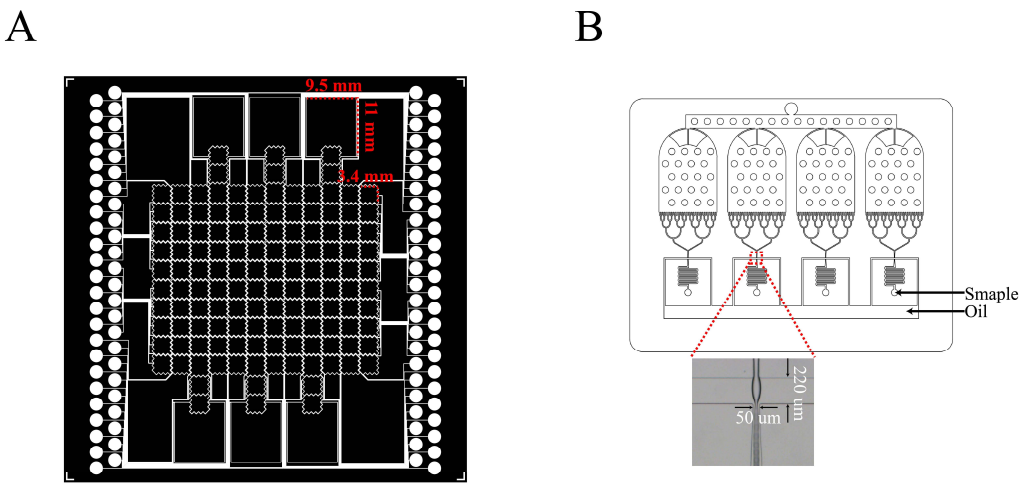

Fig. S1 Structure of the integrated microfluidic device. (A) The lower plate of the DMF module comprises 6 large electrodes (9.5 mm×11 mm) and 114 small electrodes (3.4 mm×3.4 mm); (B) Structure of the droplet microfluidics module with an enlarged view of the droplet generator.

Supplementary figure S2

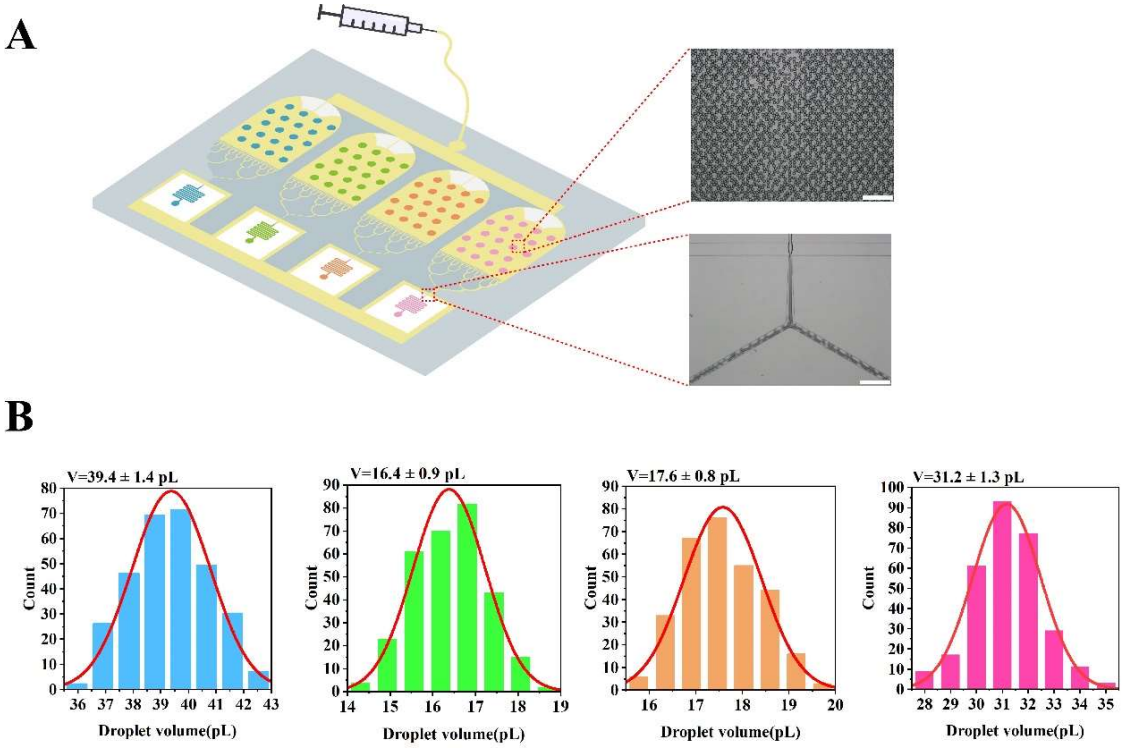

Fig. S2 Simultaneous droplet generation in parallel droplet microfluidics units. (A) Droplet generation and collection actuated by syringe vacuum (Scale bar: 500 μm); (B) Size distribution of droplets in various collection chambers.

Supplementary figure S3

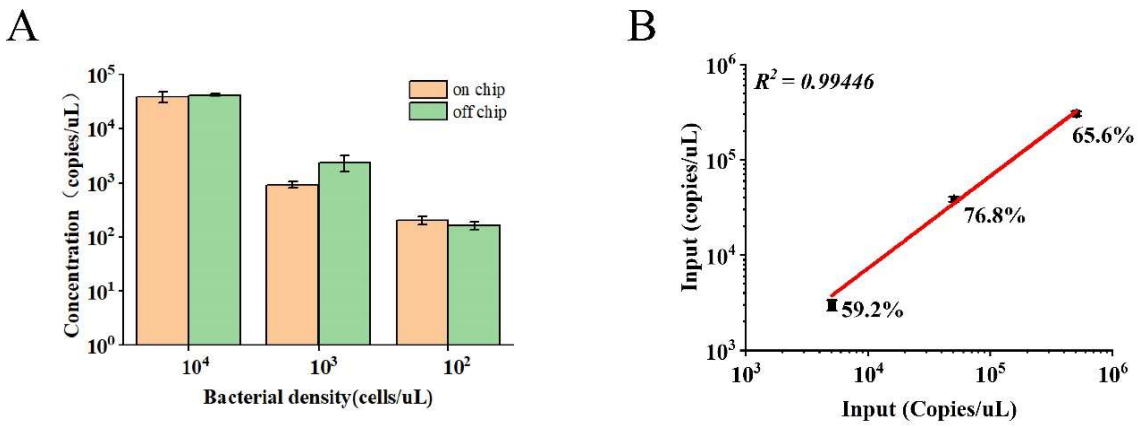

Fig. S3 Nucleic acid extraction efficiency and reproducibility.(A) Comparison of nucleic acid extraction efficiency of *E. coli* obtained by the microfluidic device and the conventional method; (B) Nucleic acid extraction with various *E. coli* input densities shows a DNA recovery of ~65.6%.

Supplementary figure S4

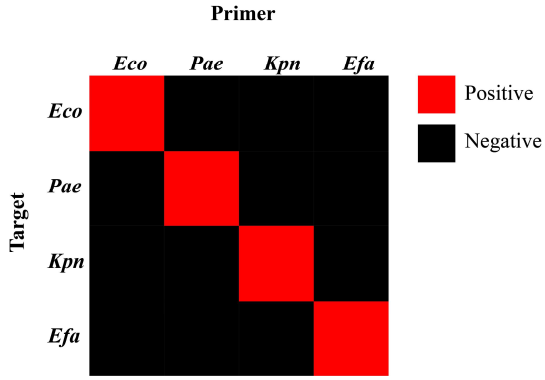

Fig. S4 Verification of specificity of the LAMP primers

Supplementary figure S5

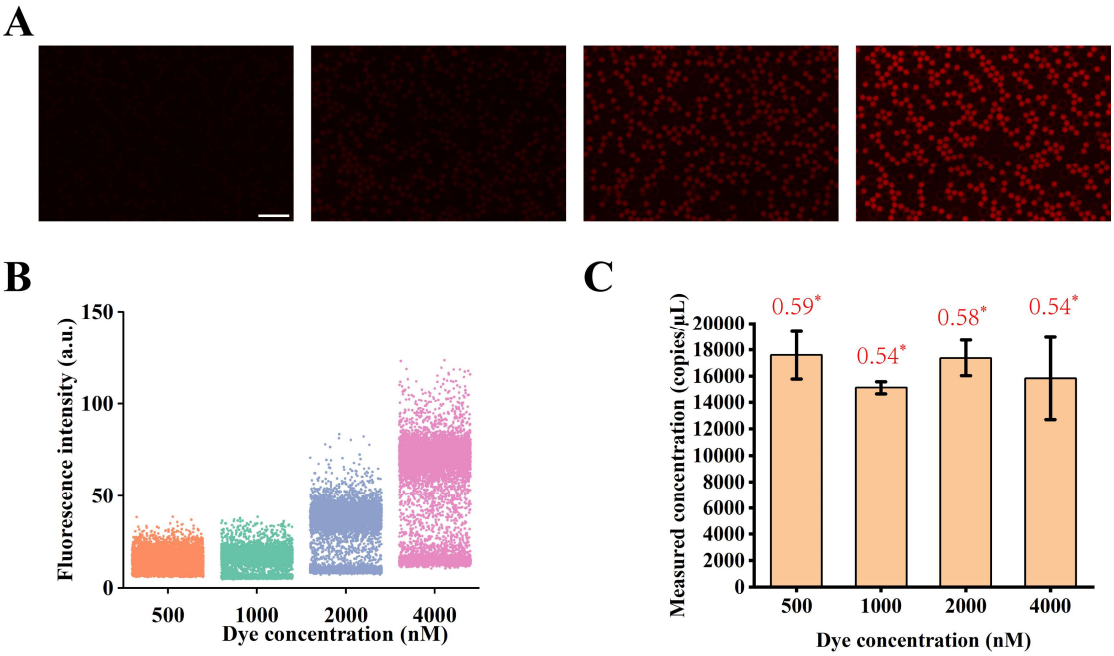

Fig. S5 Optimization of dye concentration for ddLAMP. (A) Droplet fluorescence in ddLAMP at different dye concentrations (dye concentration form left to right is 500 nmol·L^{-1}, 1000 nmol·L^{-1}, 2000 nmol·L^{-1} and 4000 nmol·L^{-1}, respectively) (Scale bar: 200 μm); (B) Distribution of droplet fluorescence in ddLAMP at different dye concentrations; (C) * the proportion of positive droplets , Quantitation of *E. coli* DNA using ddLAMP at different dye concentrations.

Supplementary figure S6

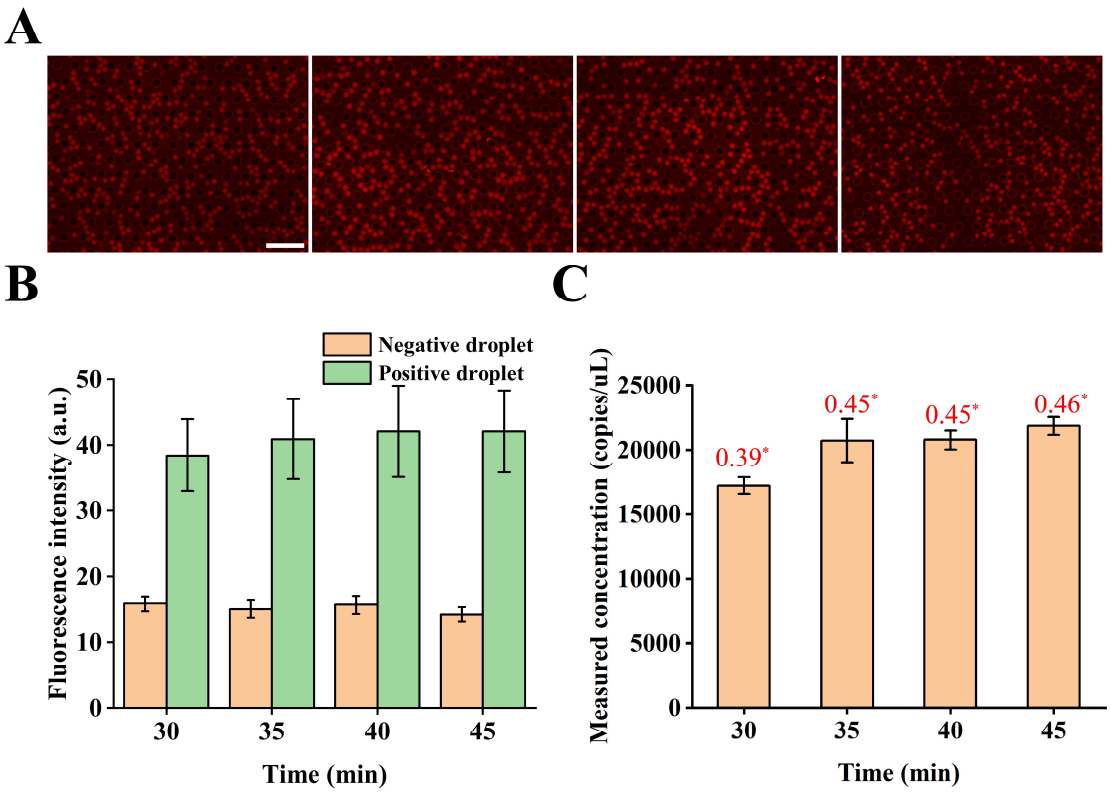

Fig. S6 Optimization of time for the droplet LAMP. (A) Droplet fluorescence detected at 30 min, 35 min, 40 min and 45 min (exposure time: 500 ms, ISO: 400; Scale bar: 200 μm); (B) Increase in fluorescence intensities of positive and negative droplets with the extension of amplification time; (C) * the proportion of positive droplets , Quantitation of *E. coli* DNA depending on the proportion of positive droplets determined at various time points.

Supplementary table S1

Table S1. Sequences of the LAMP primers

Target	Primer sequence (5'-3')
E. coli (malB gene) [1]	F3: GCCATCTCCTGATGACGC B3: ATTTACCGCAGCCAGACG FIP: CATTTTGCAGCTGTACGCTCGCAGCCCATCATGAATGTTGCT BIP: CTGGGGCGAGGTCGTGGTATTCCGACAAACACC ACGAAT T LF: CTTTGTAACAACCTGTCATCGACA LB: ATCAATCTCGATATCCATGAAGGTG
P. aeruginosa (oprI gene) [2]	F3: CTGGCTGCTGTTCTGG B3: CGCTCGTTAGCCTCGT FIP: CTGCGTCTTCGGTAGCGGGGTTGCAGCAGCCACT BIP: TCAGGCTCGCGCTGACGAAGTCTGCTGAGCTTTCTG AG LF: TCTTTGGCTTCGAGCAGACT LB: GCCTATCGCAAGGCTGACGAA
K. pneumoniae (rcsA gene) [3]	F3: GGATATCTGACCAGTCGG B3: GGGTTTTGCGTAATGATCTG FIP: CGACGTACAGTGTTTCTGCAGTTTTAAAAAACA GGAAATCGTTGAGG BIP: CGGCGGTGGTGTTTCTGAATTTTGCGAATAATG CCATTACTTTC LB: GAAGACTGTTTCGTGCATGATGA
E. faecalis (azoA gene) [4]	F3: GCCGGAAATCGATGAAGA B3: TCCAGCAACGTTGATTGT FIP: CACTTTTTGTTGTTGGTTTTCGCTTTATTATCTGCT TGGGGTGC BIP: ATCTGCAGACAA AGTAGTAATTGCTCCAAGCTT TTAAGCGTGTC LF: AAATGCTGCGCCAGCTCG LB: TCCAATGTGGAACTTAAACGTACC

Supplementary table S2

Table S2. Determination of limit of blank (LOB) and limit of detection (LOD) of the imDDNA

	False positive events			
	Eco	*Pae*	*Kpn*	*Efa*
NTC-1	0	0	0	0
NTC-2	0	2	0	1
NTC-3	1	0	0	0
NTC-4	0	0	0	0
NTC-5	0	0	0	0
NTC-6	0	1	0	1
NTC-7	1	0	1	0
NTC-8	2	0	0	1
NTC-9	0	0	0	0
NTC-10	0	0	0	0
NTC-11	0	0	0	0
NTC-12	0	1	0	0
NTC-13	2	0	0	0
NTC-14	0	1	0	1
NTC-15	0	0	0	0
NTC-16	0	0	1	1
NTC-17	2	0	0	1
NTC-18	0	1	0	0
NTC-19	0	0	0	0
NTC-20	0	0	0	0
False positive ratio (λ_{FP})[a)]	0.4	0.3	0.1	0.3
LOB[b)] (copies/uL)	2	2	1	2
LOD[b)] (copies/uL)	7	7	4	7

[a)] λ_{FP} is the mean number of false-positive events obtained in all NTC experiment;[b)] Using λFP, we calculated the LOB and LOD for the imDDNA assays with the statistical analysis method in reference [5]

Supplementary table S3

Table S3. Consistency between imDDNA and the bacterial culture assay for bacterial detection

		imDDNA		Total
		Positive	Negative	
Bacteria culture	Positive	13	0	13
	Negative	1	9	10
Total		14	9	23

Supplementary table S4

Table S4. Identification of uropathogens

Sample ID	Pathogen	Quantitation	
		Bacterial culture ($CFU \cdot mL^{-1}$)	imDDNA ($copies \cdot mL^{-1}$)
1	*E. coli*	8.0×10^4	1.64×10^6
2	*E. coli*	5.0×10^4	6.38×10^7
3	ND	Negative	Negative
4	*P. aeruginosa*	$>1.0\times10^5$	5.24×10^7
5	ND	Negative	Negative
6	ND	Negative	Negative
7	*P. aeruginosa*	1.0×10^4	6.84×10^7
	E. faecalis	1.0×10^4	9.03×10^4
8	*K. pneumoniae*	Negative	1.05×10^5
9	*E. coli*	$>1.0\times10^5$	5.89×10^7
10	*E. coli*	8.0×10^4	4.67×10^7
11	*E. coli*	$>1.0\times10^5$	4.29×10^7
12	ND	Negative	Negative
13	*P. aeruginosa*	1.0×10^4	1.31×10^7
	E. faecalis	3.0×10^4	1.14×10^6
14	ND	Negative	Negative
15	*E. faecalis*	7.0×10^4	2.27×10^6
16	*K. pneumoniae*	5.0×10^4	1.60×10^5
17	ND	Negative	Negative
18	*E. coli*	$>1.0\times10^5$	3.62×10^7
19	ND	Negative	Negative
20	*P. aeruginosa*	8.0×10^4	1.61×10^7
	E. faecalis	8.0×10^4	8.09×10^5
21	ND	Negative	Negative
22	*K. pneumoniae*	6.0×10^4	7.77×10^7
23	ND	Negative	Negative

ND: Not detected

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