Electronic Supplementary Information

Endonuclease IV and T4 ligase assisted detection of mutations in low abundance

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Materials and reagents

All the DNA sequences used in this experiment (Table S1) were synthesized and ultra-PAGE purified by Sangon Biotech Co., Ltd. (Shanghai, China). All the DNA sequences were dissolved in buffer composed of 20 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$. Endonuclease IV was purchased from HaiGene Biotech Co., Ltd. (Harbin, China). T4 DNA ligase and T4 Polynucleotide Kinase were purchased from Sangon Biotech (Shanghai, China). DNase/RNase free deionized water was purchased from Thermo Fisher Scientific Co., Ltd.

Apparatus

All enzyme involved reactions were precisely controlled on temperature with a A300 Fast Thermal Cycler (LongGene, China). PAGE was performed using BG-ver MIDI (BayGene, China). qPCR were performed using QuantStudio3 (Thermo Fisher Scientific, USA).

Preparation of phosphorylated Probe S

Typically, 15 μL reaction solution contained 1× T4 Polynucleotide Kinase buffer (140 mM Tris–HCl, 20 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.6), 10 μM Probe S, and 5 U of T4 Polynucleotide Kinase. The reaction mixture was incubated at 37 ℃ for 10 min for phosphorylation, followed by inactivation of T4 Polynucleotide Kinase by holding the reaction solution at 65 ℃ for 20 min.

Optimization of cleavage conditions

200 nM of the 34-nt template strand P, 200 nM of the CL probe, and 0.001-0.02 U of endonuclease IV were incubated at T=10- 37 ℃ for 1, 3, 5 or 10 min for cleavage. Then the mixture was incubated at 85 ℃ for 20 min to inactivate the Endonuclease IV. Cleavage samples were analyzed with electrophoresis and Image J. All experiments were repeated for at least three times.

Cleavage of strands containing AP sites

200 nM of the 34-nt template strand P, 200 nM of the CL probe, and 0.005 U of endonuclease IV were mixed and incubated at 37 ℃ for 5 min. Then the mixture was incubated at 85 ℃ for 20 min to inactivate Endonuclease IV. Cleavage samples were analyzed with electrophoresis and Image J. All experiments were repeated for at least three times.

Preparation of DNA template for qPCR reaction

200 nM of the 34-nt strand P, 200 nM of the CL probe, 200 nM of the Probe S, 0.005 U of endonuclease IV, and 0.1 U of T4 DNA Ligase were incubated at 37 ℃ for 3 min to complete enzyme cleavage, with subsequent incubation at 16 ℃ for 10 min to facilitate ligation. Then the mixture was incubated at 85 ℃ for 20 min to inactivate Endonuclease IV and T4 DNA Ligase. Results were analyzed with electrophoresis. All experiments were performed for at least three times.

PCR experiments

200 nM of mixed Temp strands (100%, 10%, 1%, 0.1%, 0.01%, 0% P to MT ratio, respectively), 200 nM of the CL probe, 200 nM of the Probe S, 0.005 U of endonuclease IV, and 0.1 U of T4 DNA Ligase were mixed and incubated at 37 ℃ for 3 min for slicing, subsequent 10 min incubation at 16 ℃ for ligation. Then the mixture was incubated at 85 ℃ for 20 min to inactivate Endonuclease IV and T4 DNA Ligase. Obtained product was diluted 1000 times before performing qPCR.

For qPCR reaction, 10 µL 2x SYBR Green PCR Mix, 2 µl diluted template, 2 µl 2 µM Forward Primer and 2 µM Reverse Primer, 4 µl DNase/RNase free deionized water were mixed together. The thermo-cycling program started with 5 min at 94 °C, followed by 45 repeated cycles of 30 s at 94 °C for DNA denature and 1 min at 60 °C for annealing, and extension at 72 ℃ for 10 min. Fluorescence signals were collected at 60 °C in each cycle. All samples were prepared in triplicate.

Supplementary tables and figures

Table S1. Sequences used in this work

Strand name	Sequence (from 5' to 3')[a]
P	ATATATGCC***A***CTGATAGCGTCATCGCCTGGAGAT
MT_a[-1]	ATATATGCC***A***ATGATAGCGTCATCGCCTGGAGAT
MT_b[-1]	ATATATGCC***A***GTGATAGCGTCATCGCCTGGAGAT
MT_c[-1]	ATATATGCC***A***TTGATAGCGTCATCGCCTGGAGAT
MT[-2]	ATATATGCC***A***CGGATAGCGTCATCGCCTGGAGAT
MT[+1]	ATATATGCA***A***CTGATAGCGTCATCGCCTGGAGAT
MT[+2]	ATATATGG***C******A***CTGATAGCGTCATCGCCTGGAGAT
CL1	ATACTGCCGCTGTGAACAGGCGATGACGCTATCAG/ids p/GGATCGG
CL2	ATACTGCCGCTGTGAACAGGCGATGACGCTATCAC/ids p/GGATCGG
CL3	ATACTGCCGCTGTGAACAGGCGATGACGCTATCAA/ids p/GGATCGG
CL4	ATACTGCCGCTGTGAACAGGCGATGACGCTATCAT/ids p/GGATCGG
Probe S	TGACATAACGACATTTAGATACGC
fp	ATACTGCCGCTGTGAACA
rp	GCGTATCTAAATGTCGT

[a]AP sites was denoted as "/ids p/". Mismatch sites were shown in red. Position '0' was shown in bold black.

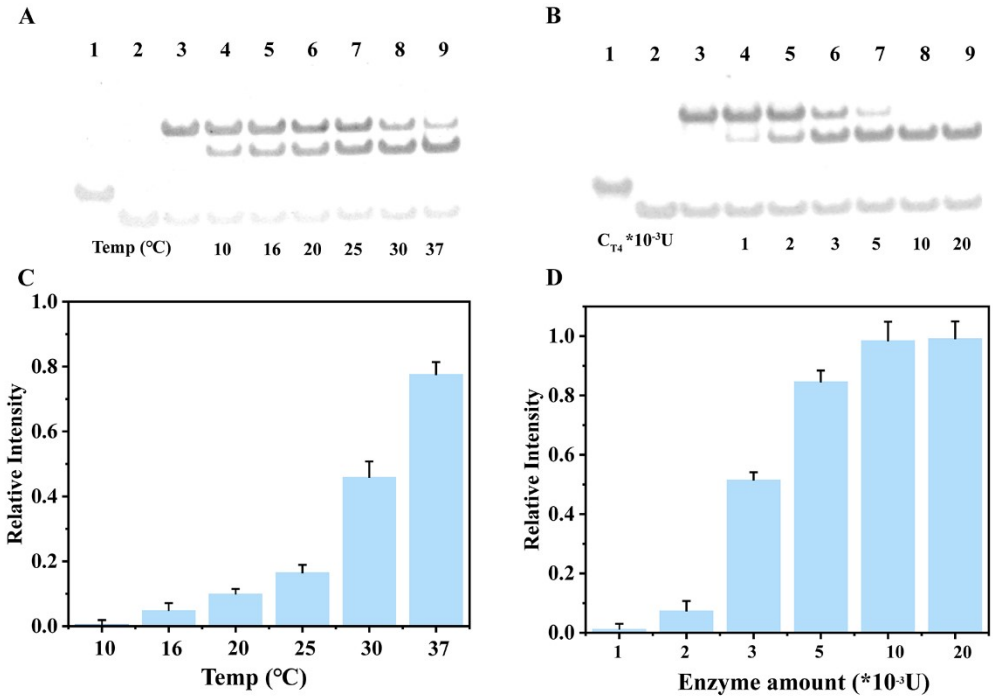

Fig. S1 Optimization of the ligation conditions. (A) The optimization of ligated temperature at 10 ℃, 16 ℃, 20 ℃, 25 ℃, 30 ℃, 37 ℃. (C) Quantification of band intensities with image J. (B) Optimization of enzyme amount applied in the experiments. Amount of 0.001 U, 0.002 U, 0.003 U, 0.005 U, 0.01 U, 0.02 U of Endo IV enzyme were investigated. (D) Quantification of products cleaved by Endo IV in (B).

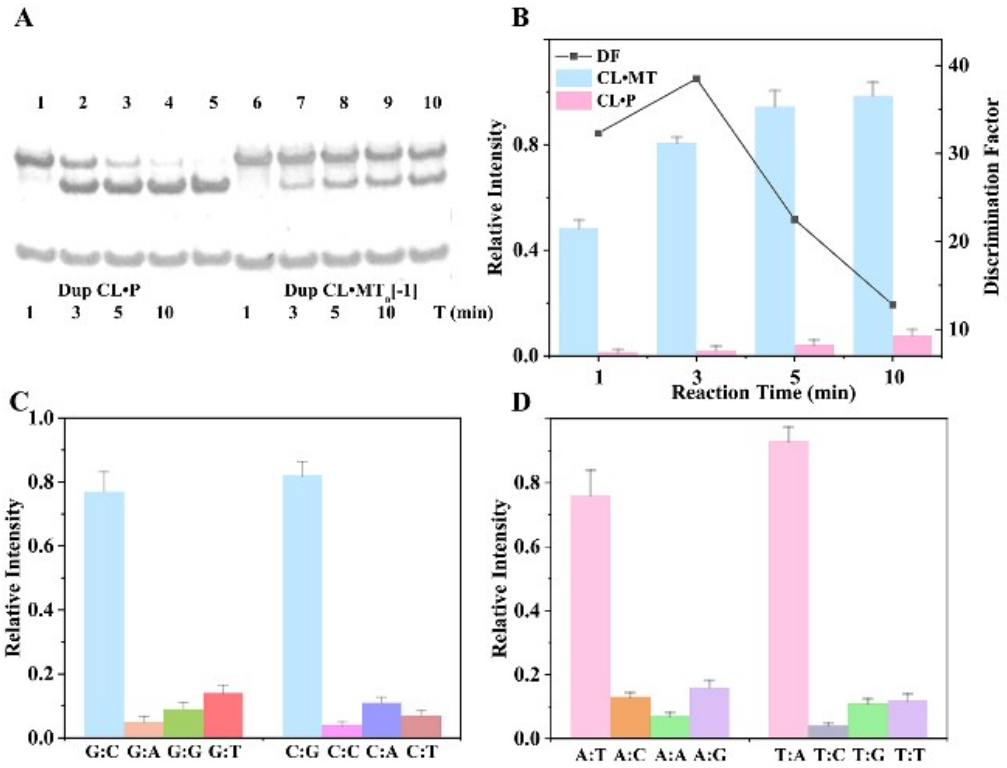

Fig. S2 (A) Time optimization of mutated target and wild type counterparts when exposed to Endo IV. (B) Quantification of endo IV cleaved products with Image J. Discrimination factor is defined by band intensity of wild targets cleaved by endo IV to band intensity of mutated target cleaved by endo IV. (C, D) Endo IV cleavage rate comparison of different mismatches at '-1' position under optimized conditions.

Table S2. List of ΔC_T values under different abundances.

Point mutation	100%	10%	1%	0.1%	0.01%
A>T	12.98	9.82	5.78	3.17	1.47
T>A	16.10	12.17	8.50	4.85	2.12
C>G	12.79	9.05	5.18	2.84	0.73
G>C	17.81	14.01	9.89	6.10	2.61
A>C	11.70	8.32	4.36	3.21	1.11
C>A	12.88	9.76	5.67	3.49	0.99
T>G	11.40	8.19	4.65	1.79	0.66
G>T	11.69	8.02	4.82	1.82	0.58
A>G	9.51	6.64	3.13	1.19	0.30
G>A	10.48	7.04	3.51	1.74	0.55
C>T	12.03	9.41	6.10	3.16	0.81
T>C	12.28	9.53	5.57	3.22	0.93