Supplementary Information

Dual detection of hypervirulent genes of *Klebsiella pneumoniae* using a single CRISPR-Cas12a system modulated by entropy-driven circuits

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Table S1 Sequences used in this work

EDC	Name	Sequence（5'-3'）
EDC p1	scaffold p1	CAATGGATGTGGCTTGACGTTTCGGAGGGCGCCG TAAGTTAGTTGGAGACGTAGG
	reporter p1	cy3-CCCTCCGAAACGTCAAGCCA-BHQ2
	trigger p1	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p1	TCTCCAACTAACTTACGGCGCCCTCCGAAACGTC AAGCCACAT
	rmpA p1	CCGAAACGTCAAGCCACATCCATTG
EDC p2	scaffold p2	GGAAAGGACAGAAAGCCAGTGGACGACCGCCCC GTAAGTTAGTTGGAGACGTAGG
	reporter p2	BHQ1-CGGTCGTCCACTGGCTTTCT- FAM
	trigger p2	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p2	TCTCCAACTAACTTACGGGGCGGTCGTCCACTGG CTTTCTGTC
	peg-344 p2	CGTCCACTGGCTTTCTGTCCTTTCC
EDC p3	scaffold p3	AGGGAAATGGGGAGGGTACAAAATGAGGGCGCC GTAAGTTAGTTGGAGACGTAGG
	reporter p3	GCCC/iBHQ2dT/CATTTTGTACC/iCy3/CTCCC
	trigger p3	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p3	TCTCCAACTAACTTACGGCGCCCTCATTTTGTACC CTCCCC
	rmpA p3	CATTTTGTACCCTCCCCATTTCCCT
EDC p4	scaffold p4	GGAGGCGCGGGAAAGGACAGAAAGCAGAGGCCC GTAAGTTAGTTGGAGACGTAGG
	reporter p4	CCTCTGC/iBHQ1dT/TTCTGTCCTT/i6FAMdT/CC
	trigger p4	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p4	TCTCCAACTAACTTACGGGCCTCTGCTTTCTGTCC TTTCCC
	peg-344 p4	GCTTTCTGTCCTTTCCCGCGCCTCC
EDC p5 (EDC1)	scaffold p5	AGGGAAATG-iBHQ2- GGGAGGGTACAAAATGAGGGCGCCGTAAGTTAGT TGGAGACGTAGG
	reporter p5	BHQ2-GCCCTCATTTTGTACCCTCCC-Cy3

	trigger p5	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p5	UCUCCAACUAACUUACGGCGCCCUCAUUUUGUA CCCUCCCC
	rmpA p5	CAUUUUGUACCCUCCCCAUUUCCCU
EDC p6 (EDC2)	scaffold p6	GGAGGCGCG-iBHQ1-GGAAAGGACAGAAAGCAGAGGCCCGTAAGTTAG TTGGAGACGTAGG
	reporter p6	BHQ1-CCTCTGCTTTCTGTCCTTTCC-FAM
	trigger p6	AGTCCTACGTCTCCAACTAACTTACGG
	fuel p6	UCUCCAACUAACUUACGGGCCUCUGCUUUCUGU CCUUUCCC
	peg-344 p6	GCUUUCUGUCCUUUCCCGCGCCUCC
	crRNA	UAGUUGGAGACGUAGGACUUAGAUGUGAAUCAU CUUUAAU
	uge-mRNA	CAAGUUCACGAAAGCGAUGCUGGAA
	fimH-mRNA	GAAACGAUCACCGACUACGUGACCC
	iroB-mRNA	GUUUGCAUCACGACCGGGAUAUCGUA

Table S2. EDCs combined with CRISPR-Cas12a for *peg-344* and *rmpA* assays

	Reagent	Volume
EDC1	scaffold1（10 μmol/L）	1 μL
	repoter1（10 μmol/L）	1 μL
	trigger（10 μmol/L）	1 μL
	fuel1（10 μmol/L）	1 μL
EDC2	scaffold2（10 μmol/L）	1 μL
	trigger（10 μmol/L）	1 μL
	repoter2（10 μmol/L）	1 μL
	fuel2（10 μmol/L）	1 μL
CRISPR-Cas12a	crRNA（10 μmol/L）	1 μL
	Cas12a（10 μmol/L）	2 μL
target (*rmpA*-mRNA or *peg-344*-mRNA）	*rmpA*-mRNA（2, 0.2, 0.02, 0.002, 0.0002, 0.00002 nmol/L）	1 μL
	peg-344-mRNA（2, 0.2, 0.02, 0.002, 0.0002, 0.00002 nmol/L）	1 μL
	10×NEbuffer r2.1	2 μL
	DEPC	6 μL

Table S3. Repeatability of EDC/CRISPR-Cas12a assay system for *rmpA*

Concentration	Fluorescence intensity（a.u.）						RSD（%）
	1	2	3	4	5	6	
100 pmol/L	4105	4288	4145	3988	4191	4379	3.30

Table S4. Repeatability of EDC/CRISPR-Cas12a assay system for *peg-344*

Concentration	Fluorescence intensity（a.u.）						RSD（%）
	1	2	3	4	5	6	
100 pmol/L	5110	4990	5219	5080	4991	4879	2.33

Table S5. Recovery rate of *rmpA* detected by EDC/CRISPR-Cas12a system

Samples	1	2	3
Concentration 1	5 fmol/L	500 fmol/L	50 pmol/L
Concentration 2	4.64 fmol/L	488.98 fmol/L	49.36 pmol/L
Recovery rate	92.80%	97.80%	98.72%

Table S6. Recovery rate of *peg-344* detected by EDC/CRISPR-Cas12a system

Samples	1	2	3
Concentration 1	5 fmol/L	500 fmol/L	50 pmol/L
Concentration 2	4.55 fmol/L	492.03 fmol/L	48.01 pmol/L
Recovery rate	91.00%	98.41%	96.02%

Table S7. Comparison of varied nucleic acids detection methods

Method	Target	Assay time	LoD	Ref.
q-PCR	*peg-344*	1-2 h	47.5 pg/μL	1
LAMP	*peg-344*	15-60 min	0.475 pg/μL	1
EDC	DNA	0.5 h	1 pmol/L	2
CRISPR-Cas12a/electrochemical biosensor	DNA	1 h	30 pmol/L	3
CRlSPR/Cas12a Coupled with Strand Displacement Amplification	miRNA	15 min	6.28 pM	4
Catalytic hairpin assembly circuit coupled with CRISPR-Cas12a	microRNA	120 min	100 fmol/L	5
Multiplex gRNAs synergically enhance detection	SARS-CoV-2 RNA	25 min	10 copies	6
EDCs/CRISPR-Cas12a	*rmpA,* *peg-344*	25 min	0.10 fmol/L, 0.17 fmol/L	This work

Designing the scaffold strand for EDCs systems In the initial EDC designs (EDC p1 and EDC p2), the triple-stranded substrate contained scaffold strands with two exposed bases at the toehold sites. To minimize non-specific binding between fuel and scaffold strands by reducing the number of exposed bases, we developed second-generation EDCs (EDC p3 and EDC p4) featuring a one-nucleotide extension of the reporter strand to block the exposed toehold site. Furthermore, to address fluorescence leakage from the reporter in the three-stranded substrate, we introduced additional modifications (EDC p5 and EDC p6) incorporating a quencher on the scaffold strand (Figure S1).

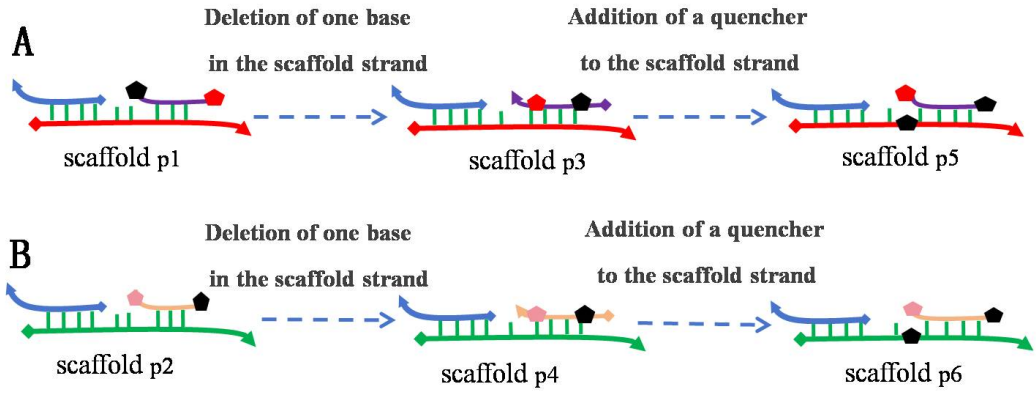

Figure S1 Design of scaffold strand for EDCs

Designing the fuel strand for EDC systems In the third-generation designs (EDCp5 and EDCp6), we replaced the DNA sequence of the fuel strand with an RNA sequence to prevent ssDNA degradation by CRISPR-Cas12a (Figure S2).

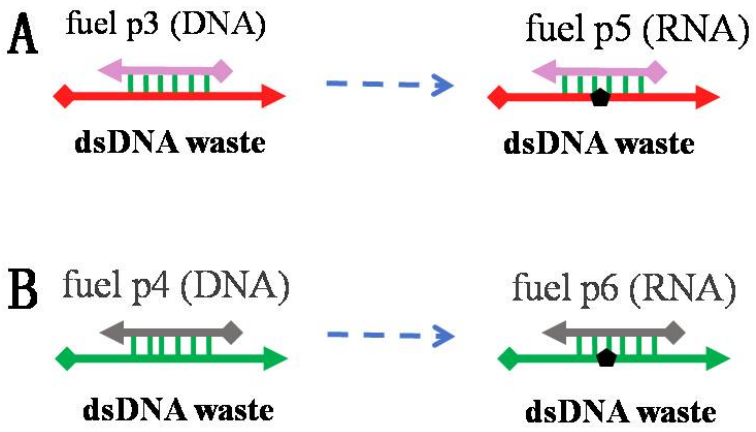

Figure S2 Design of fuel strands for EDCs

Electrophoretic characterization of the optimized EDC systems

Agarose gel electrophoresis (AGE) was employed to investigate the EDC reaction process. As shown in Figure S3, the purified strands of reporter, trigger, and scaffold displayed clear bands in lanes 1, 2, and 3, respectively. Lane 4 showed dsDNA after the assembly of the scaffold strand and reporter strand. After the hybridization of equal concentrations of reporter, trigger and scaffold strands, a three-strand structured substrate appeared in lane 5. Lane 6 showed the fuel strand. Following the addition of an equal concentration of the fuel strand to the three-stranded substrate system, lane 7 displayed a prominent band corresponding to the three-stranded substrate and a weaker band corresponding to the single-stranded fuel strand. These results indicated that the fuel strand did not react with the three-stranded

substrate in the absence of the target. Upon addition of the target, a band with faster migration appeared in lane 8 compared to that in lane 7, indicating that the three-stranded substrate had been converted into dsDNA waste composed of scaffold and fuel strands via the EDC reaction. During the EDC reaction, the reporter and trigger strands were also released, although they could not be clearly observed due to their single-stranded nature and low concentration. These results demonstrate that the EDC process proceeded as expected.

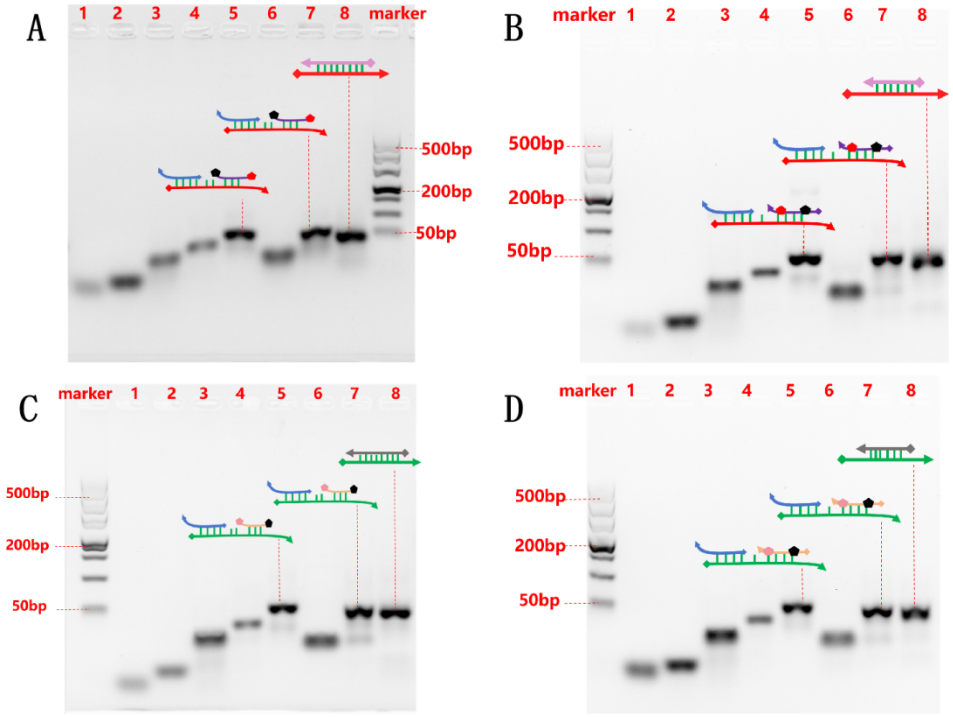

Figure S3 AGE analysis of each EDC corresponding to *rmpA* or *peg-344*. (A) EDC p1 in response to *rmpA p1*, (B) EDC p2 in response to *peg-344 p2*, (C) EDC p3 in response to *rmpA p3*, and (D) EDC p4 in response to *peg-344 p4*. Lane 1: reporter; lane 2: trigger; lane 3: scaffold; lane 4: scaffold + reporter, lane 5: three-stranded DNA complexes consisting of reporter, trigger, and scaffold strands; lane 6: fuel; lane 7: lane6 + fuel; lane 8: lane7 + target.

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