Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Sequential Logic Circuit Built on λ Exonuclease for Cross Inhibition

(Supplementary Information)

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S1. Yes gate

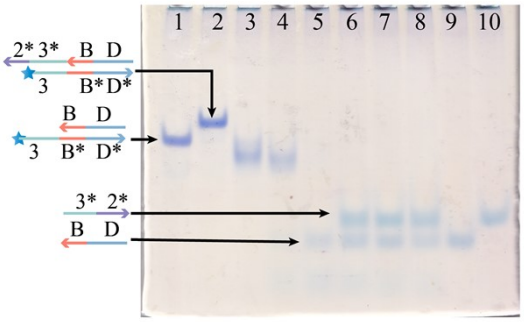

Fig. S1. PAGE gel results of P2 leakage at different concentrations of λ Exonuclease and hydrolysis of P2+Z2 (12% gel): lane 1, P2; lane 2, P2+ Z2; lane 3, P2+1U λ EXO; lane 4, P2+2U λ EXO; lane 5, P2+3U λ EXO; lane 6, P2+ Z2+1U λ EXO; lane 7, P2+ Z2+2U λ EXO; lane 8, P2+ Z2+3U λ EXO; lane 9, Inh 2; lane 10, Z2. [P2] =[Z2] =[Inh 2] = 1 μM..

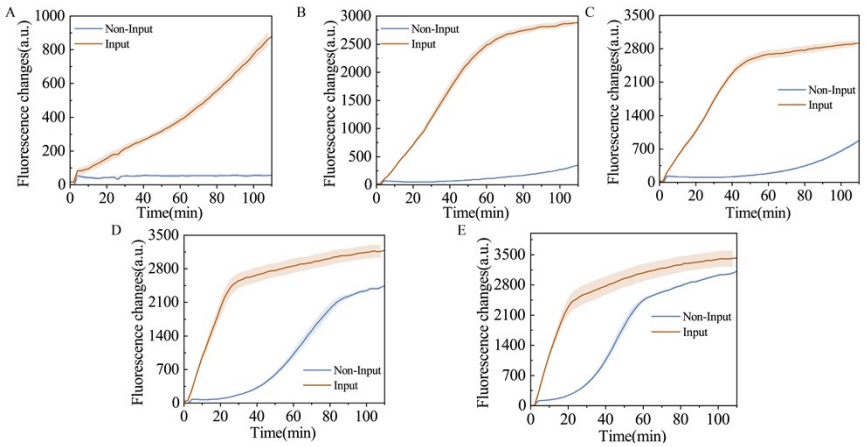

Fig. S2. Different concentrations of λ Exonuclease by the fluorescence assay. (A) Leakage in the

presence of only 0.2 μM P2 and 0.001 U/μl λ EXO and hydrolysis with the addition of 0.2 μM Z2 in the presence of 0.2 μM P2 and 0.005 U/μl λ EXO. (B) Leakage in the presence of only 0.2 μM P2 and 0.02 U/μl λ EXO and hydrolysis with the addition of 0.2 μM Z2 in the presence of 0.2 μM P2 and 0.02 U/μl λ EXO. (C) Leakage in the presence of only 0.2 μM P2 and 0.01 U/μl λ EXO and hydrolysis with the addition of 0.2 μM Z2 in the presence of 0.2 μM P2 and 0.01 U/μl λ EXO. (D) Leakage in the presence of only 0.2 μM P2 and 0.02 U/μl λ EXO and hydrolysis with the addition of 0.2 μM Z2 in the presence of 0.2 μM P2 and 0.02 U/μl λ EXO. (E) Leakage in the presence of only 0.2 μM P2 and 0.03 U/μl λ EXO and hydrolysis with the addition of 0.2 μM Z2 in the presence of 0.2 μM P2 and 0.03 U/μl λ EXO.

To investigate the effect of enzyme concentration on Yes gate, we performed a gradient test on the the enzyme concentration ranged from 0.001 U/μl to 0.03 U/μl for the fluorescence assay as in Fig S1. The results showed that the background signal was lowest when the enzyme concentration was 0.01 U/μl; there was a significant fluorescence increment when the trigger strand was added.

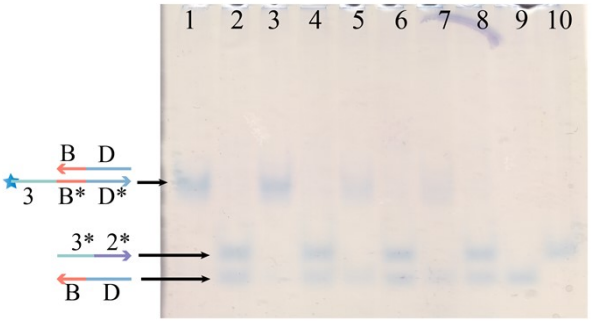

Fig. S3 PAGE gel results of the leakage of P2 at different temperatures and the hydrolysis of the complex P2+Z2(12% gel): lane 1, 3, 5, 7, P2+1U λ EXO; lane 2, 4, 6, 8, P2+ Z2+1U λ EXO. Where Lane 1, 3, 5 and 7 are the leakage of P2+1U λ EXO at different temperatures; lane 2, 4, 6 and 8 are the hydrolysis of P2 + Z2 + 1U λ EXO at different temperatures, lane 9, Inh 2; lane 10, Z2. [P2] =[Z2] =[Inh 2] = 1 μM.

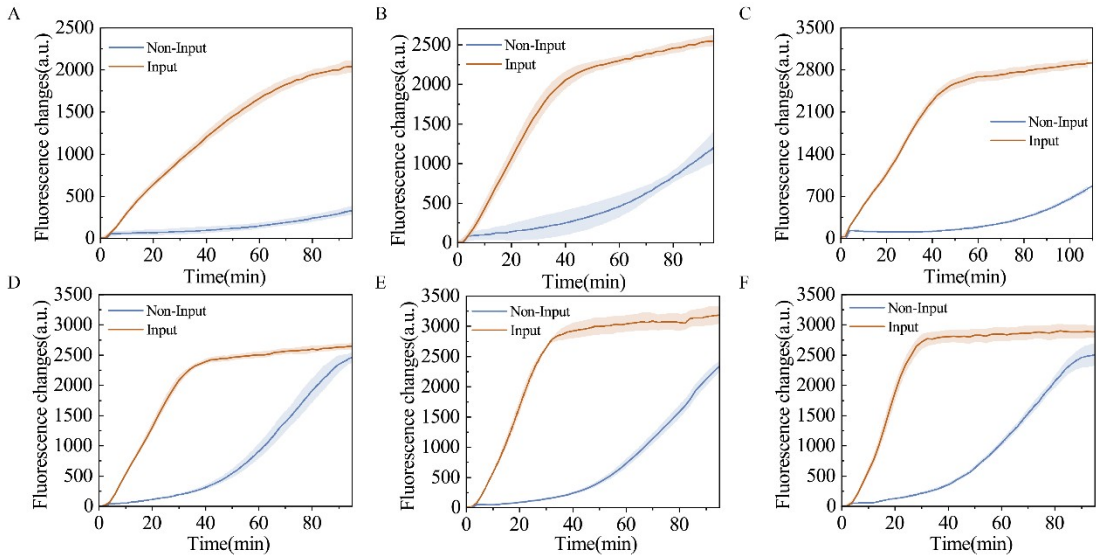

Fig. S4. Hydrolysis and leakage at different temperatures. Leakage in the presence of P2 only and hydrolysis when the trigger strand is added at 22℃(A),25℃(B),28℃(C),31℃(D),34℃(E),37℃(F).

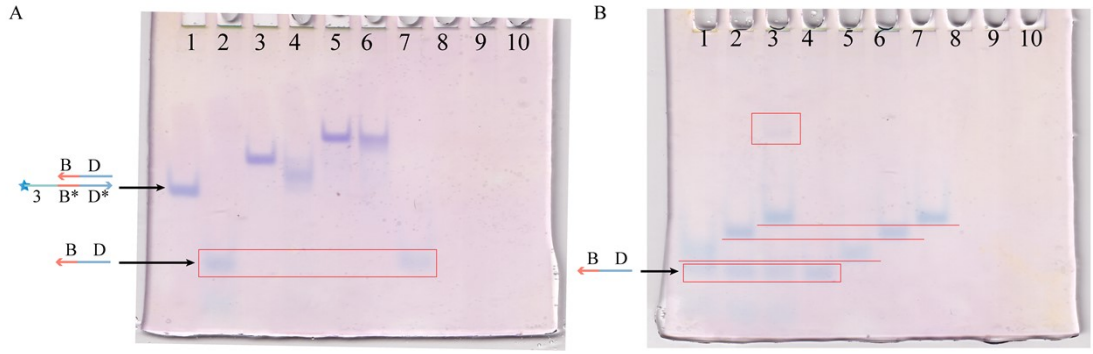

Fig. S5. Hydrolysis and leakage at different lengths of domain 3. (A) PAGE gel results of Yes gate substrate leakage at domain C lengths from 5-nt to 25-nt (12% gel): lane 1, 1 μM P2(length of domain 3 5-nt); lane 2, 1 μM P2(length of domain 3 5-nt)+1U λ EXO; lane 3, 1 μM P2(length of domain 3 15-nt); lane 4, 1 μM P2(length of domain 3 15-nt)+1U λ EXO; lane 5, 1 μM P2(length of domain 3 25-nt); lane 6, 1 μM P2(length of domain 3 25-nt)+1U λ EXO; lane 7, 1 μM Inh 2.(B)PAGE gel results of the hydrolysis at the time of adding the input is in the domain C length from 5-nt to 25-nt (12% gel):lane 1, 1 μM P2(length of domain 3 5-nt)+1U λ EXO+1 μM Z2(length of domain 3* 5-nt); lane 2, 1 μM P2(length of domain 3 15-nt)+1U λ EXO+1 μM Z2(length of domain 3* 15-nt); lane 3, 1 μM P2(length of domain 3 25-nt)+1U λ EXO+1 μM Z2(length of domain 3* 25-nt); lane 4, 1 μM Inh 2; lane 5, 1 μM Z2(length of domain 3* 5-nt); lane 6, 1 μM Z2(length of domain 3* 15-nt); lane 7, 1 μM Z2(length of domain 3* 25-nt).

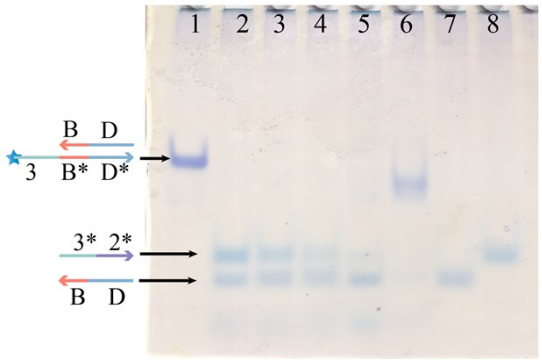

Fig. S6. PAGE gel results of P2 hydrolysis with different concentrations of Z2 (12% gel). Where Lane 2-lane 6 is at P2 + 1U λ EXO, the Z2 concentrations are 1 μM Z2, 0.75 μM Z2, 0.5 μM Z2, 0.25 μM Z2, 0.1 μM Z2 respectively, lane 7, Inh 2; lane 8, Z2. [P2] =[Inh 2] = 1 μM.

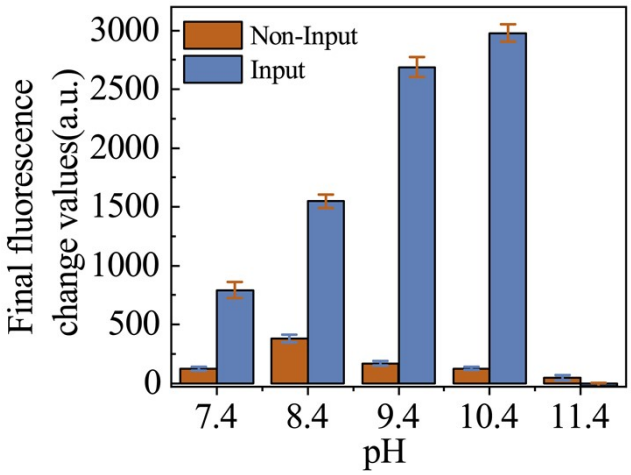

Fig. S7. Histograms of the fluorescence data for background signal and hydrolysis at different pH. Red indicates leakage at different pH containing only P2 + 1U λ EXO and blue indicates hydrolysis at different pH of P2 and 1U λ EXO to a substrate containing Z2 is added.

To investigate the effect of pH on the Yes gate, we performed a gradient test on the pH. The pH ranged from 7.4 to 11.4 for the fluorescence assay as Fig S7. The results showed less leakage and higher fluorescence increment when the pH was in the range of 9.4-10.4. and many researchers usually set the pH to 9.4, so we set the pH to 9.4.[1, 2]

S2 Inhibit gate

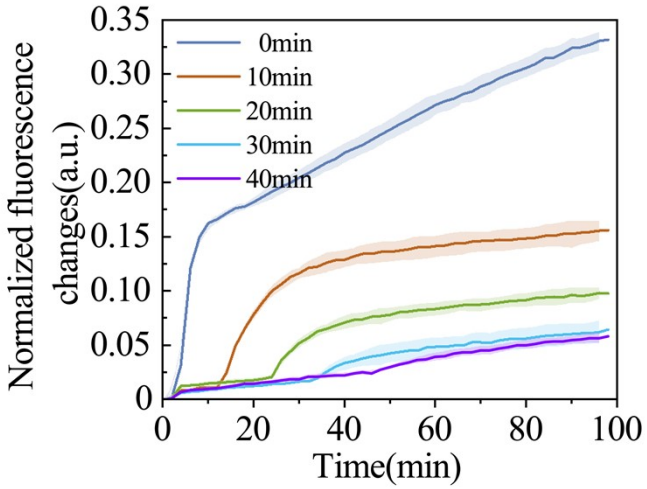

Fig. S8. Fluorescence assay monitoring the interval between the inputs. All four cases contain [S1] = 0.4 μM, [D1] = 0.2 μM, [P2] = 0.4 μM,2U λ EXO, with I2 added first and I1 added after different time intervals.

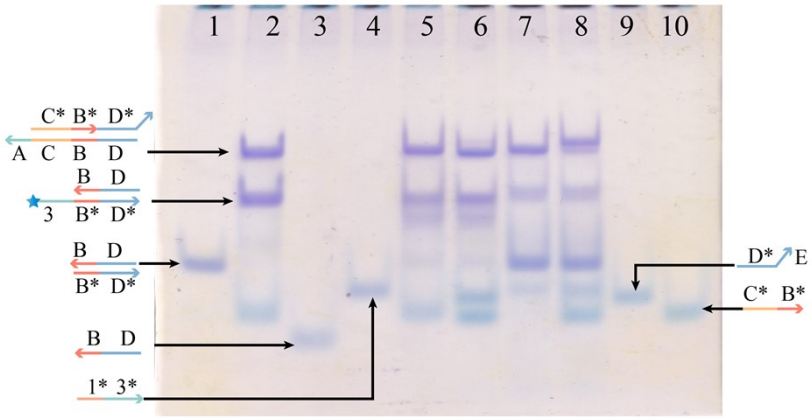

Fig. S9. PAGE gel results of Inhibit gate (12% gel): lane1, D1+ Inh 2; lane 2, P2+ D1+ S1; lane 3, Inh 2; lane 4, I2; lane 5, P2+ D1+ S1+1U λ EXO; lane 6, P2+ D1+ S1+1U λ EXO+ I1; lane 7, P2+ D1+ S1+1U λ EXO+ I2; lane 8, P2+ D1+ S1+1U λ EXO+ I2+ I1; lane 9, O1; lane 10, Z1. Where Lane 5-lane 8 are the four cases of Inhibit gate with a 40 min time interval between the control and active strands. [S1] =[D1] =[I1] =[I2] =[Inh 2] =[O1] =[Z1] =1 μM, [P2] =1.5 μM,

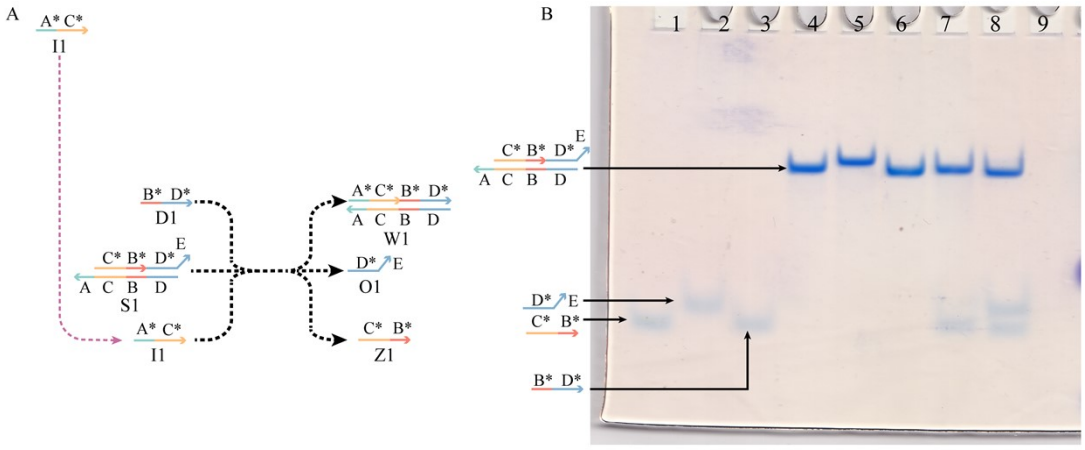

Fig. S10. PAGE gel results of DNA-based switching circuit (12% gel): lane1, 1 μM Z1; lane 2, 1 μM O1; lane 3, 1 μM D1; lane 4, 1 μM S1; lane 5, 1 μM intermediate product; lane 6, 1 μM W1; lane 7, 1 μM D1+1 μM S1; lane 8, 1 μM D1+1 μM S1 +1 μM I1.

We experimentally validated the constructed DNA-based switching circuit (Fig S10) with substrates S1 and D1. The length of domain A was 8 and the length of domain B was 4. When only the substrate was present, no output was observed (lane 7); when the input was added, output could be observed (lane 8).

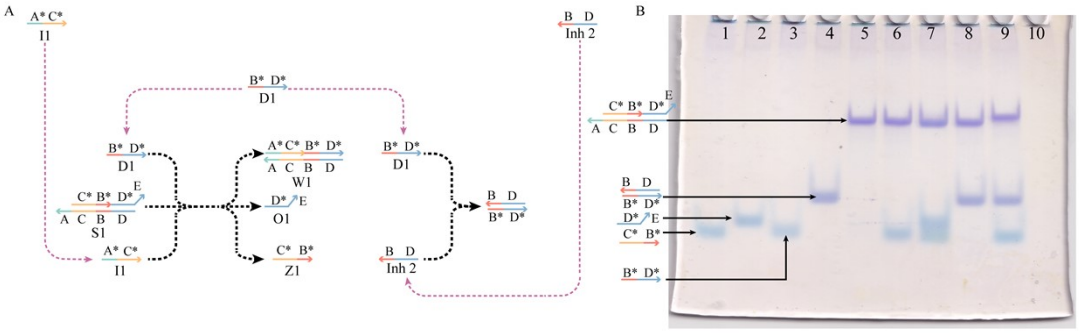

Fig. S11. (A) The design details of Inhibit gate based on DNA-based switching circuit. (B) PAGE gel results of Inhibit gate based on DNA-based switching circuit (12% gel): lane1, 1 μM Z1; lane 2, 1 μM O1; lane 3, 1 μM D1; lane 4, 1 μM D1+1 μM Inh2; lane 5, 1 μM S1; lane 6, 1 μM D1+1 μM S1; lane 7, 1 μM D1+1 μM S1+1 μM I1; lane 8, 1 μM D1+1 μM S1+1 μM Inh2; lane 9, 1 μM D1+1 μM S1+1 μM I1+1 μM Inh2.

We constructed the Inhibit gate based on the DNA-based switching circuit, in which the substrates include S1 and D1, and the inputs are the activation strand I1 and the inhibit strand Inh 2. The output could be

observed when only the activation strand I1 was added (lane 7), but otherwise no output was observed (lane 6, lane 8, lane 9).

S3 Sequential logic circuit

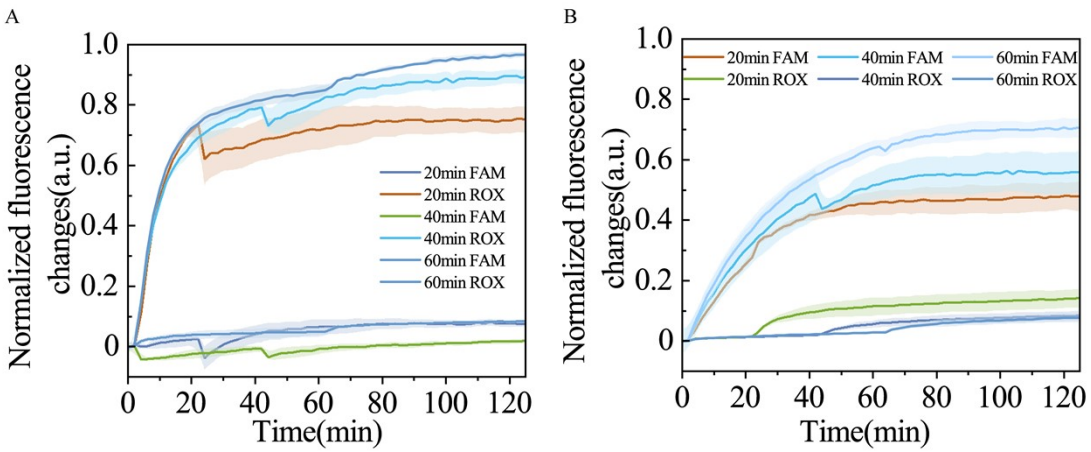

Fig. S12. Fluorescence assay monitoring the interval between the inputs. (A) All four cases contain [S1] =0.4 μM, [D1] =0.2 μM, [P1] =0.4 μM, [S2] =0.4 μM, [D2] =0.2 μM, [P2] =0.4, 2U λ EXO, with I1 added first and I2 added after different time intervals. (A) All four cases contain [S1] =0.4 μM, [D1] =0.2 μM, [P1] =0.4 μM, [S2] =0.4 μM, [D2] =0.2 μM, [P2] =0.4, 2U λ EXO, with I2 added first and I1 added after different time intervals.

We have made an investigation into the interval between the inputs of the sequential logic circuit. When an input was added, a rise in the corresponding fluorescence signal was observed, and the increase in fluorescence value gradually increased with the interval between inputs and the inhibition effect also increased (Fig S12).

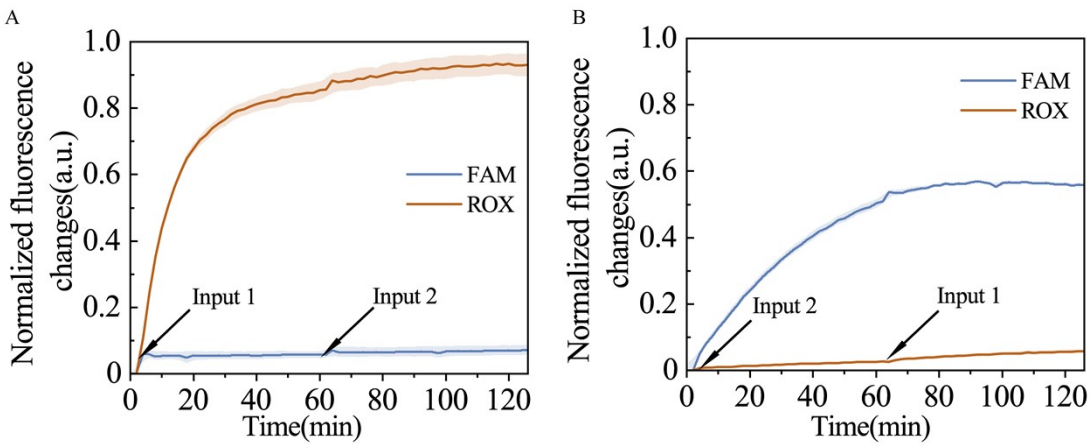

Fig. S13. Fluorescence assay to monitor Input concentration is 0.4μM of sequential logic circuit. Both cases contain substrates [S1] =0.4 μM, [D1] =0.2 μM, [P1] =0.4 μM, [S2] =0.4 μM, [D2] =0.2 μM, [P2] =0.4, 2U λ EXO. The input interval between the two inputs is 60 min. (A) I1 is added first, followed by I2 after 60 min.(B) I2 is added first, followed by I1 after 60 min.

We adjusted the input concentration and verified the inhibition effect at low concentration (Fig S13). When the input concentration was 0.4 μM, it still had a good inhibition effect.

Table S1 Criterion of optimum enzyme concentration

Enzyme concentration	fluorescence increment (I1 is added first)		Criterion (FAM/ROX)	fluorescence increment (I2 is added first)		Criterion (ROX/FAM)
	FAM	ROX		FAM	ROX	
0.002 U/μl	0.46072	0.87481	0.526652	0.81969	0.10887	0.132819
0.01 U/μl	0.10991	0.9954	0.110418	0.73591	0.14521	0.19732
0.02 U/μl	0.08257	0.96717	0.085373	0.70464	0.07759	0.110113
0.04 U/μl	0.03701	0.91795	0.040318	0.61015	0.07277	0.119266
0.06 U/μl	0.06547	0.99554	0.065763	0.52252	0.10818	0.207035

S4 DNA Sequences Design

All DNA sequences in this experiment are designed by NUPACK, and some sequences are modified manually to further ensure the robustness of the circuit. In sequence design, the Yes gate is shown in Fig 1A, to ensure that domain 3 does not produce self-hybridization. Inhibit gate shown in Fig 3A, to ensure that D1 does not self-hybridize and can hybridize with domain 3. The sequential logic circuit is shown in Fig 4B, to ensure that D1 and D2 do not self-hybridize and do not hybridize with both domain 3 and domain C, to ensure the robustness of the circuit and avoid leakage.

Table S2 Compositions of the complexes

Yes gate	P2	F-3B*D*, F-DB
	Z2	3*2*
Inhibit gate	S1	F-DBCA, C*B*, F-D*E*
	D1	B*D*
	P2	3B*D*, DB
	I1	A*C*
	I2	1*3*
Sequential logic circuit	S1	F-DBCA, C*B*, F-D*E*
	S2	F-4231, 3*2*, F-4*5
	D1	B*D*
	D2	2*4*
	P1	42, C2*4*
	P2	3B*D*, DB
	I1	A*C*
	I2	1*3*
Fig S4	P2(length of domain 3 5-nt)	3B*D*_5
	Z2(length of domain 3* 5-nt)	Z2_5
	P2(length of domain 3 15-nt)	P2
	Z2(length of domain 3* 15-nt)	Z2
	P2(length of domain 3 25-nt)	3B*D*_25
	Z2(length of domain 3* 25-nt)	Z2_25

Table S3 DNA sequences

Strand	Sequence (5’ to 3’)
DBCA	TACGAGTGATACAGTACGAATGTCTGATGTAGAGACGATGAC
C*B*	CTCTACATCAGACATTCGT
D*E*	ACTGTATCACTCGTATTTTT
B*D*	TCGTACTGTATCACTCGTA
42	TCACGAGAAGTGTCTCATC
C2*4*	P-ATGTCTGATGTAGAGGATGAGACACTTCTCGTGA
A*C*	GTCATCGTCTCTACATCAGACAT
4231	TCACGAGAAGTGTCTCATCGTCAGTGAGTCTAGTTCGTACCA
3*2*	ACTAGACTCACTGACGATG
4*5	AGACACTTCTCGTGATTTTTTTTTT
2*4*	GATGAGACACTTCTCGTGA
DB	TACGAGTGATACAGTACGA
3B*D*	P-GTCAGTGAGTCTAGTTCGTACTGTATCACTCGTA
1*3*	TGGTACGAACTAGACTCACTGAC
F-3B*D*	P-GTCAGTGAGTCTAGTTCGTACTGTATCACTCGTA-BHQ1
F-DB	TACGAG/i6FAMdT/GATACAGTACGA
F-DBCA	BHQ2-TACGAGTGATACAGTACGAATGTCTGATGTAGAGACGATGAC
F-D*E*	ACTGTATCACTCGTATTTTT-ROX
F-4231	BHQ1-TCACGAGAAGTGTCTCATCGTCAGTGAGTCTAGTTCGTACCA
F-4*5	AGACACTTCTCGTGATTTTTTTTTT-FAM
3B*D*_5	P-CTAGTTCGTACTGTATCACTCGTA
3B*D*_25	P-AGTGCAAGAGGTCAGTGAGTCTAGTTCGTACTGTATCACTCGTA
Z2_5	TTTTTTTTTACTAG
Z2_25	ACTAGACTCACTGACCTCTTGCACT

Where P stands for phosphate modification, FAM and ROX are fluorophores, and BHQ1 and BHQ2 are quencher.

1. Z. H. Qin, Y. Liu, L. H. Zhang, J. J. Liu and X. Su, *ACS Nano*, DOI: 10.1021/acsnano.2c04405, 10.
2. P. Miao, L. Ning, X. Li, Y. Shu, G. J. B. Li and Bioelectronics, 2011, **27**, 178-182.