Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Endonuclease IV recognizes single base mismatch on the eighth base 3' to the abasic site in DNA strands for ultra-selective and sensitive mutant-type DNA detection

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Experimental section

1. Materials

All the unmodified oligonucleotides and labeled probes were synthesized and purified by high-performance liquid chromatography (HPLC) by Sangon biotech (Shanghai, China). 5' was labeled with a fluorescent molecule (FAM) and 3' a quencher (BHQ1), respectively. Endonuclease IV, Exonuclease I and λ Exonuclease were purchased from Thermo Fisher Scientific, USA.

2. Sequences used in experiment

The sequence of fluorescence probe is 5'-FAM-TATCTGCAC_AGATGCACCTT-BHQ1.("_" denotes AP site. The sequence of oligonucleotides used in this paper are listed in Table S1. The first oligonucleotide perfectly matched the probe, and others are simulants of mutant mismatch target.

Table S1. Unmodified sequences used in the paper

Mismatch position	Sequence
0(Perfect match)	CGTAAGGTGCATCTAGTGCAGATAGC
+1(A:C)	CGTAAGGTGCATCCAGTGCAGATAGC
+2(G:A)	CGTAAGGTGCATATAGTGCAGATAGC
+3(A:C)	CGTAAGGTGCACCTAGTGCAGATAGC
+4(T:C)	CGTAAGGTGCCTCTAGTGCAGATAGC
+5(G:A)	CGTAAGGTGAATCTAGTGCAGATAGC
+6(C:C)	CGTAAGGTCCATCTAGTGCAGATAGC
+7(A:C)	CGTAAGGCGCATCTAGTGCAGATAGC
+8(C:C)	CGTAAGCTGCATCTAGTGCAGATAGC
+9(C:C)	CGTAACGTGCATCTAGTGCAGATAGC
+10(T:C)	CGTACGGTGCATCTAGTGCAGATAGC
+11(T:C)	CGTCAGGTGCATCTAGTGCAGATAGC
-1(C:C)	CGTAAGGTGCATCTACTGCAGATAGC
-2(A:C)	CGTAAGGTGCATCTAGCGCAGATAGC
-3(C:C)	CGTAAGGTGCATCTAGTCCAGATAGC
-4(G:A)	CGTAAGGTGCATCTAGTGAAGATAGC
-5(T:C)	CGTAAGGTGCATCTAGTGCCGATAGC
-6(C:C)	CGTAAGGTGCATCTAGTGCACATAGC
-7(T:C)	CGTAAGGTGCATCTAGTGCAGCTAGC
-8(A:C)	CGTAAGGTGCATCTAGTGCAGACAGC
-9(T:C)	CGTAAGGTGCATCTAGTGCAGATCGC
+8(C:A)	CGTAAGATGCATCTAGTGCAGATAGC
+8(C:T)	CGTAAGTTGCATCTAGTGCAGATAGC
+7(A:C)/+8(C:C)	CGTAAGCCGCATCTAGTGCAGATAGC
+7(A:C)/+9(C:C)	CGTAACGCGCATCTAGTGCAGATAGC
+8(C:C)/+9(C:C)	CGTAACCTGCATCTAGTGCAGATAGC
+1(A:C)/+8(C:C)	CGTAAGCTGCATCCAGTGCAGATAGC
+1(A:C)/+8(C:A)	CGTAAGATGCATCCAGTGCAGATAGC
+1(A:C)/+8(C:T)	CGTAAGTTGCATCCAGTGCAGATAGC
+8(C:C)/-1(C:C)	CGTAAGCTGCATCTACTGCAGATAGC

+8(C:A)/-1(C:C)	CGTAAGATGCATCTACTGCAGATAGC
+8(C:T)/-1(C:C)	CGTAAGTTGCATCTACTGCAGATAGC
+2(G:A)/+8(C:C)	CGTAAGCTGCATATAGTGCAGATAGC
+2(G:A)/+8(C:A)	CGTAAGATGCATATAGTGCAGATAGC
+2(G:A)/+8(C:T)	CGTAAGTTGCATATAGTGCAGATAGC
+3(A:C)/+8(C:C)	CGTAAGCTGCACCTAGTGCAGATAGC
+3(A:C)/+8(C:A)	CGTAAGATGCACCTAGTGCAGATAGC
+3(A:C)/+8(C:T)	CGTAAGTTGCACCTAGTGCAGATAGC
+3(T:G)	CGTAAGGTGCAGCTAGTGCAGATAGC
+3(T:G)/+8(C:C)	CGTAAGCTGCAGCTAGTGCAGATAGC
+3(A:A)	CGTAAGGTGCAACTAGTGCAGATAGC
+3(A:A)/+8(C:C)	CGTAAGCTGCAACTAGTGCAGATAGC

Prior to the experiments, all the oligonucleotides were diluted to 2.5 μM with deionized water(Tiangen).

3. Sequences used in the post-PCR assay

MT template:

CTTAATTTTTAATGGAACTGACAAAACGTAAGGTGCACCTAGTGCAGATAGCAAAA AGACTATCCCTGAGTAAAACC

MT template's complement:

PO_4-GGTTTTACTCAGGGATAGTCTTTTTGCTATCTGCACTAGGTGCACCTTACGTT TTGTCAGTTCCATTAAAAATTAAG

WT template:

CTTAATTTTTAATGGAACTGACAAAACGTAAGATGCACCTAGTGCAGATAGCAAAA AGACTATCCCTGAGTAAAACC

WT template's complement:

GGTTTTACTCAGGGATAGTCTTTTTGCTATCTGCACTAGGTGCATCTTACGTTTTGTC AGTTCCATTAAAAATTAAG

Forward primer: GGTTTTACTCAGGGATAGTCT

Reverse primer: CTTAATTTTTAATGGAACTGAC

4. Sequences used in the genomic DNA assay

FAM-TGGT_TACCTACCG-BHQ1

Forward primer: PO_4-GACCCACTCCATCGAGATTTC

Reverse primer: ACCTCACAGTAAAAATAGGTGATT

5. The protocol for discrimination

To obtain the reaction system for discrimination factors, 1 μL of the 26-nt target sequence (2.5μM) , 1 μL of the fluorescence probe (2.5μM), 2μl of 10× ThermoPol Buffer, and 0.05 unit of endonuclease IV (Thermo Scientific, USA) were added and brought up to a total volume of 20 μL by deionized water(Tiangen). The tube was put into StepOne Real-time PCR(StepOne, US) and the temperature was raised to a certain temperature immediately. Fluorescence intensity was measured once a cycle, and each cycle lasts for 10 s. The overall detection time is 16.7 min (50 cycles). All experiments were performed independently in triplicate.

6. The protocol for detection limits

To obtain the reaction system for detection limits, 1 μL of the specified abundance of mixed targets (for example, 100%, 10%, 1%, 0.05% and 0.01% MM to PM ratio, respectively), 1 μL of the fluorescence probe (2.5μM), and 0.05 unit of endonuclease IV (Thermo Scientific, USA) were added and brought up to a total volume of 20 μL by deionized water(Tiangen, China). Put the tube into StepOne real time PCR (Applied Biosystems, US) and raise the temperature to a specific value immediately. Fluorescence was read once a cycle, and each cycle lasts for 10 s. The total detection time is 41.7 min (250 cycles). All experiments were performed independently in triplicate.

7. Post-PCR assays for synthesized DNA

Two solutions were prepared for PCR experiments. Solution 1 contained 2nM of MT template and its complementary strand. Solution 2 contained 2nM of WT template and its complementary strand. To each of the solution, 5 pmol of forward primer, 5 pmol of reverse primer, 2.0 mM dNTP and 0.5 unit of Taq polymerase were added. After certain cycles of PCR, the PCR products were treated with 1.5 units of exonuclease at 37℃ for 20 min. Then the solutions were heated to 80℃ for 10 min to inactivate exonuclease. Afterwards, the products were treated with 1 units of λ Exonuclease at 37℃ for 20 min and then they were heated to 75℃ for 15 min to inactivate the enzyme. Finally, 2.5 pmol of abasic probes and 0.05 units of Endo IV were added to each of the solution and the fluorescence intensity was measured at certain temperatures at 46℃.

8. Post-PCR detection of BRAF gene single nucleotide polymophisms

MT genomic DNA were extracted from colorectal adenocarcinoma sample which was detected BRAF gene exon 15 V600G mutation by a commercial extraction kit from Tiangen Biotech Co. (Beijing, China). Then they were PCR amplified and sequenced to measure the abundance of the mutation(10%). Afterwards MT were diluted by wild-type genomic DNAs extracted from a human tissue without that mutation to prepare a series of mixed samples with the mutant-type DNA at different abundances.

2 μL of 10× ThermolPol Reaction Buffer, 1mM of dNTPs, 250 nM of forward primers, 250 nM of reverse primers, 20 ng of mixed genomic DNA samples, 0.5 units of Taq DNA Polymerase were added, and the total volume was brought up to 20 μL by deionized water. PCR procedure (94°C for 15 s, 55°C for 30 s, 72°C for 15 s, 30 cycles) was performed on StepOne Plus Real-Time PCR System. After the PCR amplification, 1.5 units of exo I was added to the amplicons at 37°C for 20 min to remove the unreacted primers, followed by inactivation at 80°C for 15 min. Then 1 units of λ exo was added to digest the strand containing 5'-PO4 in the duplex products for 20 min at 37°C. After inactivation of λ exo at 75°C for 15 min, 125 nM of the fluorescent probe, and 0.05 units of endonuclease IV were added. The detection was performed at optimized temperature.

Supplementary results

1. DFs and melting temperatures of single mismatch targets and double mismatches targets

(1) DFs and melting temperatures of DNA strands containing single mismatch

Table S2. The melting temperatures (T_m) and corresponding DFs of duplexes with single mismatch in different position

Mismatch	T_m	DF
0(perfect match)	61.7	1.00
+1(A:C)	55.2	0.53
+2(G:A)	57.8	8.93
+3(A:C)	58.2	1.62
+3(A:G)	60.3	1.22
+3(A:A)	57.4	0.91
+4(T:C)	55.7	1.44
+5(G:A)	56.2	1.55
+6(C:C)	53.9	1.61
+7(A:C)	55.4	2.44
+8(C:C)	52.4	39.55
+8(C:A)	55.6	8.87
+8(C:T)	54.3	5.26
+9(C:C)	52.1	1.74
+10(T:C)	56.8	1.56
+11(T:C)	61.4	1.56
-1(C:C)	53.5	1.35
-2(A:C)	55.4	1.05
-3(C:C)	53.9	0.84
-4(G:A)	56.2	1.08
-5(T:C)	55.6	1.42
-6(C:C)	54.2	5.97
-7(T:C)	56.6	3.48
-8(A:C)	59.2	1.54
-9(T:C)	61.8	1.80

(2) DFs and melting temperatures of DNA strands containing double mismatches

Table S3. The melting temperatures (T_m) and corresponding DFs of duplexes with different types of double mismatches

Mismatch	T_m	DF
+1(A:C)/+8(C:C)	45.9	62.3
+1(A:C)/+8(C:A)	49.1	15.11
+1(A:C)/+8(C:T)	47.8	12
-1(C:C)/+8(C:C)	44.2	127.04
-1(C:C)/+8(C:A)	47.4	32.9
-1(C:C)/+8(C:T)	46.1	16.33
-2(A:C)/+8(C:C)	46.3	61.86
-2(A:C)/+8(C:A)	49.3	18.94
-2(A:C)/+8(C:T)	48.1	9.9
+3(A:C)/+8(C:C)	48.9	877.07
+3(A:C)/+8(C:A)	52.1	501.36
+3(A:C)/+8(C:T)	50.8	258.28
+3(A:G)/+8(C:C)	51	30.79
+3(A:G)/+8(C:A)	54.2	6.07
+3(A:G)/+8(C:T)	52.9	4.87
+3(A:A)/+8(C:C)	48.1	72.71
+3(A:A)/+8(C:A)	51.3	18.79
+3(A:A)/+8(C:T)	50	12.26

2. The DFs and detection limits of all types of -3(A:C)/-8 mismatch

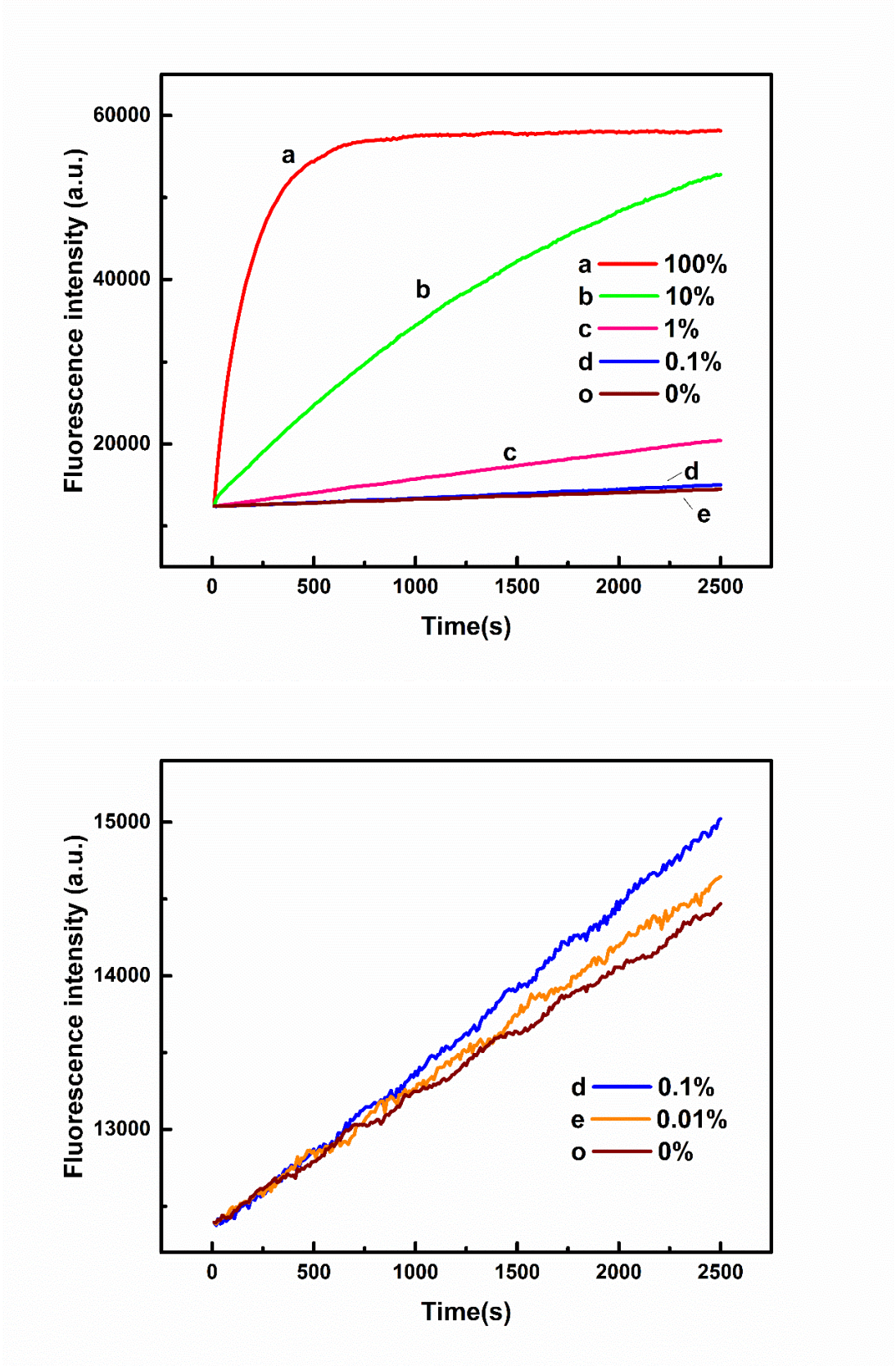
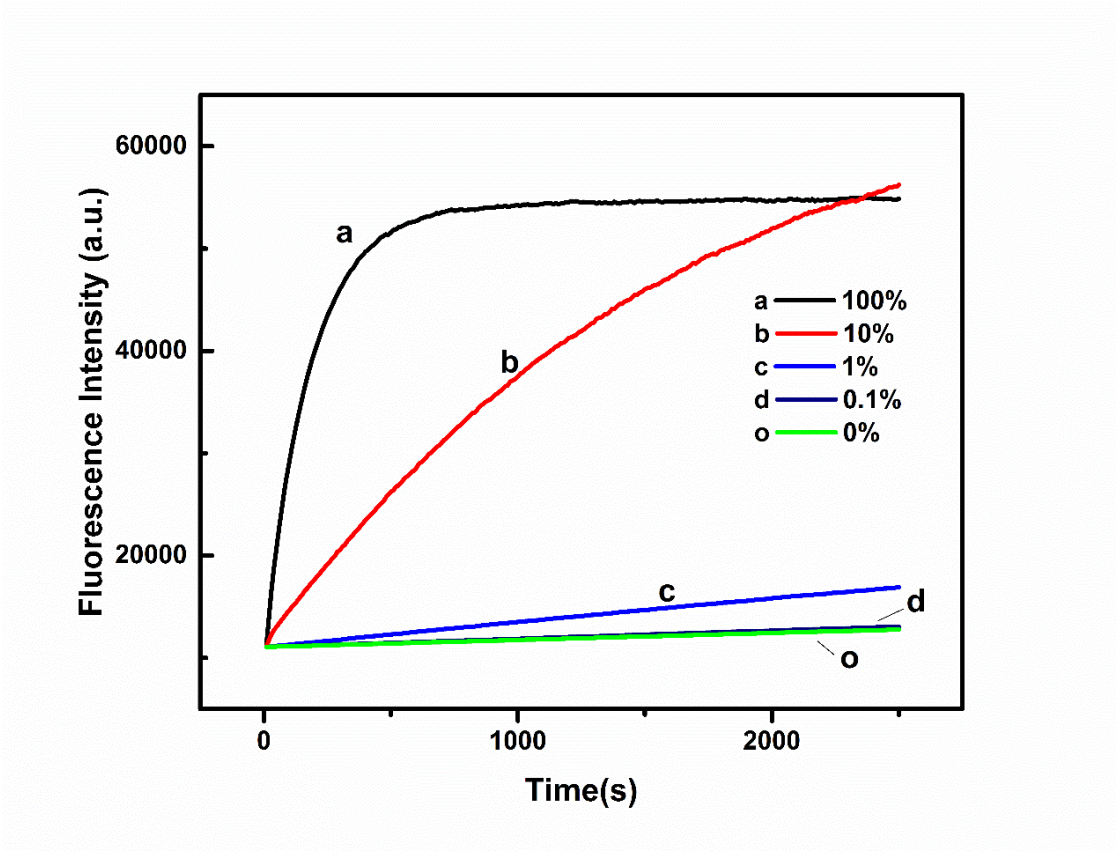


Figure S1. Detection of targets with-3(A:C)/-8(C:A) mismatch immersed in a large background of that with -3(A:C) mismatch interfering strands



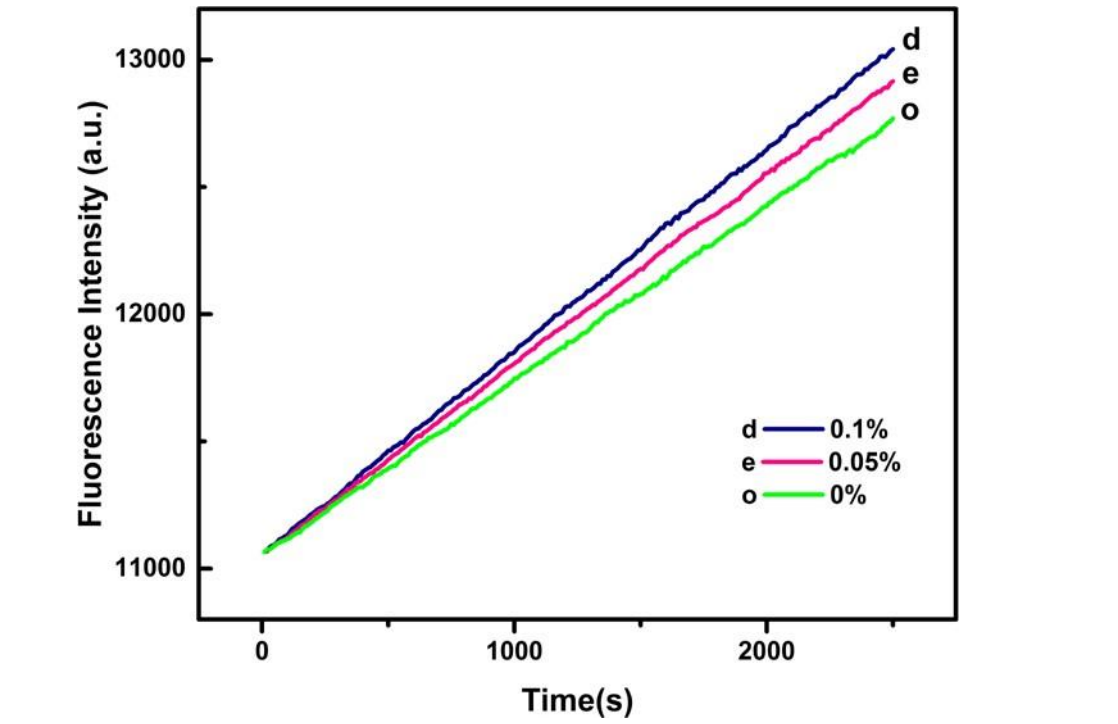


Figure S2. Detection of targets with-3(A:C)/-8(C:T) mismatch immersed in a large background of that with -3(A:C) mismatch interfering strands

3. Post-PCR assay

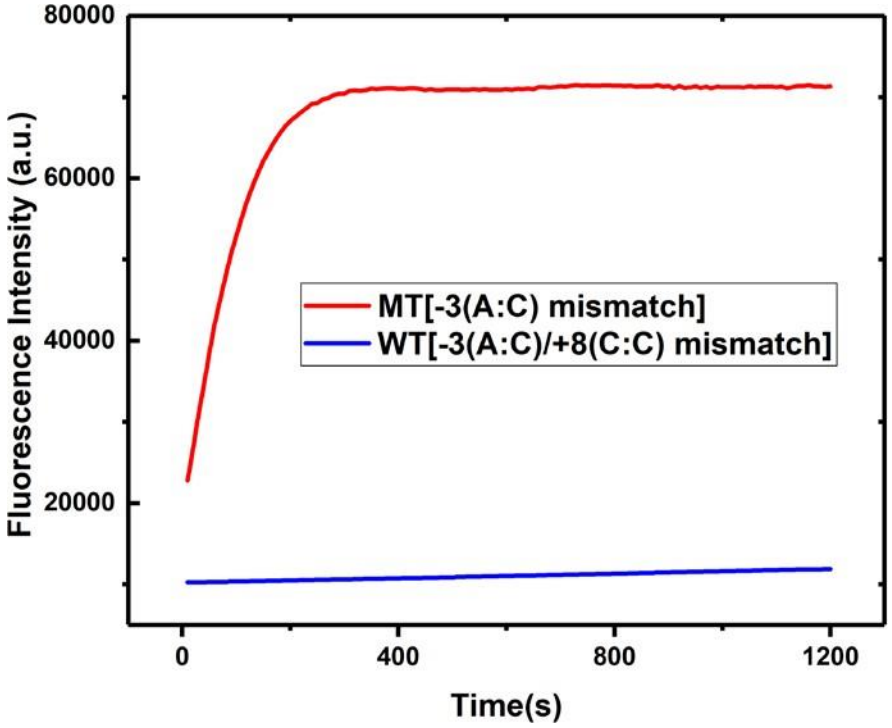


Figure S3. The result of Post-PCR assay is in consistency with synthesized DNA