Supplementary Material

Refined Design of DNA Logic Gate for Implementing DNA-based Three-level Circuit

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Table contents

SI 1. Sequences used in this work.

1. AND gate:

Segment Strand	**Sequence (from 5' to 3')**
A*	TGAAG GGA AAA[1] AGAGG TGTGG
F*	TGGTG GTTGG TAATG TGGGA GTTAA GGATG
F	CATCC TTAAC TCCCA CATTA CCAAC CACCA
O*	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
a	CCACA CCTCT
c	AA CAACT CAAAC ATACA
c*	TGTAT GTTTG AGTTG AA
b	TCCCT TCA
R	TCTTT ATCAA TCTAC CCACC
R*	GGTGG GTAGA TTGAT AAAGA
Strand name	**Sequence (from 5' to 3')**
Substrate (O*F*A*)	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGA AAA AGAGG TGTGG
Fuel (F)	CATCC TTAAC TCCCA CATTA CCAAC CACCA
Input 1 (ac)	CCACA CCTCT AA CAACT CAAAC ATACA
Input 2 (c*b)	TGTAT GTTTG AGTTG AA TCCCT TCA
FAM (R)	TCTTT ATCAA TCTAC CCACC-FAM
BHQ (R*O*)	BHQ-GGTGG GTAGA TTGAT AAAGA TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TG

Note：1. The "A" with underline are the deoxynucleotide without complementation.

2. OR gate:

Segment Strand	**Sequence (from 5' to 3')**
A*	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG
B*	TGTAT AGTGG TGATG TGAGG TGGGA GAATG

F*	TGGTG GTTGG TAATG TGGGA GTTAA GGATG
F	CATCC TTAAC TCCCA CATTA CCAAC CACCA
O*	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
W*	TGGAG TGTTA GATTG TGA
R	TCTTT ATCAA TCTAC CCACC
R*	GGTGG GTAGA TTGAT AAAGA
Strand name	**Sequence (from 5' to 3')**
Substrate 1 (O*F*A*)	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG
Substrate 2 (O*F*B*)	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGTAT AGTGG TGATG TGAGG TGGGA GAATG
Fuel (F)	CATCC TTAAC TCCCA CATTA CCAAC CACCA
Input 1 (A)	CAAAC TAAAC AACCA CACCT CTTCC CTTCA
Input 2 (B)	CATTC TCCCA CCTCA CATCA CCACT ATACA
Threshold (W*O*)	TGGAG TGTTA GATTG TGA TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
Blocker	CACAC AAACT ACATC CACA AAAA
FAM (R)	TCTTT ATCAA TCTAC CCACC-FAM
BHQ (R*O*)	BHQ-GGTGG GTAGA TTGAT AAAGA TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TG

3. AND-OR gate:

Segment Strand	**Sequence (from 5' to 3')**
A*	TGAAG GGA AAA AGAGG TGTGG
M*	TGTAT AGTGG TGATG TGAGG TGGGA GAATG
C*	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG
C	CACTA TACAC ACCCA CAACC CTAAC CATCA
F*	TGGTG GTTGG TAATG TGGGA GTTAA GGATG
F	CATCC TTAAC TCCCA CATTA CCAAC CACCA
a	CCACA CCTCT
b	TCCCT TCA
c	AA CAACT CAAAC ATACA
c*	TGTAT GTTTG AGTTG AA
O*	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
W*	TGGAG TGTTA GATTG TGA
R	TCTTT ATCAA TCTAC CCACC
R*	GGTGG GTAGA TTGAT AAAGA
Strand name	**Sequence (from 5' to 3')**
Substrate 1	TGTAT AGTGG TGATG TGAGG TGGGA GAATG

(M*F*A*)	TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGA AAA AGAGG TGTGG
Substrate 2 (O*F*M*)	TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TGTGG TGGTT GGTAA TGTGG GAGTT AAGGA TGTGT ATAGT GGTGA TGTGA GGTGG GAGAA
Substrate 3 (O*F*C*)	TGTGG ATGTA GTTTG TGTGT GGATT TGTATT GTGGT GGTTG GTAATG TGGGA GTTAA GGATG TGATG GTTAG GGTTG TGGGT GTGTA TAGTG
Fuel (F)	CATCC TTAAC TCCCA CATTA CCAAC CACCA
Input A (ac)	CCACA CCTCT AA CAACT CAAAC ATACA
Input B (c*b)	TGTAT GTTTG AGTTG AA TCCCT TCA
Input C (C)	CACTA TACAC ACCCA CAACC CTAAC CATCA
Threshold (W*O*)	TGGAG TGTTA GATTG TGA TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
Blocker	CACAC AAACT ACATC CACA AAAA
FAM (R)	TCTTT ATCAA TCTAC CCACC-FAM
BHQ (R*O*)	BHQ-GGTGG GTAGA TTGAT AAAGA TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TG

4. OR-AND gate:

Segment Strand	**Sequence (from 5' to 3')**
A*	TGTAT AGTGG TGATG TGAGG TGGGA GAATG
B*	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG
M1*	TGTAT GTTTG AGTTG TT AGAGG TGTGG
M2*	TGAAG GGA AAA AGAGG TGTGG
F*	TGGTG GTTGG TAATG TGGGA GTTAA GGATG
F	CATCC TTAAC TCCCA CATTA CCAAC CACCA
b	TCCCT TCA
c*	TGTAT GTTTG AGTTG AA
O*	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
W*	TGGAG TGTTA GATTG TGA
R	TCTTT ATCAA TCTAC CCACC
R*	GGTGG GTAGA TTGAT AAAGA
Strand name	**Sequence (from 5' to 3')**
Substrate 1 (M1*F*A*)	TGTAT GTTTG AGTTG TT AGAGG TGTGG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGTAT AGTGG TGATG TGAGG TGGGA GAATG
Substrate 2 (M1*F*B*)	TGTAT GTTTG AGTTG TT AGAGG TGTGG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGATG GTTAG GGTTG TGGGT GTGTA TAGTG
Substrate 3 (O*F*M2*)	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGA AAA AGAGG TGTGG
Fuel (F)	CATCC TTAAC TCCCA CATTA CCAAC CACCA

Input A (A)	CATTC TCCCA CCTCA CATCA CCACT ATACA
Input B (B)	CACTA TACAC ACCCA CAACC CTAAC CATCA
Input C (c*b)	TGTAT GTTTG AGTTG AA TCCCT TCA
Threshold (W*O*)	TGGAG TGTTA GATTG TGA TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
Blocker[1]	CTCAA ACATA CA
FAM (R)	TCTTT ATCAA TCTAC CCACC-FAM
BHQ (R*O*)	BHQ-GGTGG GTAGA TTGAT AAAGA TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TG

Note：1.The Blocker in OR-AND gate is partially complementary with sequence c*.

5. Voting device:

Segment Strand	**Sequence (from 5' to 3')**
S1*	TGTAT AGT AAA AGAGG TGTGG
S2*	TGAAG GGA AAA AGAGG TGTGG
S3*	TGAAG GGA AAA GGTGA TGTGA
S4*	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG
F*	TGGTG GTTGG TAATG TGGGA GTTAA GGATG
F	CATCC TTAAC TCCCA CATTA CCAAC CACCA
a1	CCACA CCTCT
a2	TCACA TCACC
b1	ACTAT ACA
b2	TCCCT TCA
J1	AA CAACT CAAAC ATACA
J1*	TGTAT GTTTG AGTTG
J2	AA CACAC TTCAA ACTCA AA
J2*	TGAGT TTGAA GTGTG
J3	CACTC ACATA CAACA AA
J3*	TGTTG TATGT GAGTG
O*	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG
R	TCTTT ATCAA TCTAC CCACC
R*	GGTGG GTAGA TTGAT AAAGA
Strand name	**Sequence (from 5' to 3')**
Complex 1 (S4*F*S1*)	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGTAT AGT AAA AGAGG TGTGG
Complex 2 (S4*F*S2*)	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGA AAA AGAGG TGTGG
Complex 3 (S4*F*S3*)	TGATG GTTAG GGTTG TGGGT GTGTA TAGTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGAAG GGA AAA GGTGA TGTGA
Substrate (O*F*S4*)	TGGAT GTAGT TTGTG TGTGG ATTTG TATTG TGGTG GTTGG TAATG TGGGA GTTAA GGATG TGATG

	GTTAG GGTTG TGGGT GTGTA TAGTG
Junction 1 (J1*J2*)	TGTAT GTTTG AGTTG TGAGT TTGAA GTGTG
Junction 2 (J1*J3*)	TGTAT GTTTG AGTTG TGTTG TATGT GAGTG
Junction 3 (J2*J3*)	TGAGT TTGAA GTGTG TGTTG TATGT GAGTG
Fuel (F)	CATCC TTAAC TCCCA CATTA CCAAC CACCA
Input A (a1J1)	CCACA CCTCT AA CAACT CAAAC ATACA
Input B (J3b2)	CACTC ACATA CAACA AA TCCCT TCA
Input C (a2J2b1)	TCACA TCACC AA CACAC TTCAA ACTCA AA ACTAT ACA
FAM (R)	TCTTT ATCAA TCTAC CCACC-FAM
BHQ (R*O*)	BHQ-GGTGG GTAGA TTGAT AAAGA TGTGG ATGTA GTTTG TGTGT GGATT TGTAT TG

SI 2. Materials and methods

1. Materials

The unlabeled DNA strands were synthesized and purified by HAP (Sangon Co., China), while the fluorophore and quencher-labeled strands were synthesized and purified by HPLC (Sangon Co., China). The sequences of all the probes and targets that have been used in this work are summarized in Table S1. The concentration of each strand was quantified by Nanodrop 2000c spectrophotometer (Thermo Fisher). RNase free deionized water from Sangon Co. was used in all the experiments. All the DNA oligonucleotides were ordered in dry format and suspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA pH 8.0). The Bst DNA Polymerase, Large Fragment (#M0275L) and dNTP (#N0447L) were all purchased from New England Biolabs (MA, USA).

2. Time-based fluorescence data acquisition.

Time-based fluorescence data were acquired using Synergy HTX Multi-Mode Microplate Reader (BioTek, USA). We set the excitation wavelength at 485nm and emission wavelength at 528nm for the FAM fluorophore. The duration of fluorescence acquisition varies. Typically, we set a maximum acquisition duration of 2 hours, but terminate the experiment early if the increasing rate of the fluorescence signal remained stable and could be calculated precisely (to facilitate more rapid collection of data). Time 0 on kinetic plots represents the moment the first data point was measured.

3. Reaction system preparation.

To prepare the reaction system, first, we mixed the Substrate strands and the Fuel strands at 1:1 ratio with 8 units Bst DNA Polymerase, 2ul dNTP, 1× Reporter and 1× ThermoPol reaction buffer (New England Biolabs); Then, we incubate the system in 37℃ for 10 min, to make the Bst DNA Polymerase extend the Output strands successfully; Finally, we add the inputs we need and start the fluorescence detection. The reporter is mixed with 1× FAM and 1× BHQ, and heated to 85 °C for 5 minutes, then to 55 °C for 3 minutes, next cooling to 25 ºC with the rate 0.1 °C/s before the experiment.

4. Kinetic experiments

To a 200μL PCR tube, the Substrate strands, the Fuel strands, the Input strands, the Reporter, the Blocker, the Bst DNA Polymerase, the dNTP and ThermoPol Buffer were added and brought up to a total volume of 50μL by deionized water. All the samples were mixed and prepared at room temperature. The solutions were immediately put into the Synergy HTX Multi-Mode Microplate Reader (BioTek, USA) for fluorescence measurement.

5. Fluorescence data analysis

All raw data acquired by the Synergy HTX Multi-Mode Microplate Reader (BioTek, USA) was exported to an EXCEL file, which was subsequently imported, analyzed, and plotted using ORIGIN. Time was linearly adjusted so that t = 0 corresponds to the first data point acquired after the PCR tubes were put into the machine after addition of reagents. For each curve, the fluorescence data were usually normalized using the background fluorescence intensity as “0” and the positive control fluorescence intensity (average of the last five data points) as “1”. The signal to noise ratio (SNR) is obtained by dividing the fluorescence signal of the desired reaction when the signal reached the plateau value by that of the leak. If the signal cannot reach the plateau value, SNR is obtained by dividing the fluorescence signal of the desired reaction at the end of the experiment by that of the leak. No additional adjustments are made to the raw fluorescence kinetics plots.

SI 3. Supplementary Tables in our study

1. The truth table of AND gate.

AND State	A	B	Output
OFF	none	none	none
ON	0	0	0
	0	1	0
	1	0	0
	1	1	1

Table S1. The truth table of AND gate. All five reasonable combinations are shown.

2. The truth table of OR gate.

OR State	A	B	Output
OFF	none	none	none
ON	0	0	0
	0	1	1
	1	0	1
	1	1	1

Table S2. The truth table of OR gate. All five reasonable combinations are shown.

3. The Sequence and proportion of leakage structure with different length of "b".

Group	Sequence "**cb**"	Sequence "**a*b***"	Length of "**b**" (nt)	Proportion of leakage
1	TGTATGTTTGAGTTG TCTTCC CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**11**	**50.4%**
2	TGTATGTTTGAGTTG CTTCC CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**10**	**44.1%**
3	TGTATGTTTGAGTTG TTCC CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**9**	**7.68%**
4	TGTATGTTTGAGTTG TCC CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**8**	**2.43%**
5	TGTATGTTTGAGTTG CC CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**7**	**1.3%**
6	TGTATGTTTGAGTTG C CTTCA	TGAAG GGAAG AGGTG TGGTT GTTTA GTTTG	**6**	**0**

Table S3. Different forming proportion of leakage structure when length of "b" changed from11nt to 6nt. Sequence "b" was marked in blue of each group. The leakage structure was shown in Figure S3a.The results were predicted by the NUPACK (www.nupack.org). NUPACK Conditions: Na^{+}: 0.05M; Mg^{2+}: 0.002M; Concentration: 100nM, 37℃.

4. The proportion of triplet with different length.

Group	Length of **"a"** (nt)	Length of **"b"** (nt)	Length of **"a+b"** (nt)	Proportion of triplet
1	13	8	21	**99.9%**
2	12	8	20	**99.8%**
3	11	8	19	**99.7%**
4	10	8	18	**99.2%**
5	9	8	17	**97.5%**
6	8	8	16	**94.2%**
7	7	8	15	**84.6%**

8	6	8	14	**66.7%**

Table S4. Different forming proportion of triplet when total length of "a+b" changed from 21nt to 14nt. Specific sequence were listed in table S5, and the triplet structure was shown in Figure S3b. The results were predicted by the NUPACK (www.nupack.org). NUPACK Conditions: Na^+: 0.05M; Mg^{2+}: 0.002M; Concentration: 100nM, 37°C.

5. The sequence of triplet with different length for each group in table S4.

Group	Sequence "**ac**"	Sequence "**c*b**"	Sequence "**a*b***"
1	CAA CCACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
2	AA CCACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
3	A CCACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
4	CCACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
5	CACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
6	ACA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
7	CA CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG
8	A CCTCT	TGTATGTTTGAGTTG	TGAAG GGAAG AGGTG
	CAACTCAAACATACA	TCC CTTCA	TGGTT GTTTA GTTTG

Table S5. Sequence of triplet for length of "a+b" changed from 14nt to 21nt. Sequence of "a" was highlighted with red, and sequence of "b" was highlighted with blue.

SI 4. Supplementary Figures in our study

1. The distinguish of signal input forms between traditional and three-level logic circuit.

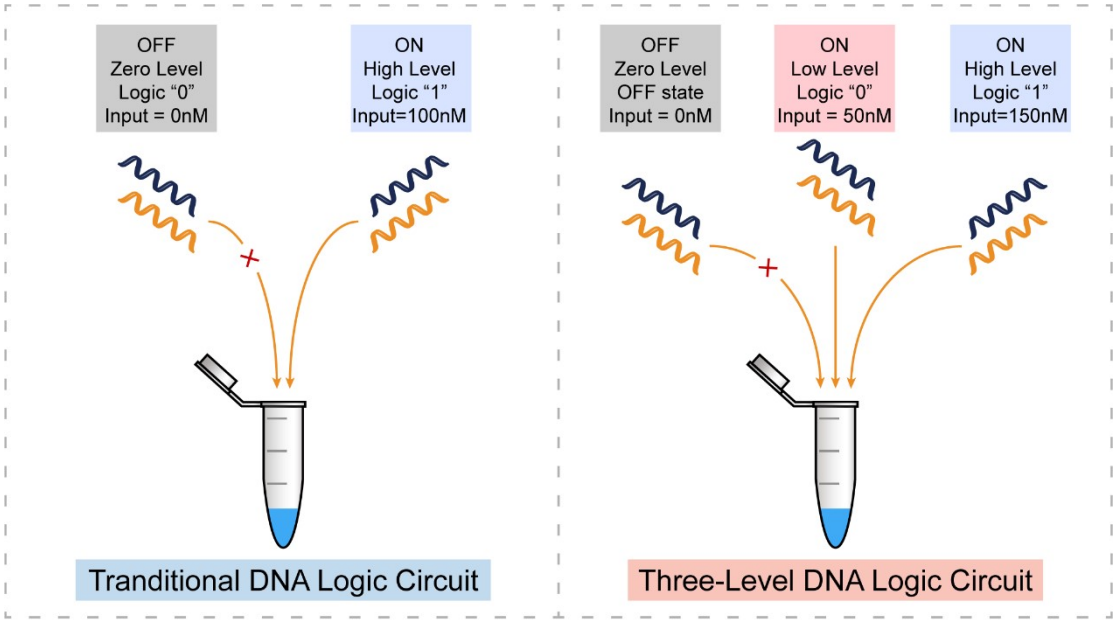

Figure S1. Signal input forms comparison between Traditional DNA Logic Circuit (left) and Three-Level DNA Logic Circuit (right). OFF, Zero Level, Logic "0", and Input = 0nM are the different expressions of the same content. So do the rest.

2. The sequence and structure of Triplet in the AND gate.

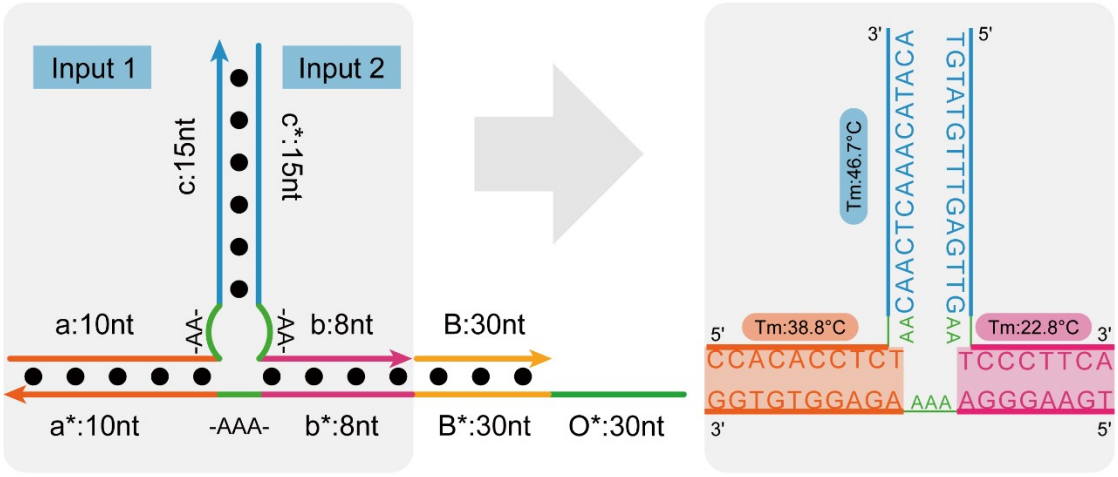

Figure S2. The detail of the Triplet in the AND gate. On the left, it shows the combining form of substrate, input1, input2 and the length of each segment; On the right, it shows the detailed information of sequence and the melt temperature of each DNA double strand.

3. Details of leakage structures and triplet structures predicted by NUPACK.

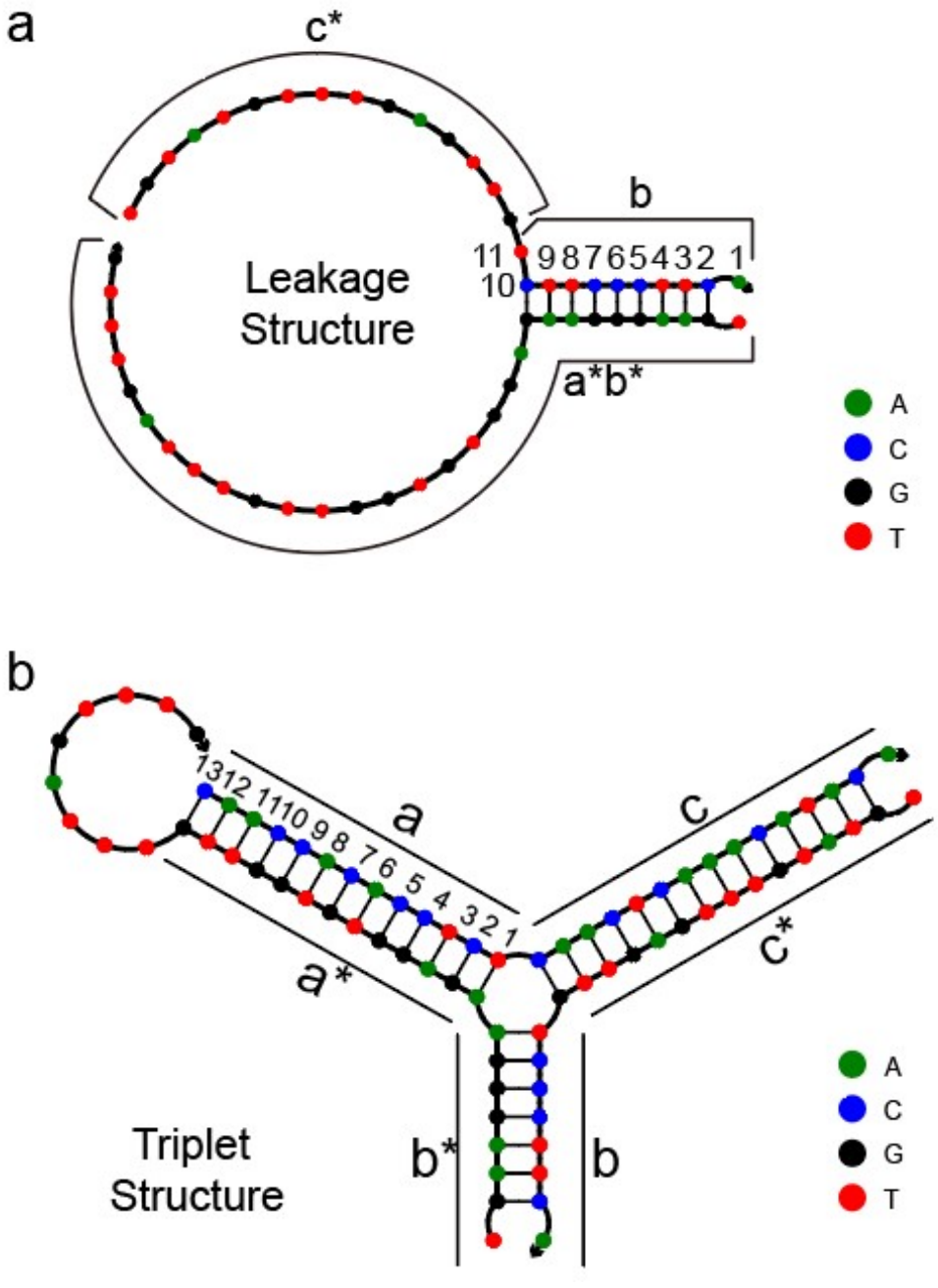

Figure S3. Prediction of leakage structure and triplet structure with NUPACK. (a) Leakage structure. When the length of b was 8nt, the base with number 1-8 would be contained, and so on. (b) Triplet structure. When the length of a was 13nt, the base with number 1-13 would be contained, and so on.

4. Optimize the length and structure of AND gate.

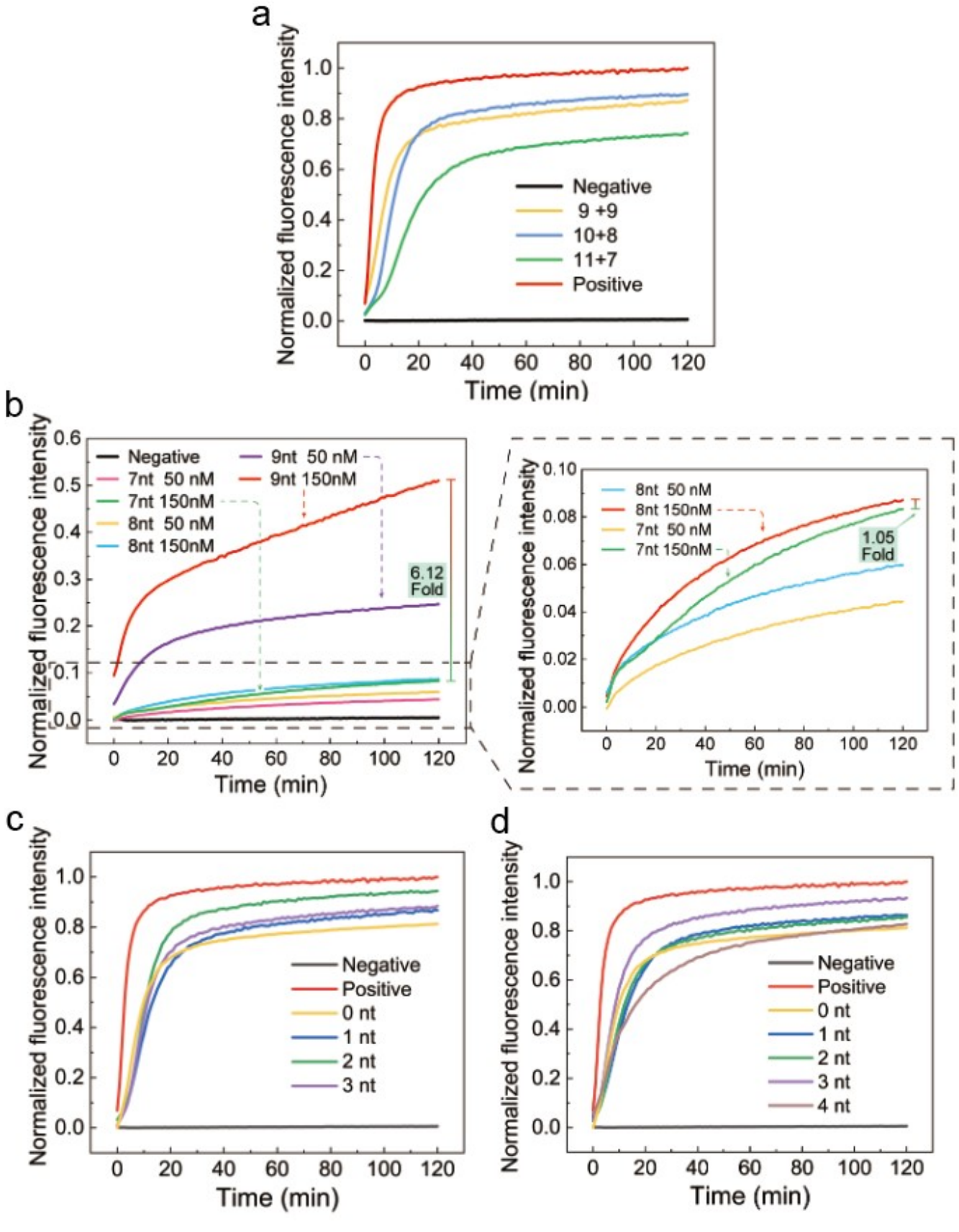

Figure S4. The data of the optimized process in the AND gate. (a) The experiment data for optimizing the length of b. It showed the normal expression of signal since input 1 and input 2 were exist. The performance of 9nt and 8nt were better than 7nt. (b) The experiment data for optimizing the length of b. It showed the leakage since only input 2 with different concentrations and lengths was exist. The data on the right was an enlargement of the data on the left. Under the concentration of 150nM, the leakage of 9nt and 8nt were 6.12 folds and 1.05 folds of 7nt. (c) Kinetic process of figure 3b. (d) Kinetic process of figure 3c. All experiments depicted in the figure were replicated at least three times.

5. Details of positive signal.

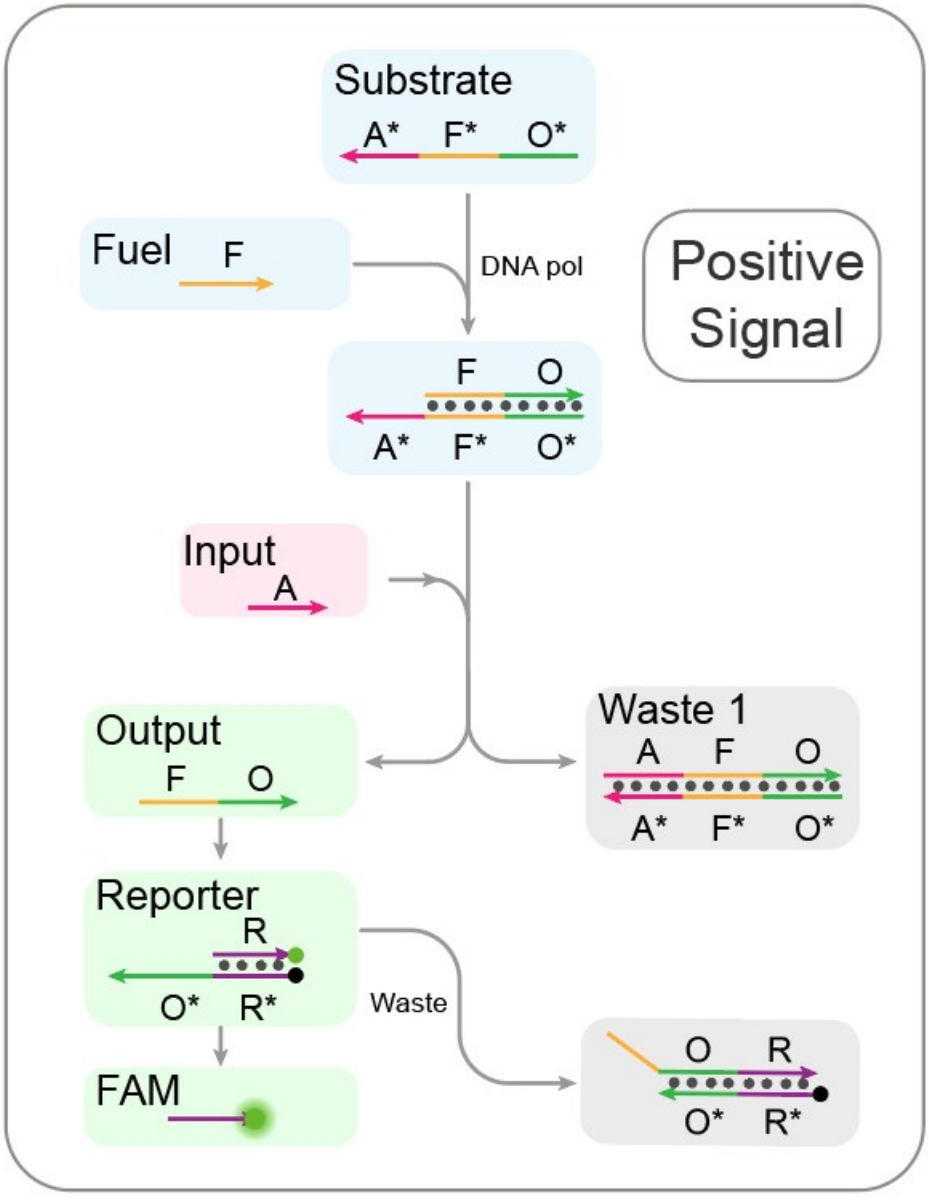

Figure S5. Details of positive signal. Fuel F binds to substrate O*F*A* to initiate the formation of "O*F*A*/F*O*" in the presence of DNA polymerase. An equimolar concentration of the input, which is fully matched with A*, is subsequently added. Under the action of polymerase, the FO strand is released and interacts with the reporting system to generate fluorescence. In this study, this output is theoretically the maximum, which we refer to as a positive signal. "DNA pol" is an abbreviation of "DNA polymerase".

6. Optimize the temperature and incubation time of AND gate.

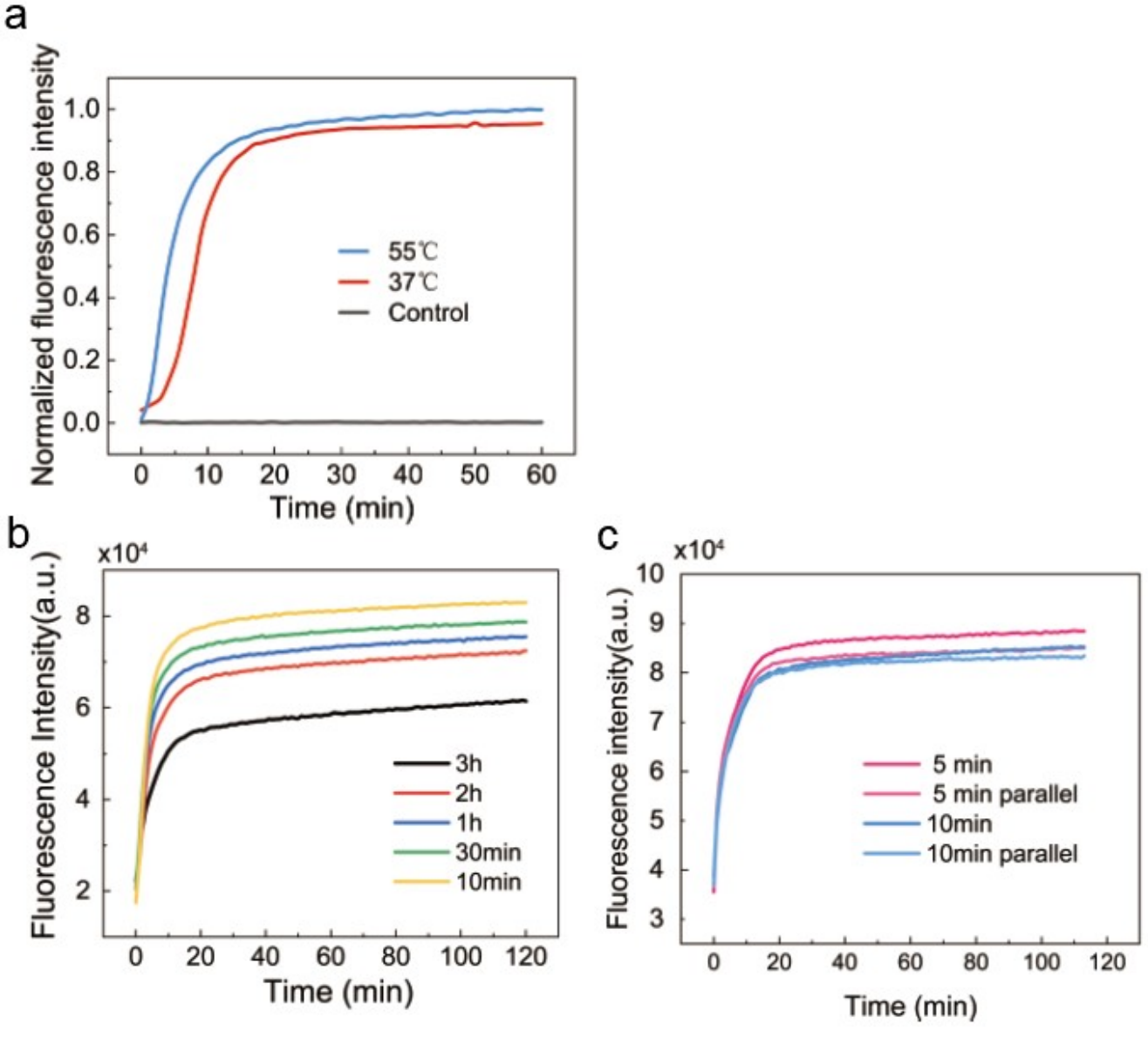

Figure S6. The data of the optimized process in the AND gate. (a) Experimental results under different temperature, 37 and 55 °C. (b) and (c) Experimental results under different incubation time, from 5min to 3h. All experiments depicted in the figure were replicated at least three times.

7. Optimize the design of OR gate.

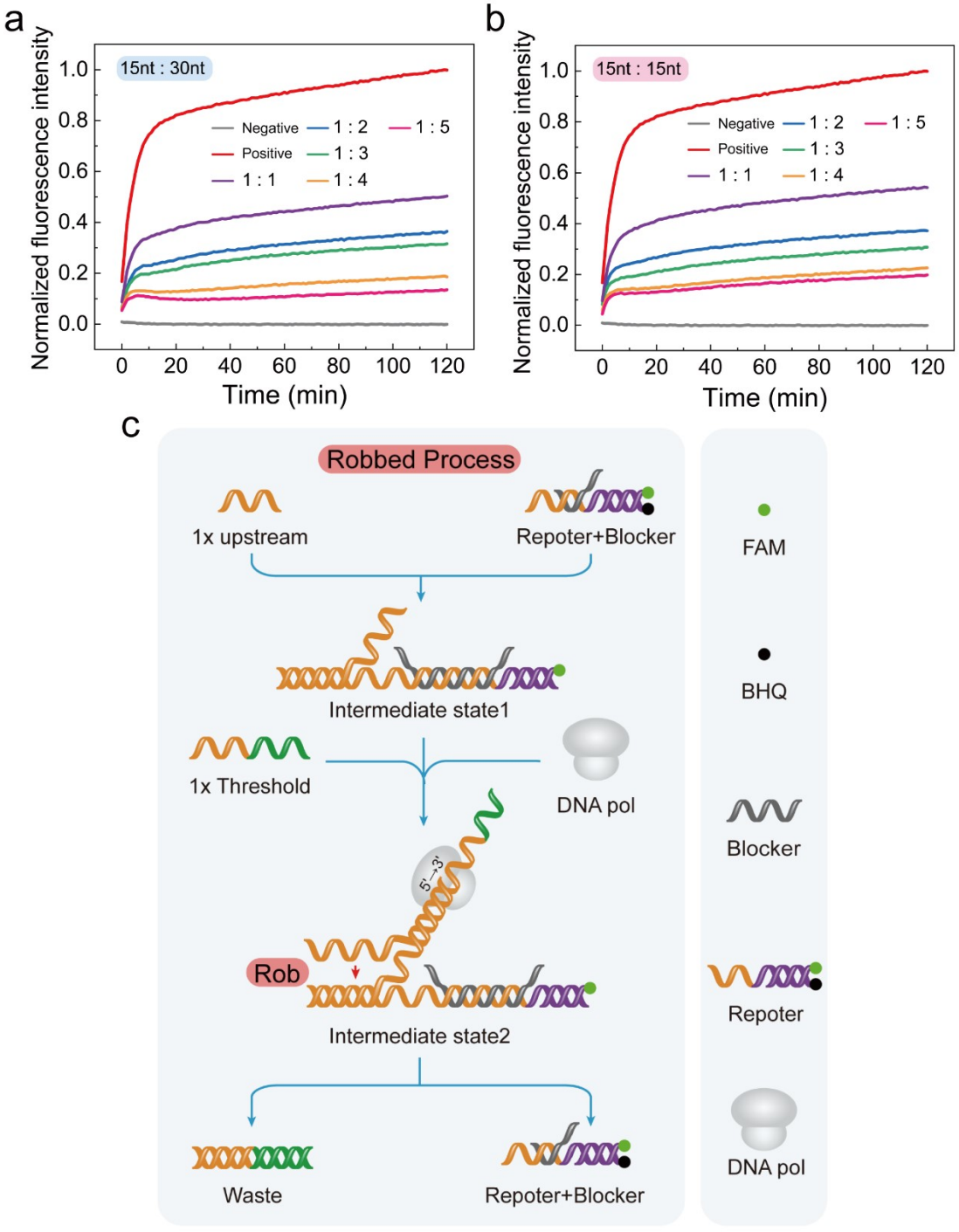

Figure S7. The data of the optimized process in the OR gate. (a) It showed the experimental data when the length of the Threshold was 30nt and the concentration ratio decreased from 1:1 to 1:5. (b) It showed the experimental data when the length of the Threshold was 15nt and the concentration ratio decreased from 1:1 to 1:5. (c) It showed the process of "Robbed" in the Figure 4d. In normal state, the input would collide with

Reporter and Threshold randomly. When colliding with Reporter firstly, it would form Intermediate state1. Then, Intermediate state 1 reacted with free Threshold. And under the work of polymerase, stable input/Threshold duplex formed, which formed intermediate state 2. Finally, Threshold robbed input from intermediate state 1, which produced waste and Reporter/Blocker complex. All experiments depicted in the figure were replicated at least three times.

8. The detail structure of voting device.

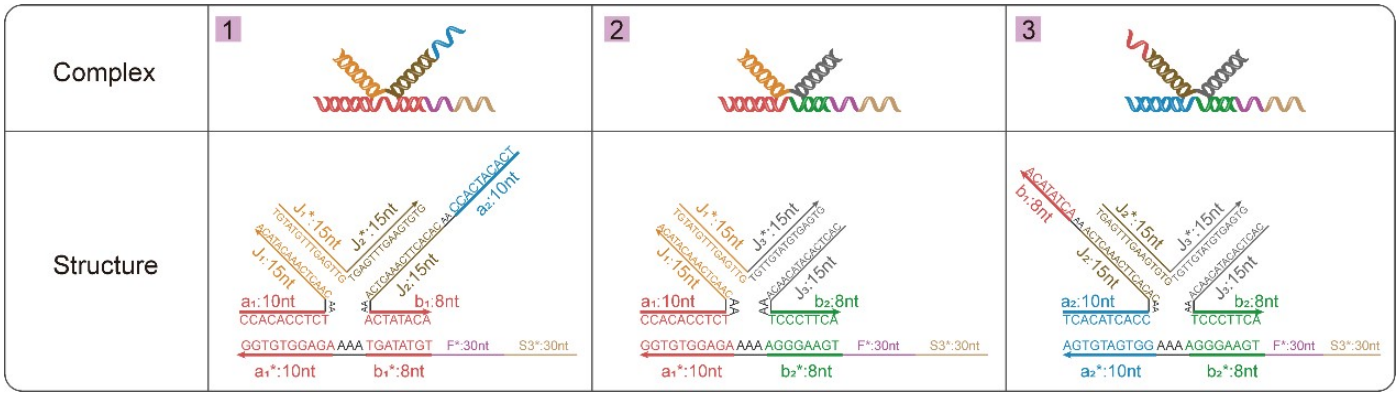

Figure S8. It showed the combining form and the detail sequence of complexes in the voting device.

9. Switches based on our design.

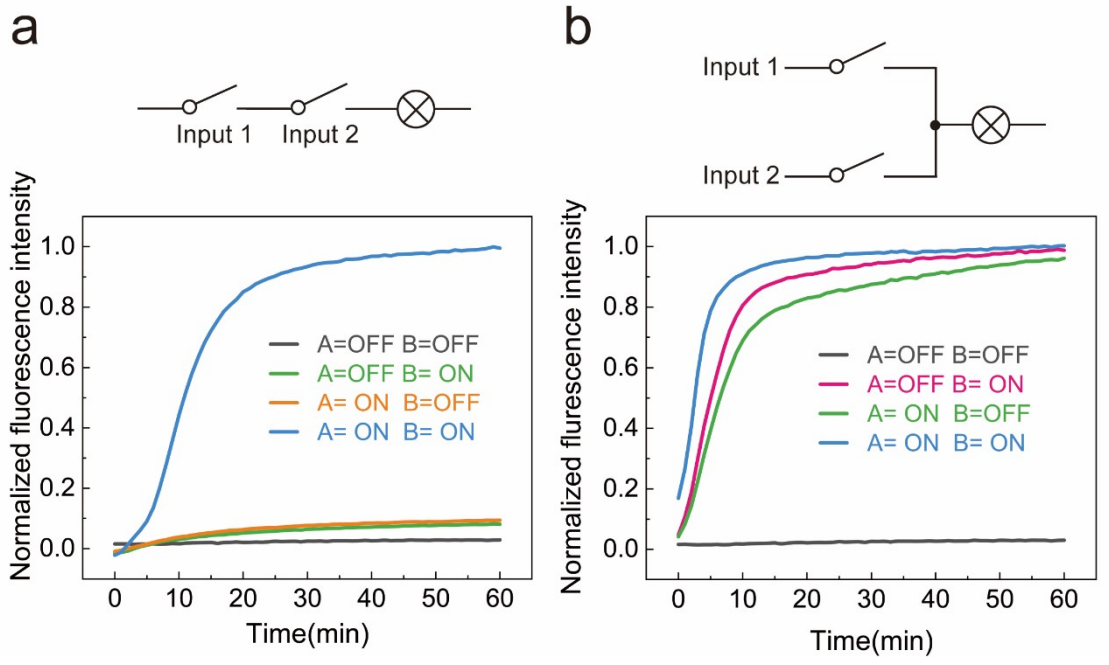

Figure S9. (a) It showed the experimental data of switches with series connection. (b) It showed the experimental data of switches with parallel connection. All experiments depicted in the figure were replicated at least three times.

10. Cascade of switches.

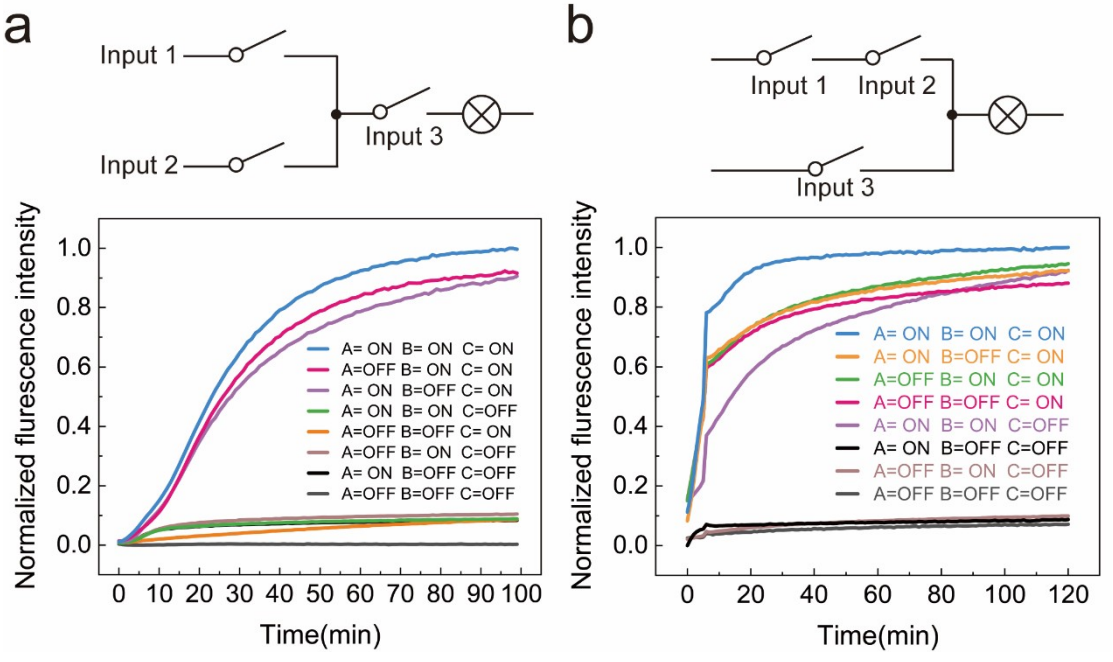

Figure S10. (a) It showed the experimental data of switches with parallel-series connection. (b) It showed the experimental data of switches with series- parallel connection. All experiments depicted in the figure were replicated at least three times.