

String-Powered Microfluidic Chip Integrating Heparin-Mediated One-Pot RT-RPA/CRISPR-Cas12a for Multiplex Detection of HFMD Viruses

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Table S1. Primers for plasmid linearization containing the T7 promoter

Target	ID	Sequences (5'-3')
EV-A71	EV-A71-F	GAAATTAATACGACTCACTATAGGGGCAGTTGAATTGG TGGATGAG
	EV-A71-R	TCGTCCTTGACATAAGTGGAGTAA
CV-A16	CV-A16-F	GAAATTAATACGACTCACTATAGGGATCCCATTGCTGA CATGATCG
	CV-A16-R	GTACTGGTACACTTGATGTCATTCC
CV-A6	CV-A6-F	GAAATTAATACGACTCACTATAGGGCAAATGCAGTAGA AAGCGCTGTGA
	CV-A6-R	TGGCTGTATGTCGGATAGTTCTTGA
CV-A10	CV-A10-F	GAAATTAATACGACTCACTATAGGGTACCACTCCCAGT TCACACCGAC
	CV-A10-R	GCTTGCTTGATACTGGAACGGTC

Table S2. Sequences of RT-RPA amplification primers

Target	ID	Sequences (5'-3')
EV-A71	F-1	CACTATATGAGCCTGACGAATACATCAAAGAG
	R-1	TGACATAAGTGGAGTAAGGGAGATCAAGAC
	F-2	GGAAATACACTATATGAGCCTGACGAATAC
	R-2	GGAGTAAGGGAGATCAAGACCATATTTATC
	F-3	GATGAGCAAGGAGTCAACCTGGAACATA
	R-3	CTTACCACTGAGATTCAAGAAGCCATACTG
	F-4	GCAAGGAGTCAACCTGGAACATAACC
	R-4	ACCGCCAATGTGAATACCAATAACCT
CV-A16	F-1	TGTGAATAACCAAGTGAACCGCTCCTTA
	R-1	CTAAAGAAATTCCCGATGGCTGTCTCCT
	F-2	GTACACAGAACACAGACGGTTACGTCAACTG
	R-2	AACGAATCTCTGGAAGTGGGTTTCGGAGCC
	F-3	GAATAACCAAGTGAACCGCTCCTTAACC
	R-3	CTAAAGAAATTCCCGATGGCTGTCTCCT
	F-4	GTGAATAACCAAGTGAACCGCTCCT
	R-4	CGTAACCGTCTGTGTTCTGTGTACC
CV-A6	F-1	GATAGCAGGAAGTCATACCAATGGCAGACT
	R-1	CAAGCTCTCACGTGCTTAATTCTCATGTA
	F-2	GCCATTTTGCTATCCGAACAGTCAGTGAAT
	R-2	TAATCCGTGGTGGTTATGCTTGACGATCAG
	F-3	AGCCAGATAGCAGGAAGTCATACCAATG
	R-3	GTAGATTGTTTGTGTTACCAAAATGTAGGG
	F-4	CGCTTGCCGACACCACAATATCC
	R-4	CCTTCACCTCCACAACCTCCTACCA
CV-A10	F-1	GGTATGCTACATGGGACATAGACATTATGGG
	R-1	CTGGTGACATGAAAGGAACTGAGACTTGT
	F-2	ACTTTCGGCCAGCACCCGGAGACCTCAAACACAAC
	R-2	CTTGGGACCCAGGCCCTCACATGCTTAA
	F-3	CCGTTCTGGATTAGTGGGAGTGATTAAC
	R-3	AGTGAGTTTGACAAAGACTGATGGGTAG
	F-4	TGCCACTTCTAATGCCACAGATGAG
	R-4	GTACGGACGAGCCTCTCCATTCT

Table S3. Sequences of crRNA and ssDNA reporters

Oligonucleotide	Sequences (5'-3')
EV-A71	UAAUUUCUACUAAGUGUAGAUGGGUAUCACCAAGUUCAUC
CV-A16	UAAUUUCUACUAAGUGUAGAUGGCACUGGUGUUGUCCCAGC
CV-A6	UAAUUUCUACUAAGUGUAGAUCAAGCCGCAGAAACGGGAGC
CV-A10	UAAUUUCUACUAAGUGUAGAUCAGUGAGAGUUGUUAGUAAA
FAM-ssDNA-BHQ	FAM-TTATT-BHQ1

Table S4 PCR amplification primers

Target	ID	Sequences (5'-3')
EV-A71	F	GCAAGGAGTCAACCTGGAACCTAACC
	R	ACCGCCAATGTGAATACCAATAACCT
CV-A16	F	GTGAATAACCAAGTGAACCGCTCCT
	R	CGTAACCGTCTGTGTTCTGTGTACC
CV-A6	F	CGCTTGCCGACACCACAATATCC
	R	CCTTCACCTCCACAACCTCCTACCA
CV-A10	F	TGCCACTTCTAATGCCACAGATGAG
	R	GTACGGACGAGCCTCTCCATTCT

Table S5. Evaluation of one-pot assay strategies

Name	Method category	Core strategy	LOD	Time	Cost/1000 reactions (\$)	Pros	Cons	Ref
SHERLOCK	Two-step	Amplification first, followed by addition of the cleavage system	0.1 aM	> 1 h	625.8	High sensitivity without competitive inhibition	Aerosol contamination ; laborious operation	Gootenberg ¹
DETECTR	Two-step		1 aM	> 1 h	442.8			Chen ²
NA	Physical separation	Glycerol phase separation	100 aM	1 h	452.26-509.33	Low reagent cost with good universality	Increased system viscosity; requires careful handling	Lin ³
SPOC	Physical separation	Paraffin encapsulation of lyophilized CRISPR reagents	1 copy/ μ L	22 min	1770	Low sensitivity and short reaction time	Requires specific operation and precise detection instruments	Hu ⁴
NA	Enhanced RPA efficiency	RNase H enhances RPA efficiency	200 copy/ μ L	5-30 min	532.34-961.72	Broad applicability	Reaction system requires fine optimization	Feng ⁵
CORDS v2	Enhanced RPA efficiency	System optimization leads to reduced Cas12a activity	6 copy/ μ L	1 h	NA	Rapid design of amplification primers and crRNA	Integrated device does not involve sample pretreatment	Lin ⁶
NA	crRNA structural modification	crRNA-modified ssDNA	5 aM	35 min	NA	Excellent visual detection performance	Complex crRNA design increases synthesis cost	Zeng ⁷
NA	crRNA structural modification	Hybridization-protective nucleotides in crRNA	10 copy/ μ L	30 min	NA	Cas12a activity controlled by LED illumination	High requirements for reagent storage and operation	Hu ⁸

sPAMC	crRNA structural modification	Use of suboptimal PAM sequences to reduce Cas12a activity	2.34 fM	<20 min	439.66	No special reagents required	Limited universality due to target sequence dependence	Lu ⁹
DESCRIBER	crRNA structural modification	DNase-mediated signal amplification cascade	0.5 copy/μL	1 h	NA	Extremely high specificity	Complex design with high cost	Huang ¹⁰
NA	One-pot assay	Heparin sodium regulates Cas12a activity	10 aM	1 h	439.7	Low cost; reagents require only simple mixing	Limited shelf life of pre-mixed reagents	This study

Note: The reagent cost estimations for the SHERLOCK, DETECTR, and the present study were primarily derived from the work of Cheng et al.¹¹ For studies involving additions of components such as glycerol or RNase H to the reaction system, the cost of heparin sodium used in Cheng et al. was replaced with the corresponding reagent. Price ranges of these substituted reagents were retrieved from commercial suppliers' databases, and all values were converted using the 2025 exchange rate. For studies employing suboptimal PAM sequences to regulate Cas12a activity, the cost of heparin sodium was excluded, and potential losses from crRNA validation or optimization procedures were not considered. All other cost data were directly obtained from the corresponding reports.

Table S6. Performance comparison of centrifugal microfluidic chip centrifuges and simple manual spinning devices

Device	Drive Mechanism	Power Source	Max Speed/RCF	Directional Control	Speed Stability	Cost (\$)	Ref
Custom centrifugal device	Electromagnetic	Battery	10,000	Bidirectional alternating rotation	Constant-speed type	5,802	YoungChip-CP360
	Electromagnetic	Battery	10,000	Bidirectional alternating rotation	Constant-speed type	16,680	CentriDx [®] 200
	Electromagnetic	Battery	900	Bidirectional alternating rotation	Constant-speed type	2707	RSB120AT-T3A
Hand-driven fan	Gear Train	Human Power	3,500	Unidirectional rotation	Constant-speed type	2.17	Xu, Li ^{12,13}
Fidget spinner	Ball Bearing	Human Power	1,000	Unidirectional rotation	Deceleration-controlled type	2.9	Michael ¹⁴
Hand-held spinning	Pull-string / Torsional Recoil	Human Power	Not reported (estimated 10 ³ -10 ⁴)	Unidirectional rotation	Pulsed rotation type	1.74	Li ¹⁵
Paperfuge	Supercoils	Human Power	125,000	Bidirectional alternating rotation	Pulsed rotation type	0.87	Bhamla ¹⁶
HICAS-Chip	Supercoils	Human Power	About 5,000-8,000	Bidirectional alternating rotation	Pulsed rotation type	0.87	This study

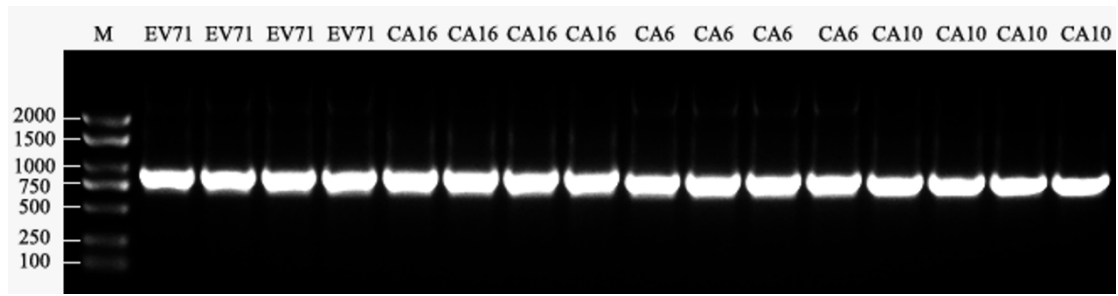


Figure S1. Plasmid linearization and gel purification. The forward primer contained a T7 promoter sequence recognizable by T7 RNA polymerase, enabling plasmid linearization through PCR amplification. The PCR products were analyzed and purified by agarose gel electrophoresis. Only bright, single bands consistent with the expected plasmid size were selected for subsequent recovery and in vitro transcription experiments. PCR-amplified linearized plasmids were separated on a 1.2% agarose gel under electrophoresis conditions of 120 V for 40 min.

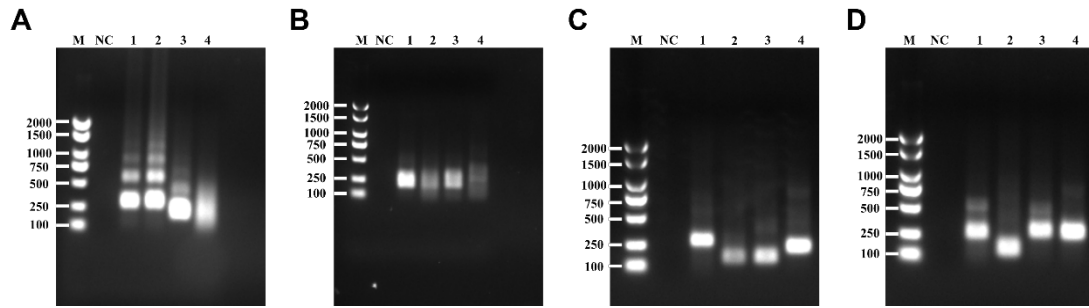


Figure S2. Screening of RT-RPA primers targeting four enteroviruses (EV-A71, CV-A16, CV-A6, and CV-A10). RT-RPA products were purified using an equal volume of phenol/chloroform (1:1) and analyzed via 1.5% agarose gel electrophoresis for 40 min. (A-D) Amplification performance of various primer sets for the four viruses. Results indicated that the third primer set for EV-A71 (A) and the first primer set for CV-A16 (B) yielded distinct, sharp bands without significant smearing; thus, these were selected as the target regions for subsequent crRNA design. For CV-A6 (C) and CV-A10 (D), despite multiple primer sets showing high amplification efficiency, the fourth and second primer sets were respectively chosen based on the higher density of potential crRNA recognition sites within their amplified regions.

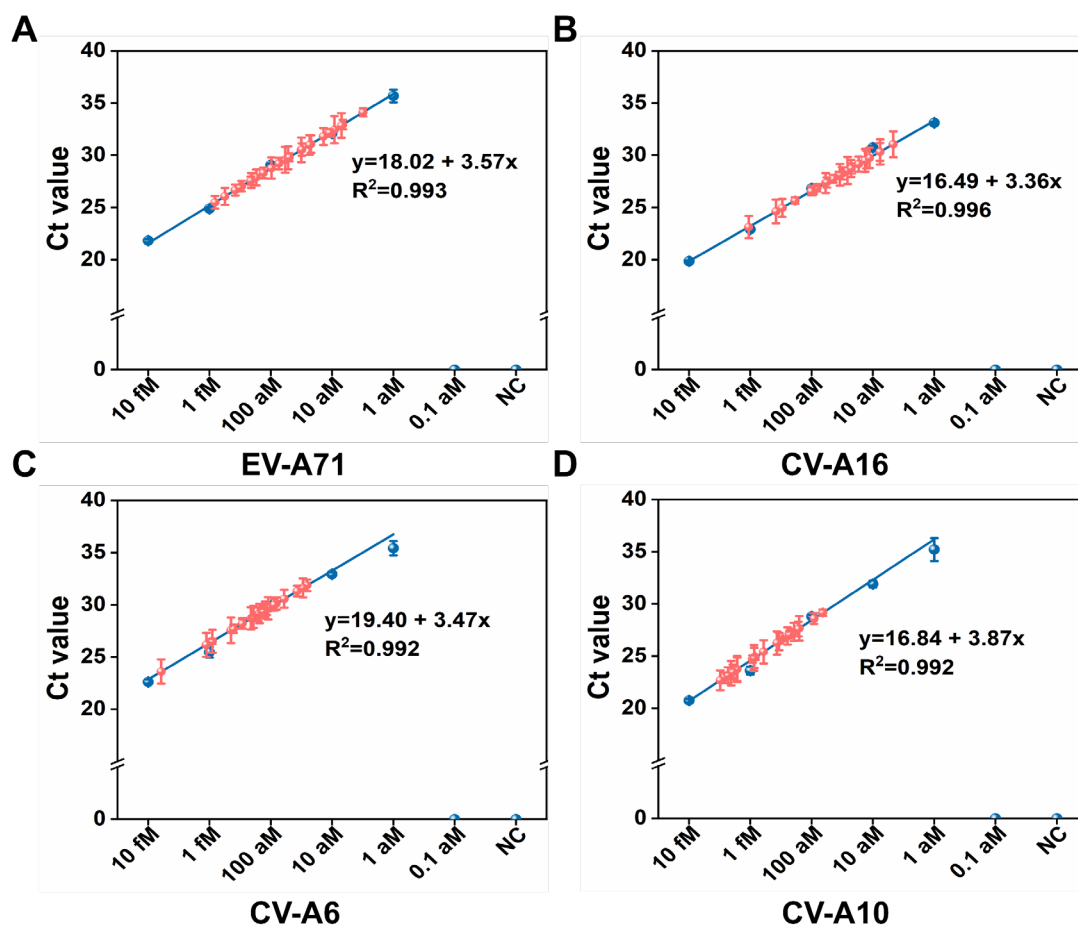


Figure S3. PCR standard curves and detection of actual wastewater samples. Standard plasmids corresponding to four enterovirus subtypes-EV-A71, CV-A16, CV-A6, and CV-A10-were serially diluted to establish PCR standard curves within the concentration range of 10 fM to 0.1 aM. A total of 24 wastewater samples were subsequently analyzed using the same assay conditions. The results showed that the concentrations of these four viral subtypes in wastewater were predominantly distributed within the range of 1 aM to 1 fM. (A) EV-A71 PCR standard curve and detection results. (B) CV-A16 PCR standard curve and detection results. (C) CV-A6 PCR standard curve and detection results. (D) CV-A10 PCR standard curve and detection results. Data are presented as mean $\pm$ SD from three independent experiments.

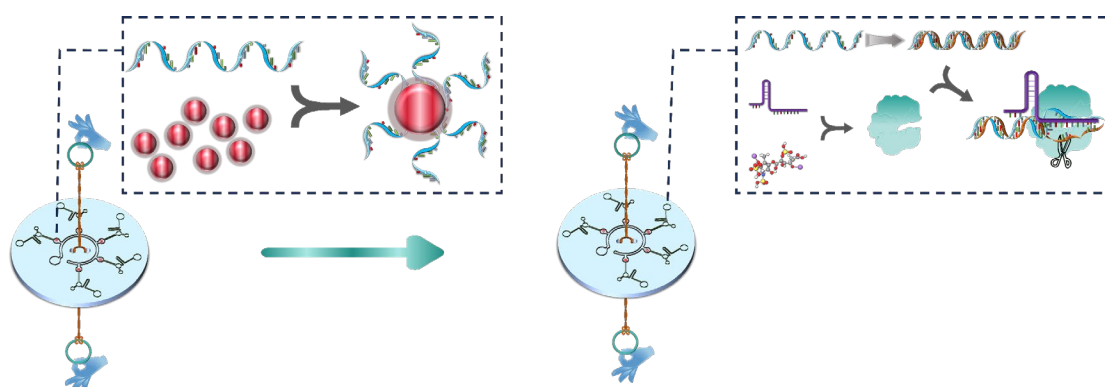


Figure S4. Schematic illustration of the one-pot assay workflow on chip. Purified nucleic acids captured by magnetic beads are eluted into the reaction chamber, where they are mixed with preconfigured one-pot RT-RPA/CRISPR-Cas12a reagents. The subsequent reaction generates fluorescence signals, enabling rapid identification and typing of HFMD viral targets.

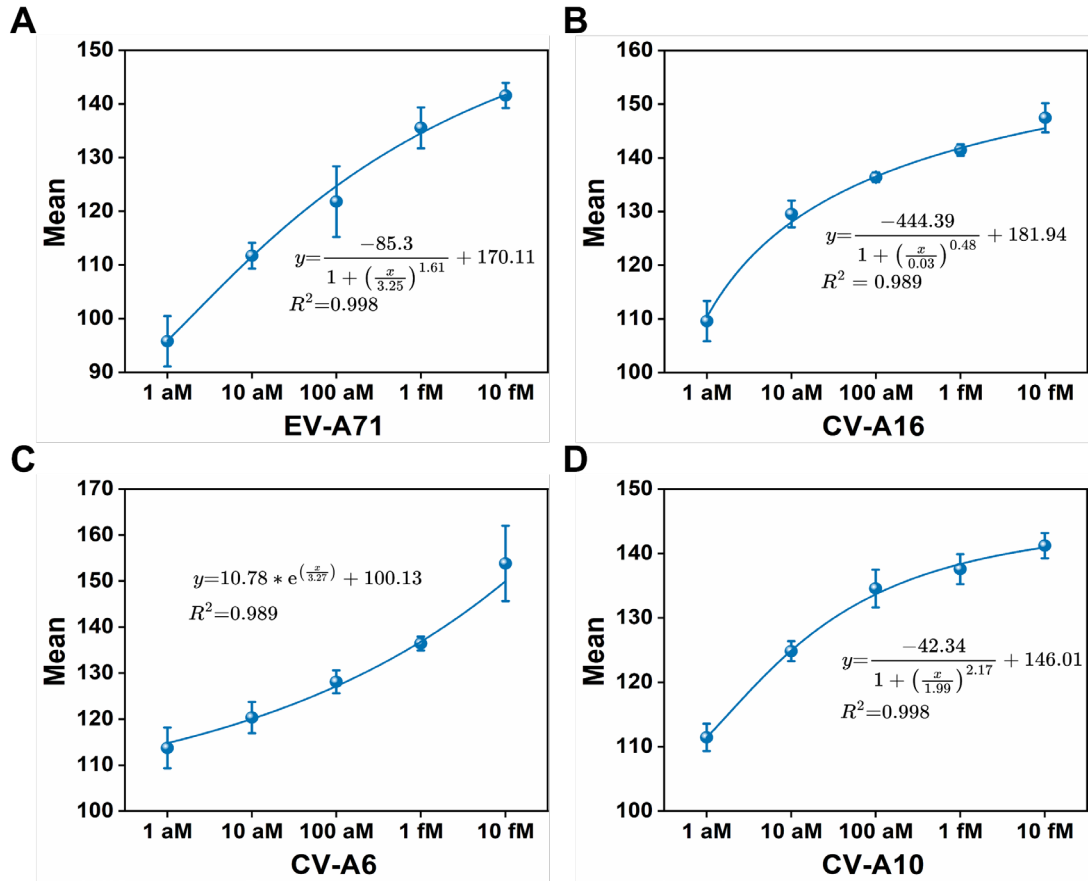


Figure S5. Standard curves of the HICAS-Chip assay. In vitro-transcribed RNAs of EV-A71, CV-A16, CV-A6, and CV-A10 were used as templates. After quantification, the RNA samples were serially diluted from 10 fM to 1 aM and subjected to one-pot assay on the HICAS-Chip. Fluorescence intensities were quantified using ImageJ software, and standard curves were established through nonlinear regression fitting. (A) EV-A71 standard curve. (B) CV-A16 standard curve. (C) CV-A6 standard curve. (D) CV-A10 standard curve. Data are presented as mean $\pm$ SD from three independent experiments.

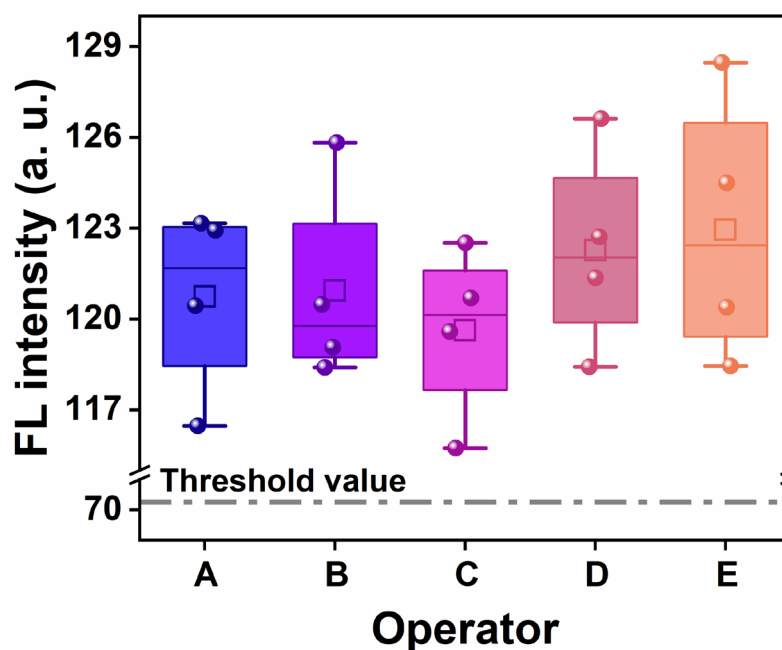


Figure S6. Inter- and intra-assay variability of HICAS-Chip. Five independent operators (three males and two females, aged 20-40 years) performed the detection procedure using the same RNA sample (EV-A71, 100 aM), with four replicate experiments conducted by each operator ($n = 4$). One negative control was included for threshold determination. Fluorescence intensities were quantified from the reaction chambers using ImageJ based on mean RGB values. The dashed line indicates the detection threshold defined as the mean fluorescence intensity of the negative control plus three standard deviations. All twenty measurements produced fluorescence signals above the threshold, corresponding to a success rate of 100%, with an overall CV of 2.74%.

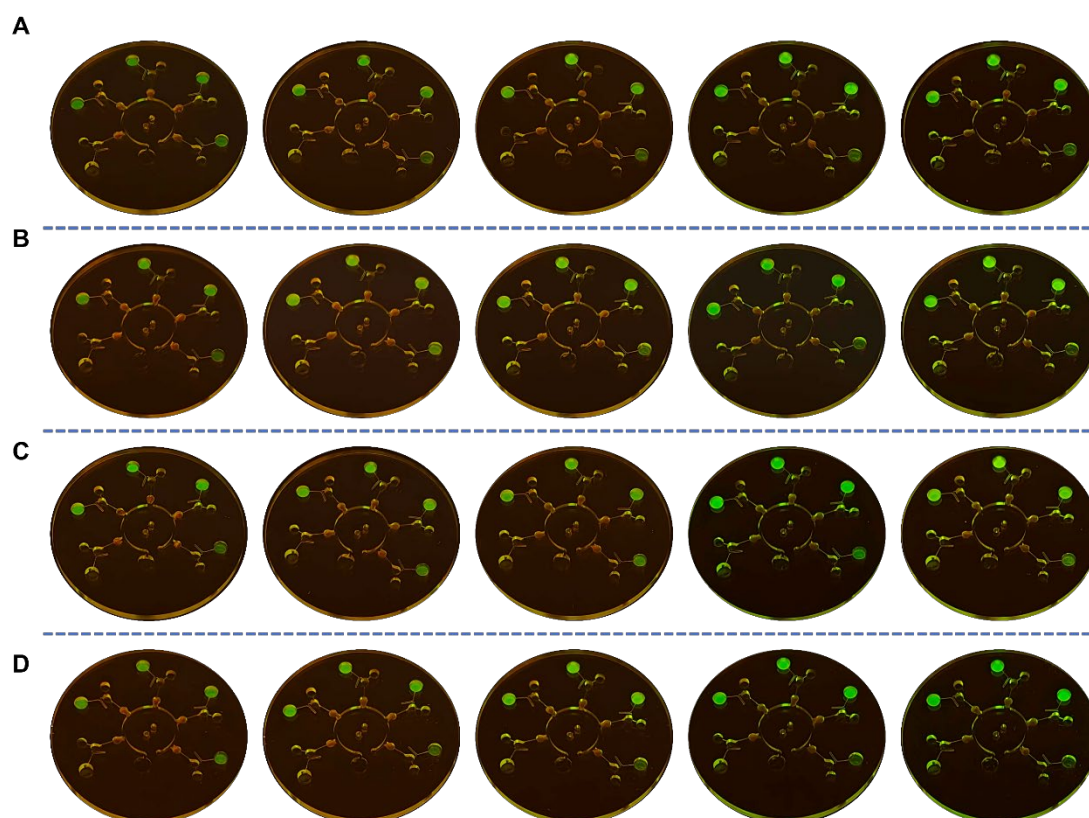


Figure S7. Visualization of HICAS-Chip detection at different target concentrations. Fluorescence images show the detection performance of the HICAS-Chip for four enterovirus subtypes across a concentration range from 1 aM to 10 fM. (A) Detection of EV-A71 from 1 aM to 10 fM. (B) Detection of CV-A16 from 1 aM to 10 fM. (C) Detection of CV-A6 from 1 aM to 10 fM. (D) Detection of CV-A10 from 1 aM to 10 fM.

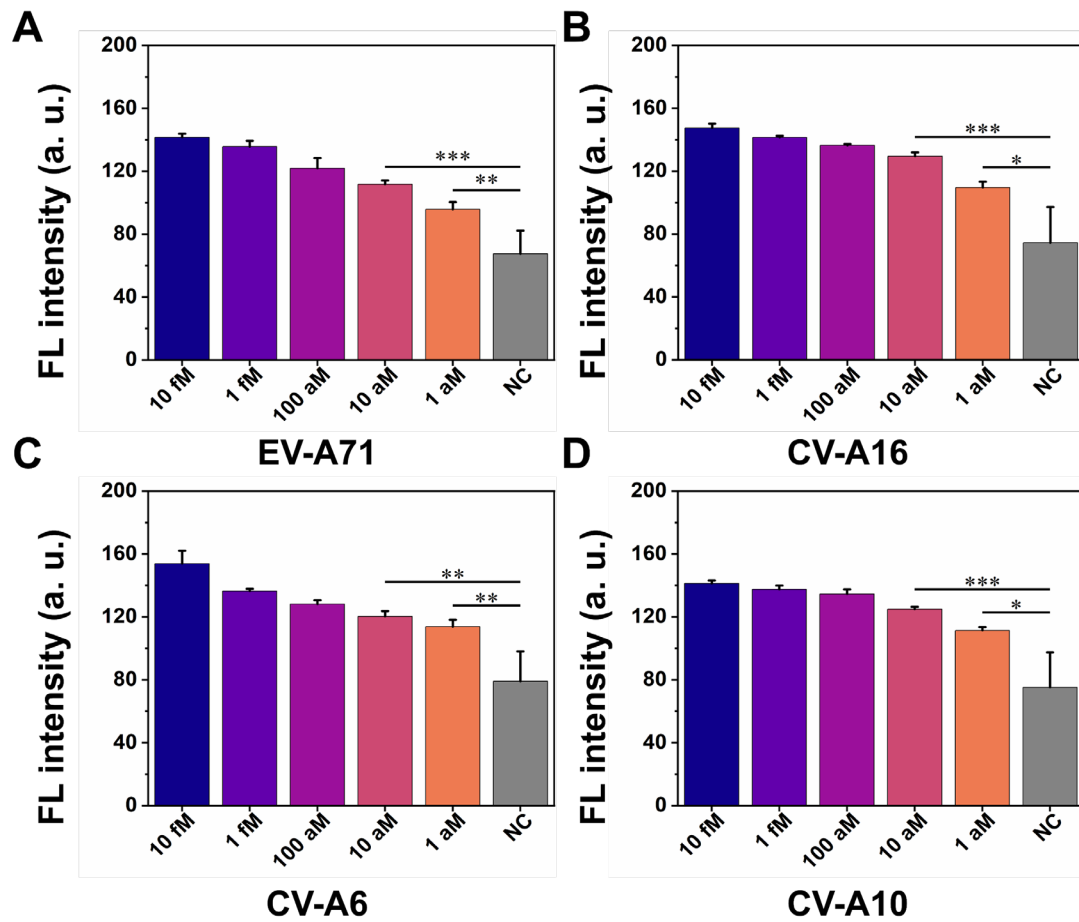


Figure S8. Quantitative fluorescence analysis of HICAS-Chip detection at different target concentrations. Fluorescence images were analyzed using ImageJ, and fluorescence intensity was calculated using the weighted RGB formula ($0.299R + 0.587G + 0.114B$). (A-D) Quantitative fluorescence intensities of HICAS-Chip detection for target concentrations ranging from 10 fM to 1 aM for (A) EV-A71, (B) CV-A16, (C) CV-A6, and (D) CV-A10. The negative control (NC) is presented as mean $\pm$ 3SD. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance compared with the negative control is indicated as: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

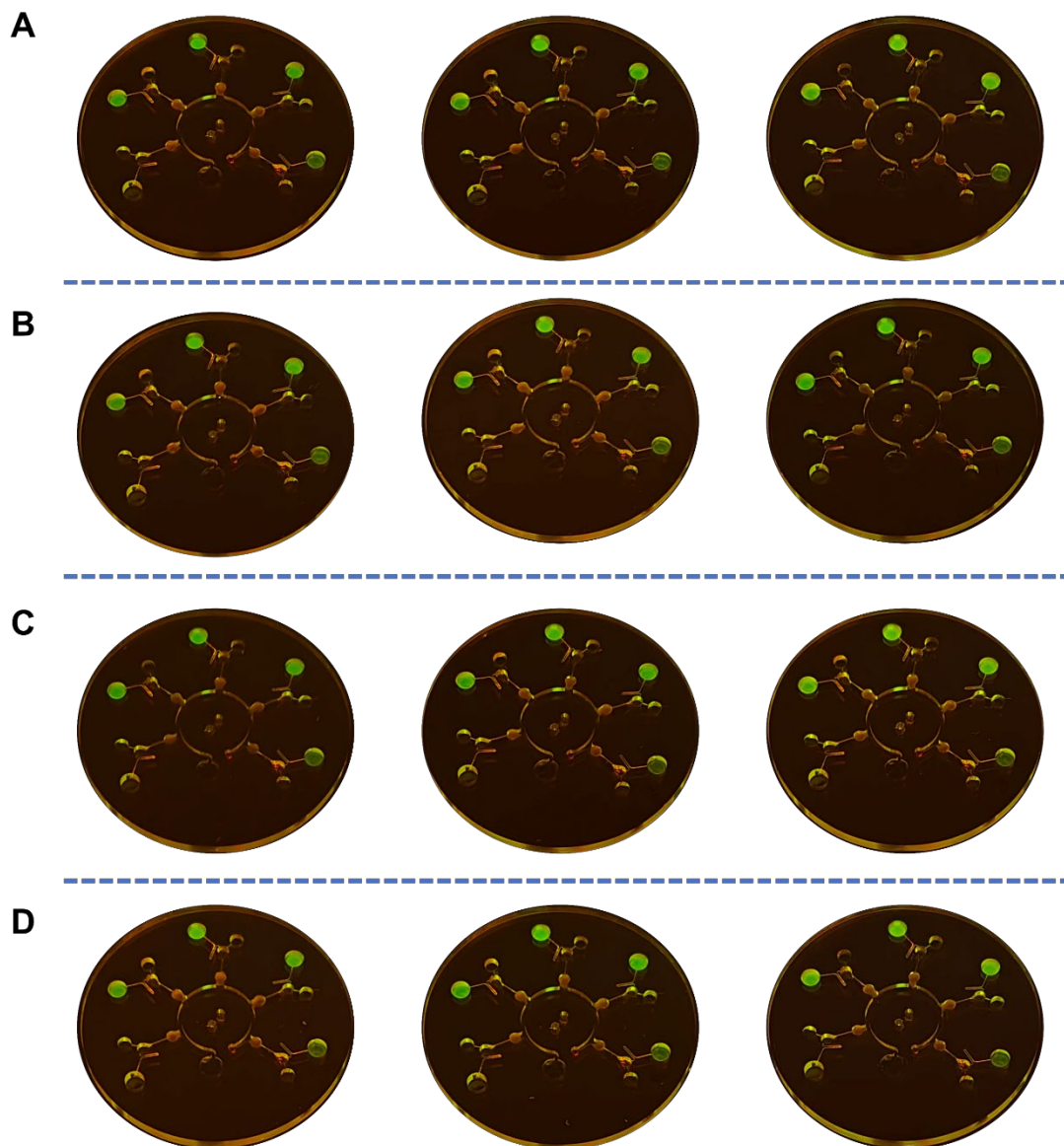


Figure S9. Fluorescence visualization of HICAS-Chip spike-and-recovery tests. Fluorescence images illustrate the detection performance of the HICAS-Chip for four enterovirus subtypes (EV-A71, CV-A16, CV-A6, and CV-A10) in January wastewater samples spiked with RNA at concentrations of 10 aM, 100 aM, and 1 fM. (A) EV-A71 recovery from 10 aM to 1 fM. (B) CV-A16 recovery from 10 aM to 1 fM. (C) CV-A6 recovery from 10 aM to 1 fM. (D) CV-A10 recovery from 10 aM to 1 fM.

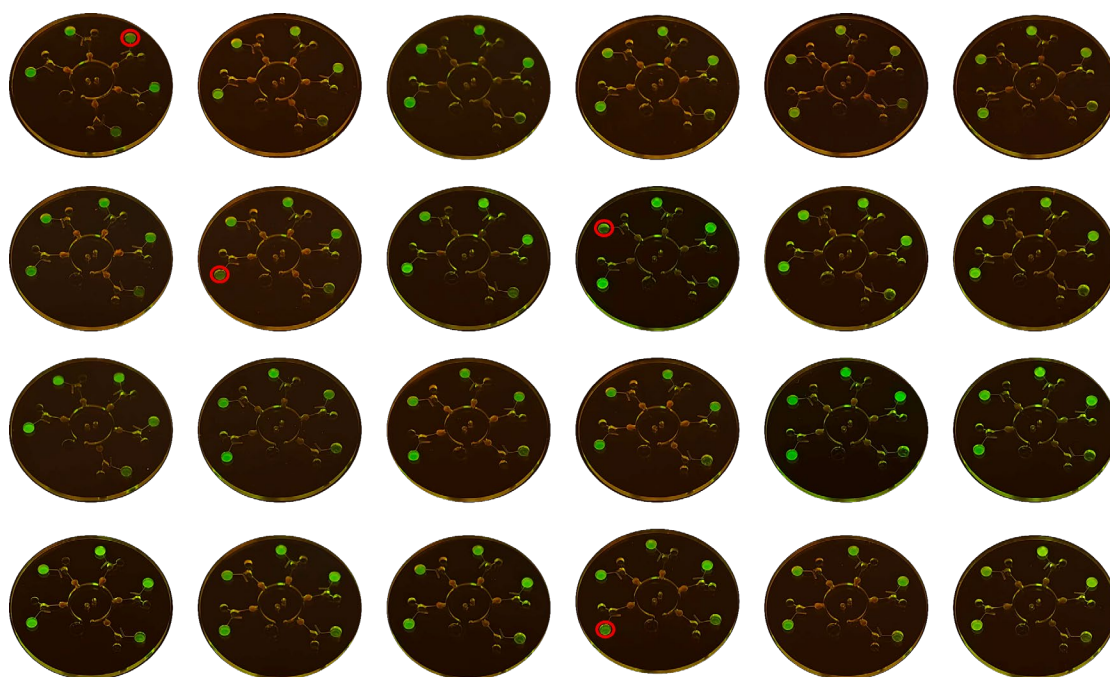


Figure S10. Detection of 24 wastewater samples using the HICAS-Chip. Fluorescence imaging results of 24 wastewater samples analyzed with the HICAS-Chip. Samples showing no detectable fluorescence signal were identified as negative and are marked with red circles for clarity.

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