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Supporting Information for

Enzyme-Mediated Single-Nucleotide Variation Detection at Room Temperature with high Discrimination Factor

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Experimental Section

Materials

Lambda exonuclease (λ exo), Exonuclease I (exo I), Lambda Exonuclease Buffer and ThermoPolReaction 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 1 and Table S1. DNase/RNase free deionized water from Tiangen Biotech Co. was used in all the experiments.

Detection of target ssDNA (Figure 1, Figure 2, Figure S1 and Figure S2)

To a 200 μL PCR tube, 39 μL of water, 5 μL of 10× ThermolPolReaction Buffer, 2 μL of probe (10 pmol) and 1 μL of target ssDNA (5 pmol) were added and mixed well. The solution was heated to 85 ^{o}C and then gradually cooled down to 37 ^{o}C (for Figure 2B, 25°C, 30 ^{o}C and 37°C, respectively). Then 3 μL of λ exo (0.83 U) was added and the detection was performed at 37 ^{o}C (for Figure 2B, 25°C, 30 ^{o}C and 37°C, respectively) on a Rotor-Gene Q 5plex HRM Instrument (QIAGEN, Hilden,Germany) with gain level of 7. Fluorescence intensity was measured once a cycle (5 s per cycle) for 240 cycles. The excitation and emission wavelengths were set to 470 nm and 510 nm, respectively. The rate of fluorescence increase was determined by the slope of the linear portion of the time curve.

Detection of SNP (Figure 3A)

To a 200 μL PCR tube, 37 μL of water, 5 μL of 10× ThermolPolReaction Buffer, 1 μL of KCl (100 mM), 1 μL of $(NH_4)_2SO_4$ (100 mM), 2 μL of probe (10 pmol) , and 1 μL of target ssDNA (5 pmol) were added and mixed well. The

solution was heated to 85 ^oC and then cooled down to 25^oC. Then 3 μL of λ exo (5 U) was added and the detection was performed at 25 ^oC in the same manner as described above.

Detection of low abundance point mutation (Figure 3B and Figure S4)

To a 200 μL PCR tube, 37 μL of water, 5 μL of 10× ThermolPol Reaction Buffer, 1 μL of KCl (100 mM), 1 μL of $(NH_4)_2SO_4$ (100 mM), 2 μL of probe (10 pmol), and 1 μL of mixed target ssDNA (total amount 5 pmol) were added and mixed well. After the same heating-annealing procedure as above, 3 μL of λ exo (5 U) was added and the detection was performed at 25 ^oC in the same manner as described above.

Determination of the sensitivity of this method (Figure S3)

To a 200 μL PCR tube, 37 μL of water, 5 μL of 10× ThermolPolReaction Buffer, 1 μL of KCl (100 mM), 1 μL of $(NH_4)_2SO_4$ (100 mM), 2 μL of probe (10 pmol) , and 1 μL of target ssDNA (1 pmol, 100 fmol, 10 fmol and 1.5fmol) or 1 μL of water as the blank were added and mixed well. After the same heating-annealing procedure as above, 3 μL of λ exo (5 U) was added and the detection was performed at 25 ^oC in the same manner as described above (gain=7.67).

Detection of the low abundance V617F mutation in the *JAK2* gene using synthetic template strands (Figure S5)

To a 200 μL PCR tube, 36 μL of water, 5 μL of 10× ThermolPol Reaction Buffer, 1.5 μL of KCl (100 mM), 1.5 μL of $(NH_4)_2SO_4$ (100 mM), 2 μL of probe (10 pmol), and 1 μL of mixed target ssDNA (total amount 5 pmol) (see Table S1 for sequence) were added and mixed well. After the same heating-annealing procedure as above, 3 μL of λ□exo (1.25 U) was added and the detection was performed at 25 ^oC in the same manner as described above (gain=9).

Detection of low abundance *JAK2*V617F mutation in PCR amplicons (Figure 4)

To a 200 μL PCR tube, 25.5 μL of water, 5 μL of 10× ThermolPol Reaction Buffer, 4 μL of dNTPs (10 nmol), 1 μL of forward primers (12 pmol), 1 μL of reverse primers (12 pmol), 1 μL of mixed ssDNA templates (total amount 1 fmol, 32 pg) (see Table S1 for sequence), 0.5 μL of Taq (1.25 U) were added and mixed well. PCR procedure (94^oC for 30 s, 60 ^oC for 30 s, 72 ^oC for 20 s, 25 cycles) was performed on a Rotor-Gene Q 5plex HRM Instrument (QIAGEN, Hilden, Germany). After the PCR amplification, 1 μL of exo I (5 U) was added to the amplicons remove the unreacted primers, followed by inactivation at 85 ^oC for 10 min. Then 3 μL of λ exo (5 U) was added to digest the strand containing 5'-PO_4 in the duplex products for 20 min at 37 ^oC. After inactivation of λ exo at 85 ^oC for 10 min, 1.5 μL of KCl (100 mM), 1.5 μL of $(NH_4)_2SO_4$ (100 mM) and 2 μL of probe (10 pmol) were added and mixed well. After the same heating-annealing procedure as above, 3 μL of λ exo (1.25 U) was added and the detection was performed at 25 ^oC in the same manner as described above (gain=8).

Table S1. DNA sequences used in the work that are not included in **Table 1**

Name	Sequence(5'->3')
Probe sequences	
P-4FAM	PO_4-TCTT(-FAM)CACAGACACATACTCCA-BHQ1
P-10FAM	PO_4-TCTCCACAGT(-FAM)CACATACTCCA-BHQ1
P-15FAM-4C	PO_4-TCTCCACAGACACAT(-FAM)ACTCCATAATTTAA-BHQ1
P-3digoxin-15FAM-4C	PO_4-TCT(-digoxin)CCACAGACACAT(-FAM)ACTCCATAATTTAA-BHQ1
P-*JAK2*-CC	PO_4-TCT(-FAM)TCCTGTGGAGACGAGAGT-BHQ1
P-*JAK2*-DD	PO_4-TDT(-FAM)TDCTGTGGAGACGAGAGT-BHQ1[a]
[b]Tested target sequences for P-3FAM-4C, P-15FAM-4C and P-3digoxin-15FAM-4C	
Perfect match	GTTTTAAATTATGGAGTATGTGTCTGTGGAGACGAGAGTAAG
(-II/-I)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTGGA**AC**CGAGAGTAAG
(-I/0)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTGG**CA**ACGAGAGTAAG
(-I/+I)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTG**C**A**A**ACGAGAGTAAG
(-I/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGT**T**GA**A**ACGAGAGTAAG
(-II/-I/0)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTGG**CAC**CGAGAGTAAG
(-II/-I/+I)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTG**T**A**AC**CGAGAGTAAG
(-II/-I/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGT**T**GA**AC**CGAGAGTAAG
(-I/0/+I)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGTG**TCA**ACGAGAGTAAG
(-I/0/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGT**T**G**CA**ACGAGAGTAAG
(-I/+I/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTGT**TC**A**A**ACGAGAGTAAG
Tested target sequences for P-4FAM	
(-I/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTG**C**GA**C**GACGAGAGTAAG
(-I/+I/+II)-mismatch	GTTTTAAATTATGGAGTATGTGTCTG**CT**A**C**GACGAGAGTAAG
Tested target sequences for P-10FAM	
(-I/+II)-mismatch	GTTTTAAATTATGGAGTATG**C**GA**A**TGTGGAGACGAGAGTAAG
(-I/+I/+II)-mismatch	GTTTTAAATTATGGAGTATG**CC**A**A**TGTGGAGACGAGAGTAAG
Tested target sequences for P-*JAK2*-DD and P-*JAK2*-CC	
JAK2-mutant	AGTTTTACTTACTCTCGTCTCCACAG**A**AA**C**ATACTCCATAA
JAK2-wild	AGTTTTACTTACTCTCGTCTCCACAG**AC**A**C**ATACTCCATAA
Sequences used in the PCR procedure	
Forward-primer	PO_4-GCAGCAAGTATGATGAGCAA
Reverse-primer	GGCATTAGAAAGCCTGTAGTT
Wild type	GCAGCAAGTATGATGAGCAAGCTTTCTCACAAGCATTTGGTTTTAAATTATG GAGTATGT**G**TCTGTGGAGACGAGAGTAAGTAAAACTACAGGCTTTCTAATG CC
V617F mutant type	GCAGCAAGTATGATGAGCAAGCTTTCTCACAAGCATTTGGTTTTAAATTATG GAGTATGT**T**TCTGTGGAGACGAGAGTAAGTAAAACTACAGGCTTTCTAATG CC

[a] D stands for the artificial base 5-nitroindole.

[b] The bases shown in bold and underlined in the tested targets indicate that they are mismatched with the opposite bases in the probe.

[c] The point mutation in the sequences of wild-type and V617F mutant type of *JAK2* is indicated in red.

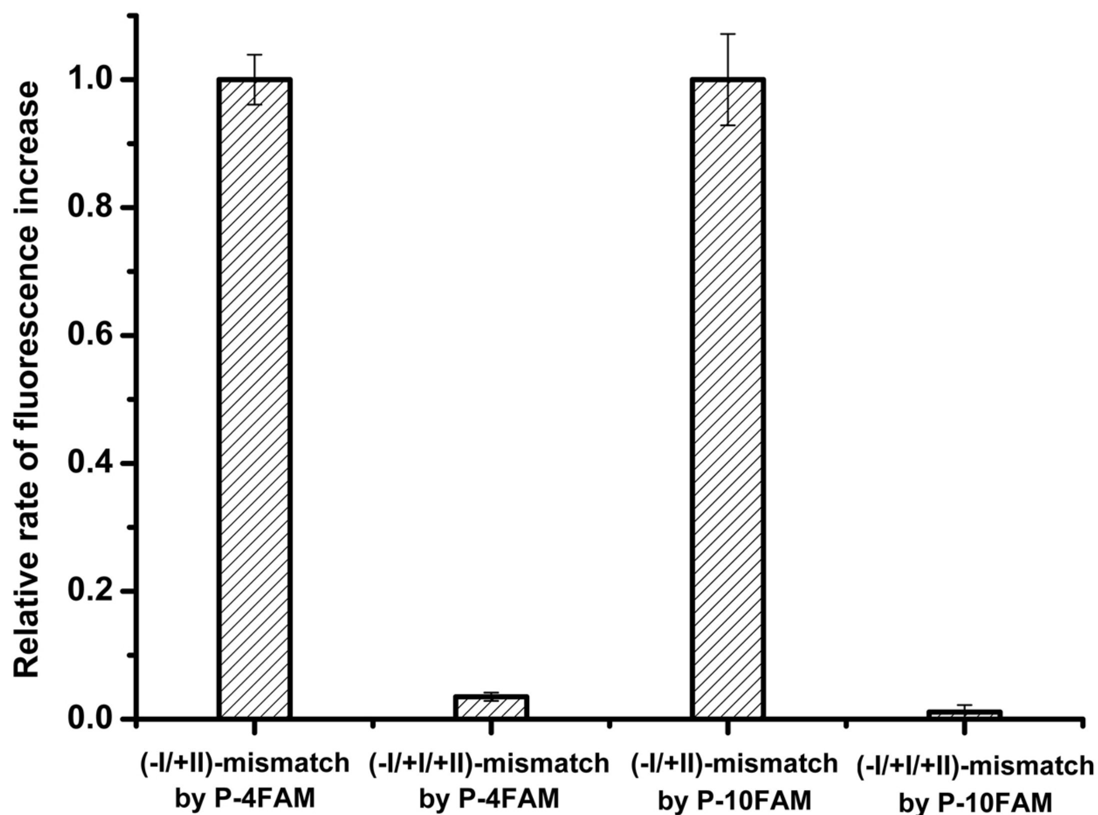


Fig. S1 Investigation on the discrimination capability of two single-base different DNA strands by using probes P-4FAM and probe P-10FAM, respectively (see Table S1). The sequence of Probe P-4FAM is 5'PO_4-TCTT(-FAM)CACAGACACATACTCCA-BHQ1, in which the fluorophore (FAM) is labeled at the fourth nucleoside from 5' end. For Probe P-4FAM, the sequences of (-I/+II)-mismatch and (-I/+I/+II)-mismatch are GTTTTAAATTATGGAGT-ATGTGTCTG**C**GA**C**GACGAGAGTAAG and GTTTTAAATTATGGAGTATGTGTCTG**CT**A**C**GACGAGAGTAAG, respectively. The sequence of Probe P-10FAM is 5'PO_4-TCTCCACAGT(-FAM)CACATACTCCA-BHQ1, in which the fluorophore (FAM) is labeled at the tenth nucleoside from 5' end. For Probe P-10FAM, the sequences of (-I/+II)-mismatch and (-I/+I/+II)-mismatch are GTTTTAAATTATGGAGTATG**C**GA**A**TGTGGAGACGAGAGTAAG and GTTTTAAATTATGGAGTATG**CC**A**A**TGTGGAGACGAGAGTAAG, respectively.

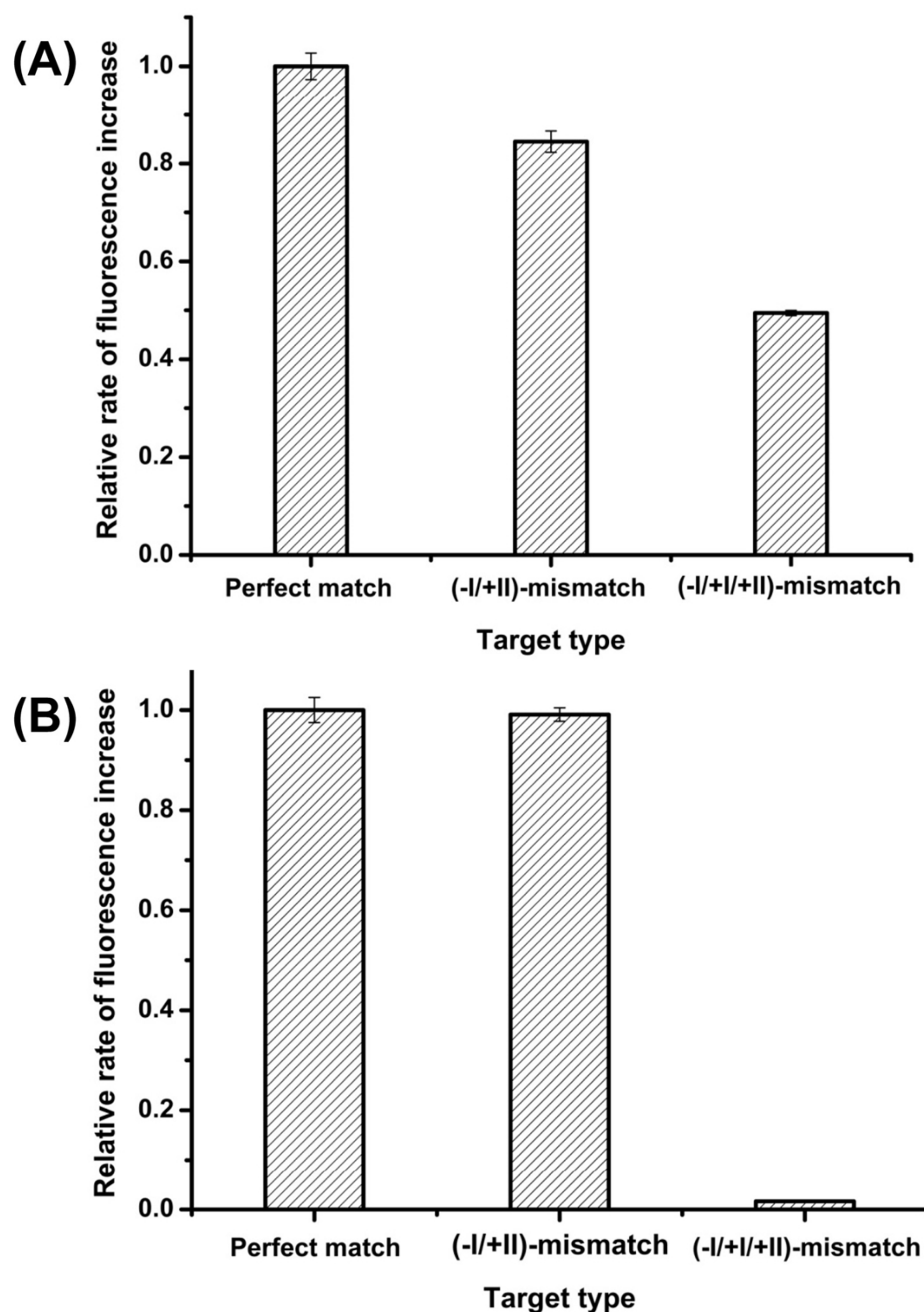


Fig. S2 Investigation on the contribution of the covalently labled group at Position 0 to the discrimination power of the system against mispaired bases at Position +I. (A) P-15FAM-4C (B) P-3digoxin-15FAM-4C. The detailed sequences of the probes and target strands are listed in Table S1.

Table S2. Discrimination factor for C:T mismatch type as N:M for P-3FAM-4C under different buffers.

Buffer	DF(for C:T type)
1× Lambda ExonucleaseBuffer	9.1
0.5×ThermoPol	13
1×ThermoPol	50
2×ThermoPol	78
3×ThermoPol	129
4×ThermoPol	65
5×ThermoPol	9.7
1× ThermoPol+4 mM $MgSO_4$	36
1× ThermoPol+0.2% Triton-X 100	44
1× ThermoPol+10 mM KCl+10 mM $(NH_4)_2SO_4$	122
1× ThermoPol+20 mM KCl+20 mM $(NH_4)_2SO_4$	140
1× ThermoPol+30 mM KCl+30 mM $(NH_4)_2SO_4$	103
1× ThermoPol+40 mM KCl+40 mM $(NH_4)_2SO_4$	101

ThermoPol is short for ThermoPolReaction Buffer.

To a 200 μL PCR tube, 2 μL of P-3FAM-4C (10 pmol) and 1 μLof (-I/+II)-double-mismatch or (-I/+I/+II)-triple-mismatch target ssDNA (5 pmol), different buffers and water (to make the total volume 47 μL) were mixed. After heating-annealing procedure mentioned above, 3 μL of lambda exonuclease (0.83 U) was added and other settings were the same as described above.

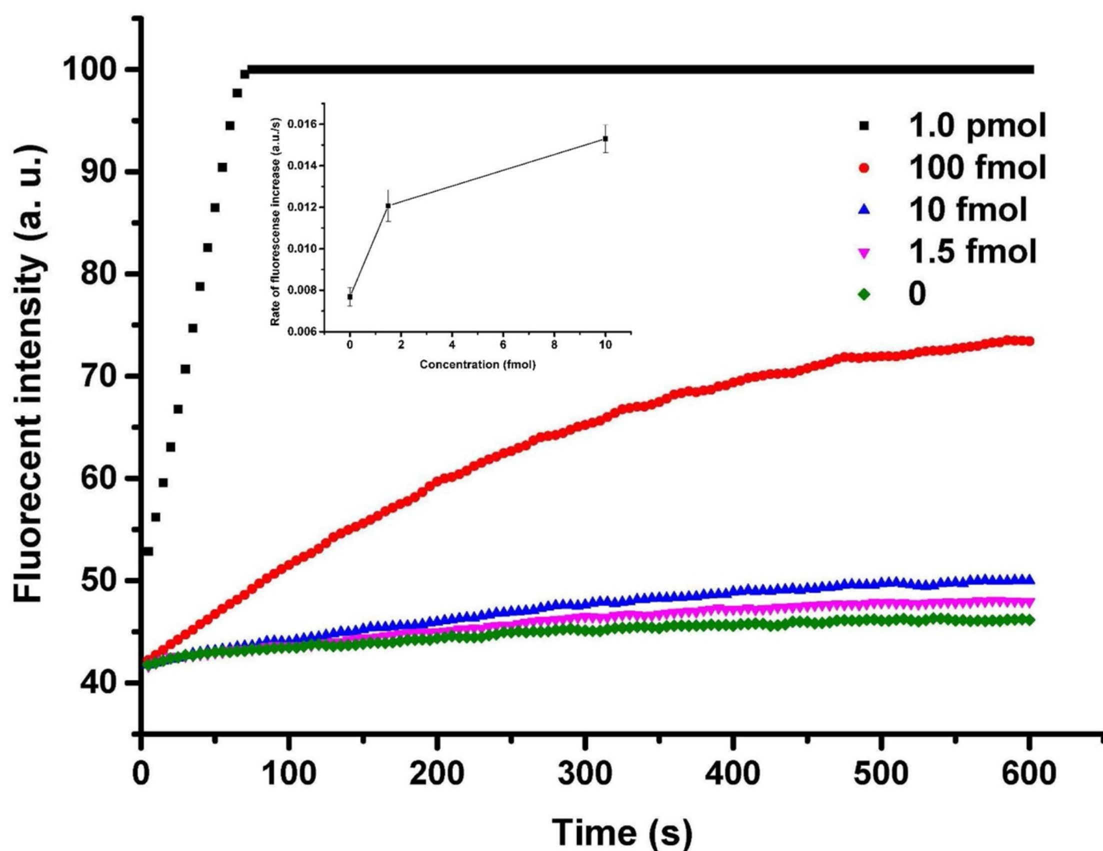
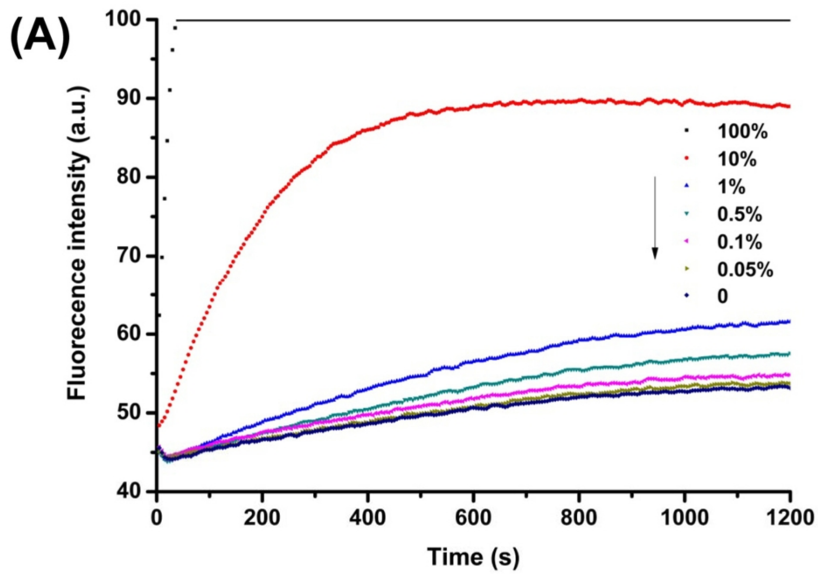
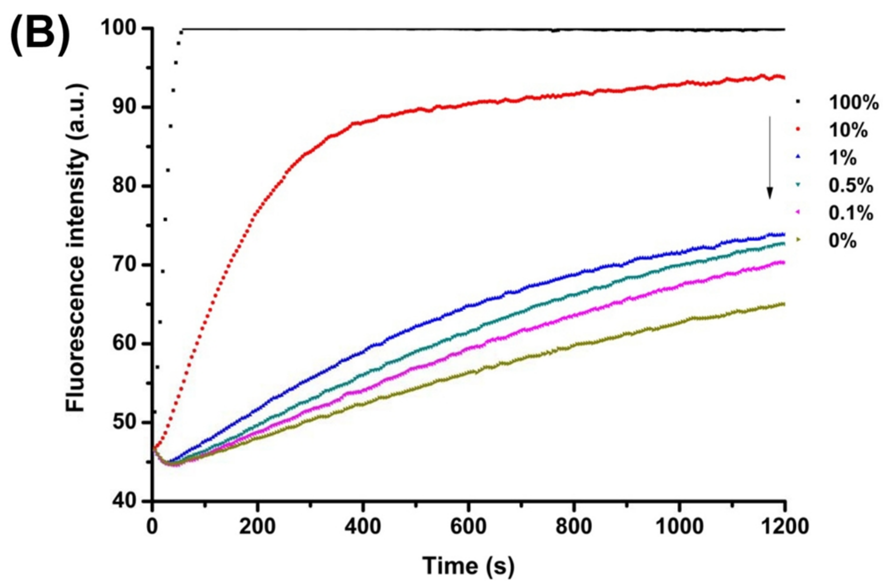
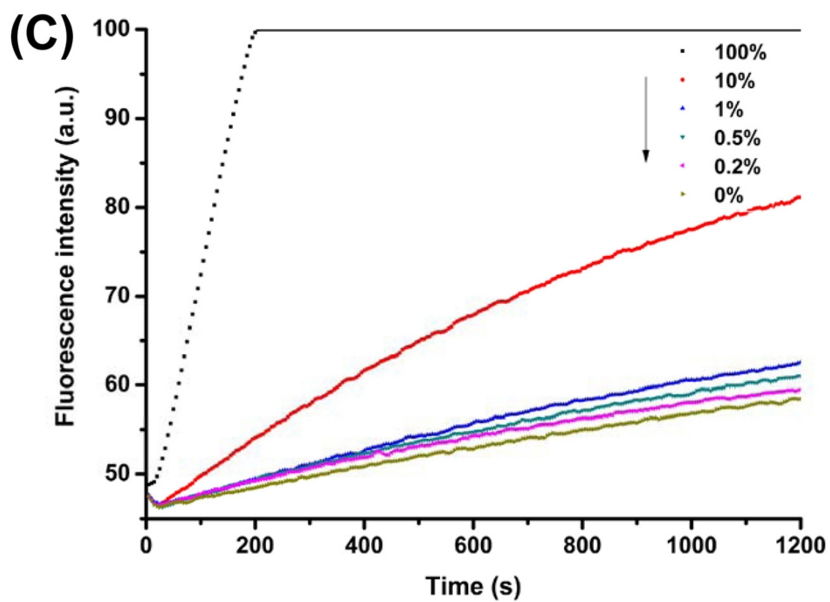


Fig. S3 Determination of the assay sensitivity. Probe: P-3FAM-4C; Target: 5'-GTTTTAAATTATGGAGTATGTGTCT-GT**T**GA**A**ACGAGAGTAAG. The inset shows the mean values and standard deviations of the rate of fluorescence increase within 100-500 s for the background solution (0 fmol) and the two tested solutions with the lowest concentrations (i.e. 1.5 fmol and 10 fmol). The limit of detection of the assay is found to be 1.5 fmol (S/N=4.9>3).


(A)
Fluorecence intensity (a.u.)
Time (s)
100%
10%
1%
0.5%
0.1%
0.05%
0


(B)
Fluorecence intensity (a.u.)
Time (s)
100%
10%
1%
0.5%
0.1%
0%


(C)
Fluorescence intensity (a.u.)
Time (s)
100%
10%
1%
0.5%
0.2%
0%

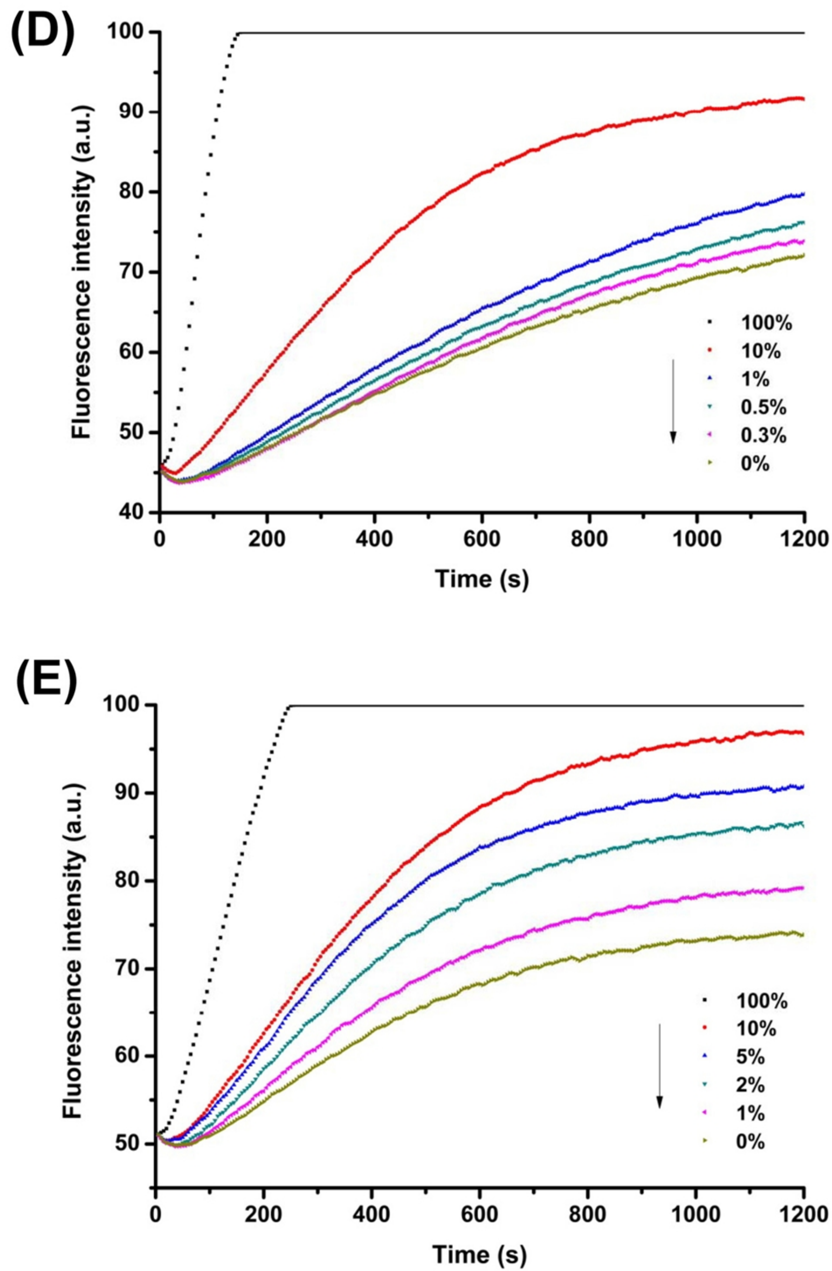


Fig. S4 Fluorescence intensity responses of the designed probes to different types of DNA point mutations at different abundances. The probe sequence is 5'PO_4-TCT(-FAM)**N**CACAGACACATACTCCA-BHQ1. The target sequence is 5'GTTTTAAATTATGGAGTATGTGTCTGTT**M**AAACGAGAGTAAG. 100% means the target strands are all mutant strands (N:M match). 0% means the target strands are all wild-type strands (N:M mismatch). (A) Mutant strands (N:M = C:G); Wild-type strands (N:M = C:A), Selectivity: 0.1% (defined as S/N ≥3); (B) Mutant strands (N:M = C:G); Wild-type strands (N:M= C:T), Selectivity: 0.1%; (C) Mutant strands (N:M = T:A); Wild-type strands (N:M= T:C), Selectivity: 0.2%; (D) Mutant strands (N:M = T:A); Wild-type strands (N:M= T:T), Selectivity: 0.5%; (E) Mutant strands (N:M = A:T); Wild-type strands (N:M= A:C), Selectivity: 1%.

Table S3. Discrimination factors for *JAK2*V617F and two other tested targets against their single-base different DNA strands by using probes P-JAK2-DD and P-JAK2-CC, respectively

Targets	P-*JAK2*-DD	P-*JAK2*-CC
T-1 (*JAK2*V617F)	26	9.3
T-2	17	13
T-3	67	56

The sequences of the three tested targets are:

T-1: AGTTTTACTTACTCTCGTCTCCACAG**A***M*A**C**ATACTCCATAA

M=A (matched) or C (mismatched)

T-2: AGTTTTACTTACTCTCGTCTCCACAG**A***M*A**T**ATACTCCATAA

M=A (matched) or C (mismatched)

T-3: AGTTTTACTTACTCTCGTCTCCACAG**T***M*A**T**ATACTCCATAA

M=A (matched) or C (mismatched)

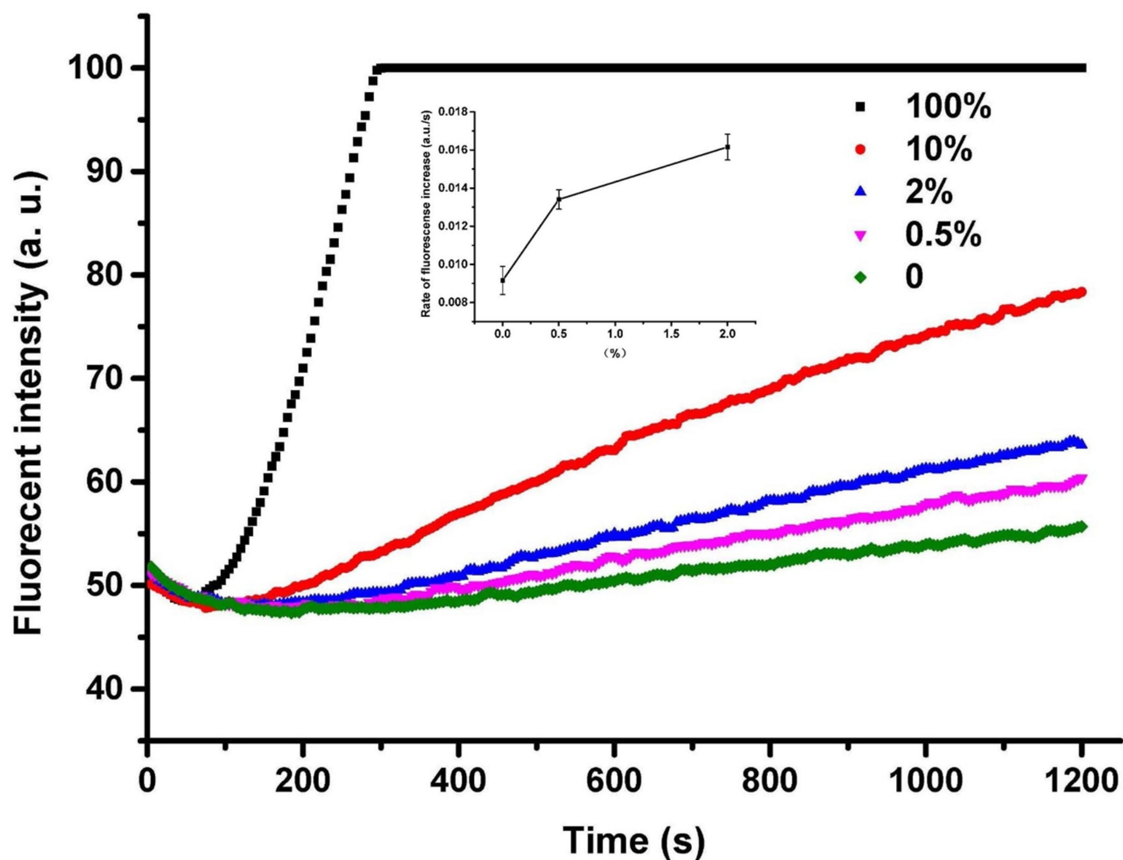


Fig. S5 Fluorescence intensity responses of P-*JAK2*-DD to detect *JAK2*V617F mutant-type at different abundances in synthetic target solutions. 100% means the tested strands are all *JAK2*V617F mutant-type. 0% means the tested strands are all *JAK2* wild-type. The inset shows the mean values and standard deviations of the rate of fluorescence increase within 300-1200 s for the two tested solutions with the lowest-abundance (i.e. 0.5% and 2%).