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Supporting Information for

Endonuclease IV based competitive DNA probe assay for differentiation of low-abundance point mutations by discriminating stable single-base mismatches

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Table S1. Sequences used in this work:

Strand name	Sequence (from 5' to 3')[a]
Probe-0	5'FAM-ACG C_A GCA GCT-3'BHQ1
PM-1	TTG GAG CTG CTG GCG TAG G
MM-1	TTG GAG CTG ATG GCG TAG G
MM-2	TTG GAG CTG GTG GCG TAG G
MM-3	TTG GAG CTG TTG GCG TAG G
Blocker-1	ACG CCA TCA GC
Blocker-2	ACG CCA CCA GC
Blocker-3	ACG CCA ACA GC
Probe-1	5'FAM-TAC GC_ AAC AGC TC-3'BHQ1
MT-1	TTG GAG CTG TTG GCG TAG G
WT-1	TTG GAG CTG GTG GCG TAG G
Blocker-WT-1	CTA CGC CAC CAG C
Blocker-WT-1-12nt	CTA CGC CAC CAG
Blocker-WT-1-14nt	CTA CGC CAC CAG CT
Probe-2	5'FAM-ACG CC_ CTA GCT CC-3'BHQ1
MT-2	GTT GGA GCT AGT GGC GTA G
WT-2	GTT GGA GCT GGT GGC GTA G
Blocker-WT-2	ACG CCA CCA GCT C
Probe-3	5'FAM-TAC GC_ AGC AGC TC-3'BHQ1
MT-3	TTG GAG CTG CTG GCG TAG G
WT-3	TTG GAG CTG GTG GCG TAG G
Blocker-WT-3	CTA CGC CAC CAG C
Forward primer	GCC TGC TGA AAA TGA CTA
Reverse primer	5' PO_4-ATT CGT CCA CAA AAT GAT TCT G
Probe-S	5'FAM-CTGCAC_AGATGC-3'BHQ1
PM-S	GCATCTAGTGCAG
MM-A	GCATATAGTGCAG
MM-T	GCATTTAGTGCAG
MM-G	GCATGTAGTGCAG

[a] AP sites was denoted as "_". Mismatch sites were shown in red.

Table S2. Melting temperatures of involving duplexes

Duplexes	Melting temperatures (°C)
Probe-0/PM-1	47.0
Probe-0/MM-1	36.5
Probe-0/MM-2	37.7
Probe-0/MM-3	35.0
Blocker-1/PM-1	31.8
Blocker-1/MM-1	44.1
Blocker-2/PM-1	28.0
Blocker-2/MM-2	48.1
Blocker-3/PM-1	31.8
Blocker-3/MM-3	45.0
Probe-1/MT-1	43.9
Probe-1/WT-1	39.7
Blocker-WT-1/MT-1	33.1
Blocker-WT-1/WT-1	49.1
Probe-2/MT-2	50.0
Probe-2/WT-2	46.0
Blocker-WT-2/MT-2	37.7
Blocker-WT-2/WT-2	51.6
Probe-3/MT-3	47.6
Probe-3/WT-3	41.1
Blocker-WT-3/MT-3	36.5
Blocker-WT-3/WT-3	49.1
Probe-S/PM-S	47.5
Probe-S/MM-A	38.5
Probe-S/MM-T	36.2
Probe-S/MM-G	39.0

Materials and methods

1. Materials

Lambda exonuclease (λ exo), Exonuclease I (exo I), Lambda Exonuclease Buffer and ThermoPol reaction buffer were all purchased from New England Biolabs (MA, USA). Taq DNA polymerase was purchased from Tiangen Biotech Co. (Beijing, China). DNA strands were synthesized and purified by HPLC (Sangon Co., China). The sequences of all the probes and targets that have been studied in this work are summarized in Table S1. DNase/RNase free deionized water from Tiangen Biotech Co. was used in all the experiments. Endonuclease IV was purchased from Thermo Fisher Scientific, USA.

2. Determination of the melting temperature

To a 100 μL PCR tube, 125 nM of the duplexes to be measured, 2μL of 10× ThermoPol Buffer, and 1 μL of Eva Green (Biotium, US) were added and brought up to a total volume of 20 μL by deionized water (Tiangen Biotech, Beijing). The tube was put into StepOne Plus Real-Time PCR Systems (Applied Biosystems, US). Fluorescence intensity was measured from 20 ºC to 60 ºC with an increasing step of 0.5 ºC, and every step held for 10s.

3. Determination of the discrimination factors

To a 100 μL PCR tube, 125 nM of the 19-nt target sequence, 125 nM of the fluorescent probe, 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 Biotech, Beijing). The tube was put into StepOne Plus Real-Time PCR Systems (Applied Biosystems, US). Fluorescence intensity was measured every 10s. The overall detection time was 1000s. All experiments were performed independently in triplicate.

4. Detection of low-abundance point mutations

To a 100 μL PCR tube, 250 nM of mixed strands (100%, 10%, 1%, 0.01%, 0.005%, 0.003% and 0.001% MM to PM ratio, respectively), 125 nM of the fluorescent probe,

an optimized amount of blockers, and 0.05 unit of endonuclease IV were added, and the total volume was brought up to 20 μL by deionized water. Put the tube into StepOne Plus Real-Time PCR System and raise the temperature to an optimized value. Fluorescence intensity was measured every 10s. The total detection time is 50min (300 cycles). All experiments were performed independently in triplicate.

5. Post-PCR detection of low-abundance point mutations from genomic DNA sample

The mutant-type genomic DNA (KRAS G12V) were extracted from SW480 cell-lines using a commercial extraction kit bought from Tiangen Biotech Co. (Beijing, China). The extracted genomic sample was then PCR amplified, and the PCR products were sequenced to measure the abundance of KRAS G12V mutation. According to the abundance level, the genomic sample was diluted by specified amount of normal genomic DNAs brought from Promega (USA) to prepare mixed genomic DNA samples with the abundance of KRAS G12V at different levels.

To a 100 μL PCR tube, 2 μL of 10× ThermolPol Reaction Buffer, 1mM of dNTPs, 500 nM of forward primers, 500 nM of reverse primers, 20 ng of mixed genomic DNA samples (see Table S1 for sequence), 0.5 units of Taq DNA Polymerase were added, and the total volume was brought up to 20 μL by deionized water then mixed well. 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, 10 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 5 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, optimized amount of the blockers, and 0.05 units of endonuclease IV were added. The detection was performed at optimized temperature in the same manner as described above.

Supplementary figures

1. Generality of the recognition property of Endo IV

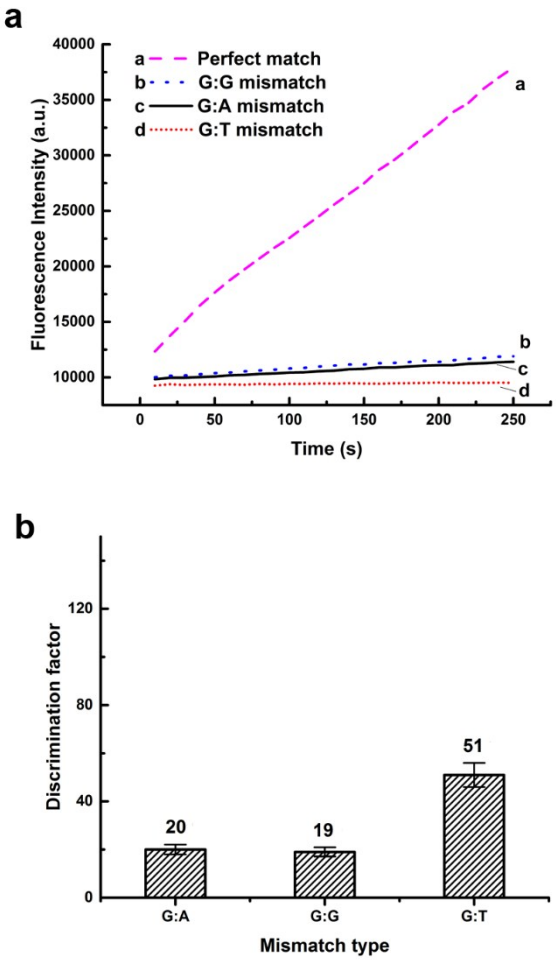

Figure S1. (a) Fluorescence intensity responses of solutions comprising of Endo IV and DNA duplexes with different stable single-base mismatches at position +2. DNA duplexes were formed by Probe-S and corresponding target strands (See their sequences in Table S1: Probe-S, PM-S, MM-A, MM-T, MM-G). All the experiments were conducted under 32ºC. (b) Discrimination factors of Endo IV toward three types of stable single-base mismatches. The DFs were calculated from the curves in Figure S1a.

2. Optimization of the length of blocker-1

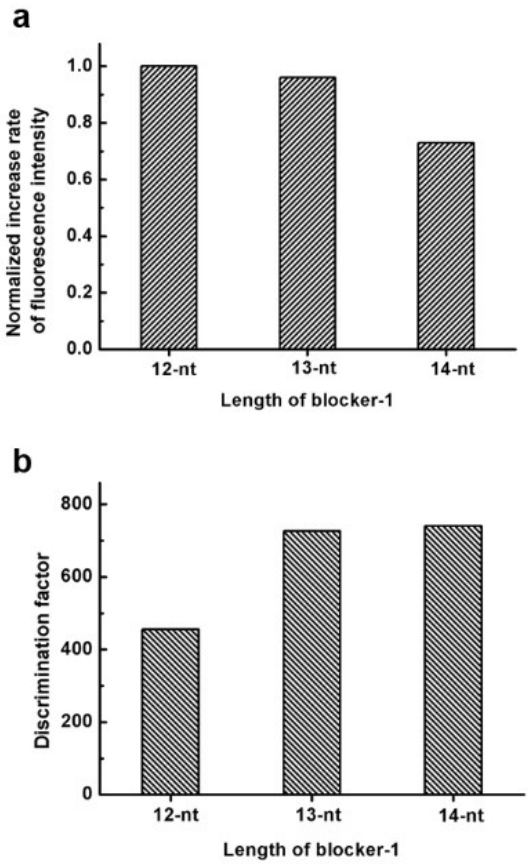

Figure S2. Optimization of the length of blocker-1. The amount of blocker-1 was 1.125μM, which was 9 times of the probe and the target sequences. The increase rate of fluorescence intensity of MT-1 with addition of 11-nt blocker was set to be 1.

3. Optimization of the concentration of blocker-1

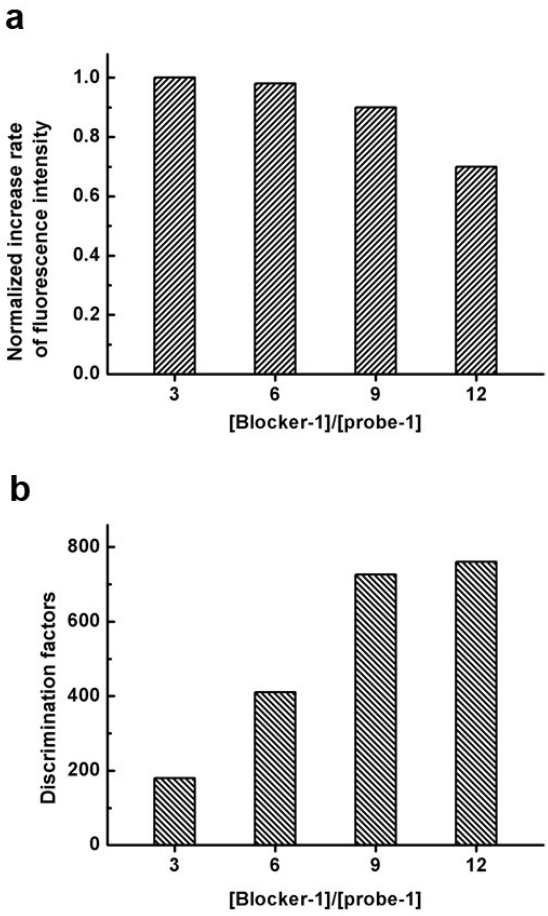

Figure S3. Optimization of the concentration of blocker-1. The concentration of probe-1 and target sequences were set at 125 nM.

4. Detection of low-abundance KRAS G12V mutations in synthesized DNA samples

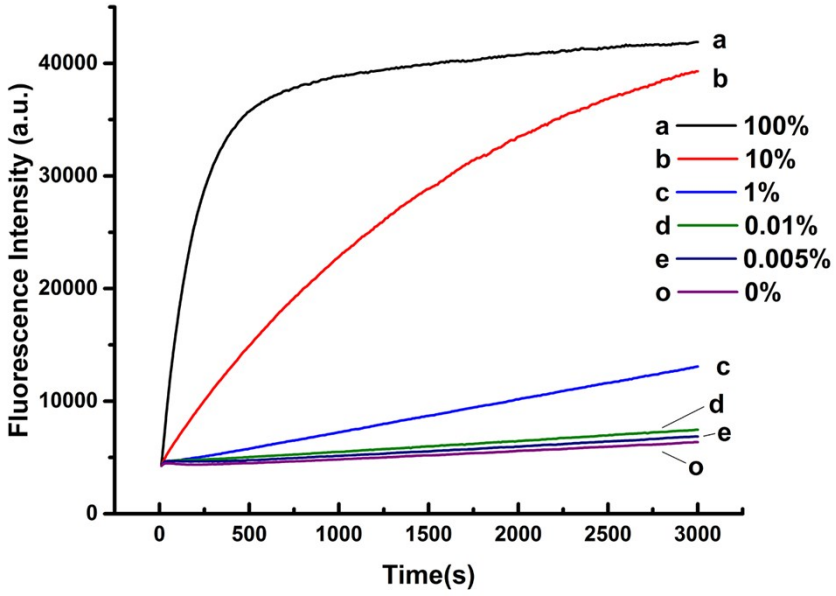

Figure S4. Detection of low-abundance KRAS G12V mutations in synthesized DNA samples.

5. Detection of low-abundance KRAS G12S mutations in synthesized DNA samples

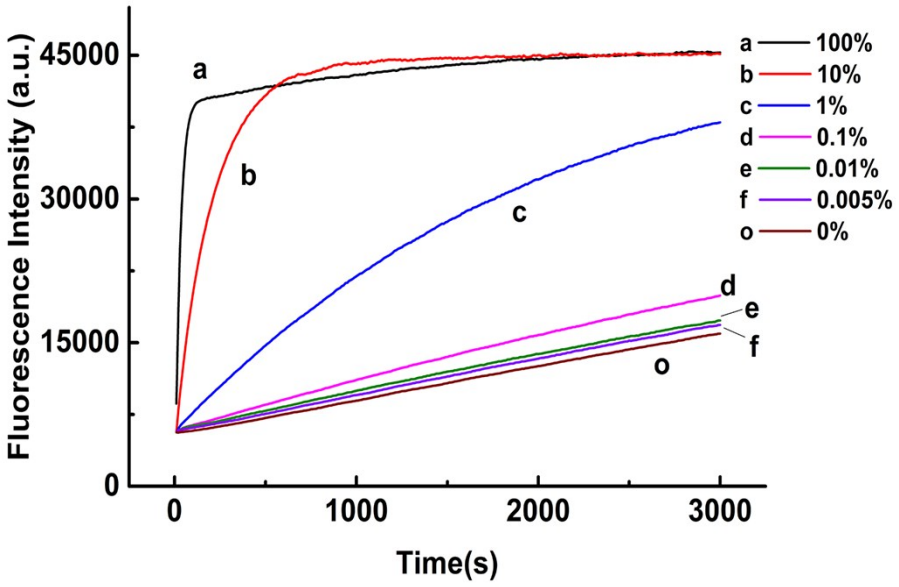

Figure S5. Detection of low-abundance KRAS G12S mutations in synthesized DNA samples.

6. Detection of low-abundance KRAS G12A mutations in synthesized DNA samples

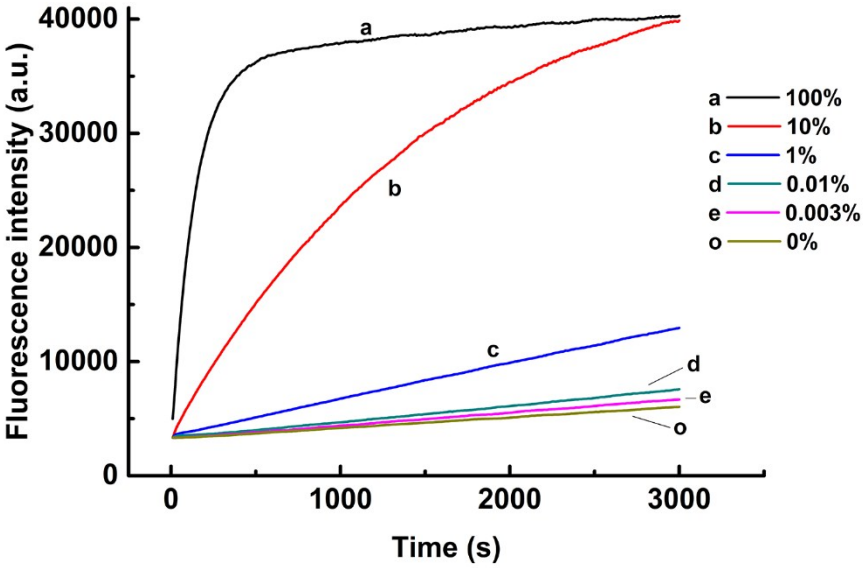

Figure S6. Detection of low-abundance KRAS G12A mutations in synthesized DNA samples.

7. The relationship between the increase rates of fluorescence intensity and mutation abundances

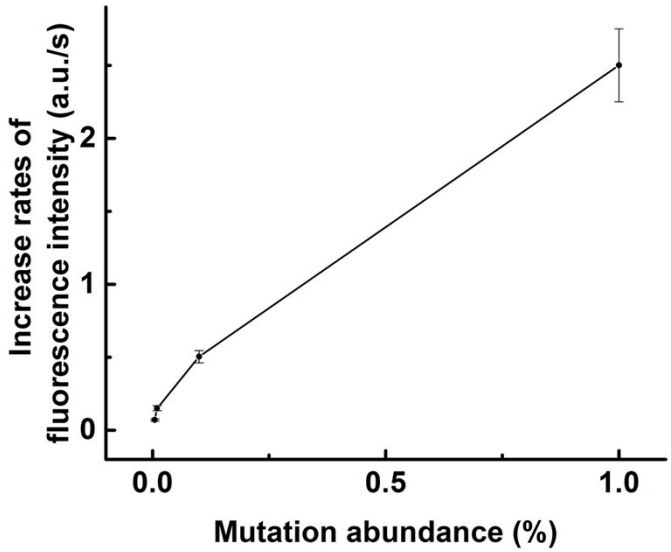

Figure S7. Increase rates of fluorescence intensity of curves in Figure 3 against mutation abundances.