Supporting information

Ultrasensitive detection of 17 β - estradiol (E2) based on multi-step isothermal amplification

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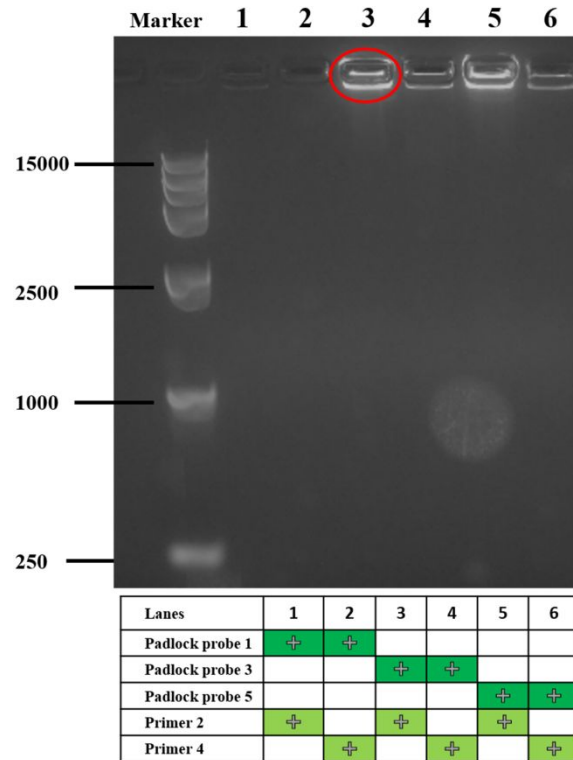| Lanes	1	2	3	4	5	6
Padlock probe 1	+	+				
Padlock probe 3			+	+		
Padlock probe 5					+	+
Primer 2	+		+		+	
Primer 4		+		+		+

Figure S1.　The Punnett method was used to selected padlock probes and primer by analyze the looping efficiency of different padlock probes and primer probes and the amount of products generated by the rolling circle amplification of the　circular DNA. The sequences in each lane was shown in the table below the picture.

A crossover experiment was designed to verify the efficiency of different padlock probes to construct circ-DNA under different primer probes. Padlock and primer probe sequences were screened by the amount of product generated after RCA of circ-DNA. After the electrophoresis, the long single-stranded DNA (LssDNA) generated by RCA would be jammed in the spotting port of the polyacrylamide gel. As showed in Figure S1, the padlock probe 3 and primer probe 2 groups produced the most products. So in this experiment, the P3 and P2 were selected as the padlock probe and primer probe
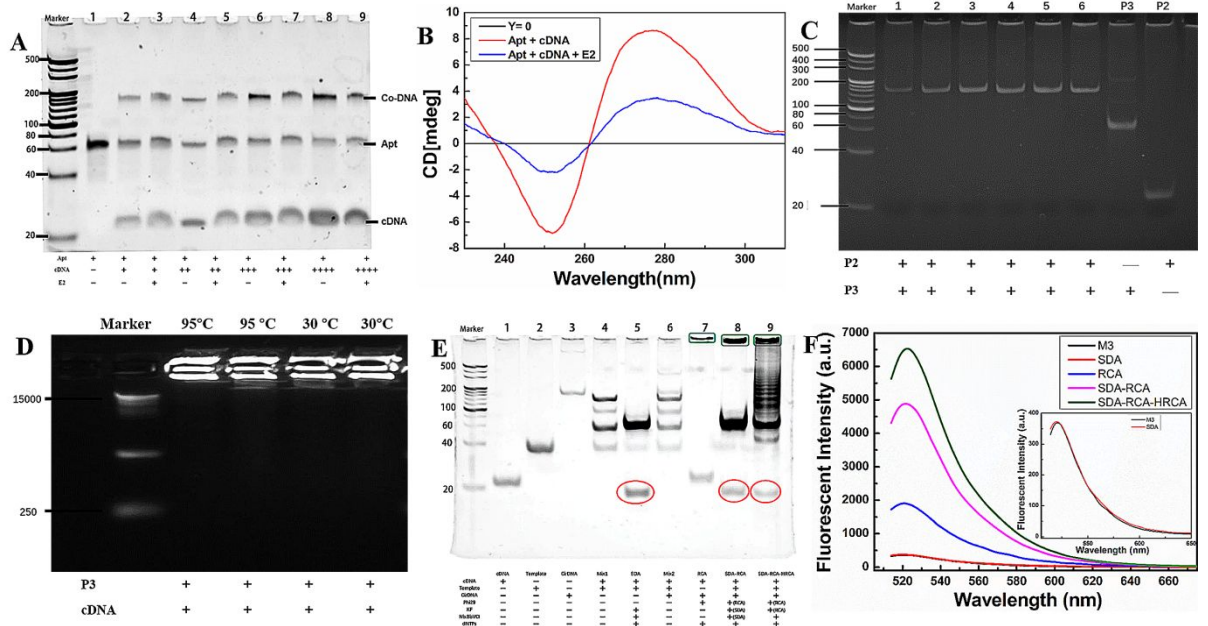
Figure S2. The result of the feasibility verification of the scheme. The E2 aptamer and cDNA were hybridized at room temperature, while E2 could bind to its aptamer and causes the release of the cDNA. (Lanes1: Apt, Lanes2: Apt(1μM)+cDNA (1μM), Lanes3: Apt(1μM)+cDNA (1μM) +E2(100ng/mL), Lanes4: Apt(1μM)+cDNA (2μM) , Lanes5: Apt(1μM)+cDNA (2μM) +E2(100ng/mL), Lanes6: Apt(1μM)+cDNA (3μM), Lanes7: Apt(1μM)+cDNA (3μM) +E2(100ng/mL), Lanes8: Apt(1μM)+cDNA (4μM), Lanes7: Apt(1μM)+cDNA (4μM) +E2(100ng/mL).) This phenomenon were characterized by the electrophoretic characterization(A) and circular dichroism(B), and the looping effect of the selected padlock probe and primer probe was verified though electrophoretic (C), and compare the effect of triggering RCA reaction under different temperature (D), electrophoresis characterization of the feasibility of multi-step isothermal expansion (E), comparison of fluorescence results between single-step isothermal expansion and multi-step isothermal expansion (F).
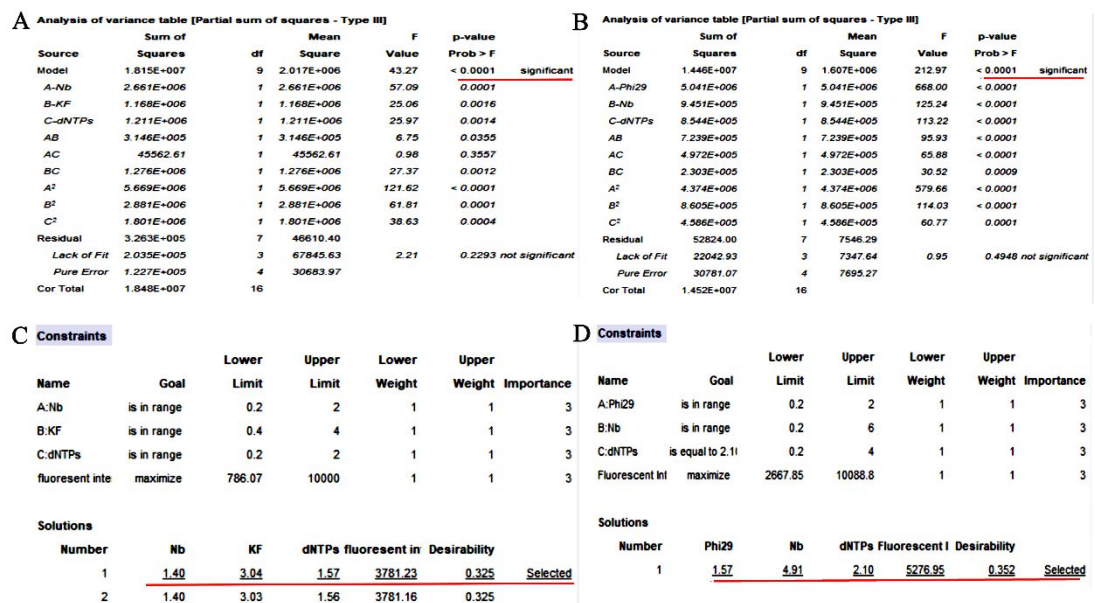

Figure S3. The dose of different reagents in the SDA and RCA-HRCA was optimized by the response surface methodology. The fluorescence value of each group in the response surface experiment was analyzed by analysis of

variance （ANOVA）(A-B). And the recommended dosage of reagents in the experiment(C-D)

As shown in Figure S3, the amount of the dose of different reagents in the SDA and RCA-HRCA was optimized by the response surface methodology. The results of the optimization experiment were significantly different between groups by analysis of variance (ANOVA) ($p<0.01$), indicating that the differences between different groups were statistically significant (Figure S 3A-B). The fluorescence value of SDA (A)and RCA-HRCA(B) was analyzed by analysis of variance （ANOVA）, the P values of Both them are less than 0.01, indicating that the differences between different groups are statistically significant. According to the results of the response surface methodology, the recommended dosage of different reagents in the SDA(C) and RCA-HRCA was given(D).

Table S1. Oligonucleotide sequences of linear ssDNA

strand	Sequence
Padlock probe(P3)	5'-Phosphate-ATTGAATTACACCTCAGCCCCTACCATTATTAATAGACTG CCTCAGCCACCATCACCTTTGCTATTTAACCTCAGCGCTTCCAGCTT-3'
Primer(P2)	5'-TGTAATTCAATAAGCTGGAAGC-3'
Aptamer	5'-GCTTCCAGCTTATTGAATTACACGCAGAGGGTAGCGGCTCTGCGCAT TCAATTGCTGCGCGCTGAAGCGCGGAAGC-Biotin-3'
cDNA	5'-AAAATTTAAAATGTAATTCAATAAGCTGGAAGC-3'
Template	5'-GCTTCCAGCTTATTGAATTACACCTCAGCTTCCAGCTTATTGAATTA CA-3'
Molecular beacon(M3)	5'-6-FAM-ATGACTACACCATCACCTTTGCTATTTAATAGTCAT-BHQ1-3'
The random ssDNA	5'-GGGTGGTCAGGTGGGATAGCGTTCCGCGTATGGCCCA-Biotin-3'
The cDNA of random ssDNA	5'-CCTGACCACCCAAAAAAAAAA-3'

Table S2. Oligonucleotide sequence of padlock probe and primer probe

strand	Sequence
Padlock probe 1(P1)	ATTGAATTACACCTCAGCGCTTCCAGCTTATTGAATTACACCTCAG CGCTTCCAGCTTATTGAATTACACCTCAGCGCTTCCAGCTT
Primer 2(P2)	**TGTAATTCAATAAGCTGGAAGC**
Padlock probe 3(P3)	**ATTGAATTACACCTCAGCCCCTACCATTATTAATAGACTGCCTC AGCCACCATCACCTTTGCTATTTAACCTCAGCGCTTCCAGCTT**
Primer 4(P4)	CGAAGGTCGAATAACTTAATGT
Padlock probe 5(P5)	GAATTACACCTCAGCCCCTACCATTATTAATAGACTGCCTCAGCCA CCATCACCTTTGCTATTTAACCTCAGCGCTTCCAGCTTATT