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Controllable extension of hairpin-structured flaps to allow low-background cascade invasive reaction for sensitive DNA logic sensor of mutation detection

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Experimental section

Sample collection and DNA purification

Samples and clinical data were collected from patients at Jinling Hospital (Nanjing, China) with informed consent forms and ethics committee approval. Tissue samples DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Cell free DNA from plasma was extracted using an EasyPure Viral DNA/RNA Kit (TransGen) according to the manufacturer's instructions.

Conventional invasive reaction

Twenty microliters of a reaction mixture contained 10 mM MOPS (pH 7.5), 0.05% (v/v) NP-40, 0.05% (v/v) Tween-20, 12 μg/mL BSA, 7.5 mM $MgCl_2$, and 6.7 ng/μL *Afu* flap endonuclease prepared in our lab, 50 nM invasive oligo, 600 nM downstream probe, 500 nM FRET probe, 1 μL of target. The cascade invasive reaction was performed at 63 °C for 240 min. The time-course curves were automatically recorded by StepOne® Real-Time PCR System (Applied Biosystems). The reaction rates were calculated based on kinetics of cascade reaction. Sequences of oligonucleotides were listed in **Table S1**.

Controllable extension enabled invasive reaction

Twenty microliters of a reaction mixture contained 10 mM MOPS (pH 7.5), 0.05% (v/v) NP-40, 0.05% (v/v) Tween-20, 12 μg/mL BSA, 7.5 mM $MgCl_2$, and 6.7 ng/μL *Afu* flap endonuclease prepared in our lab, 500 nM FRET probe, 0.005 U of *KlenTaq*® DNA polymerase, 10 μM of dGTP and dTTP, 50 pM flap or extended flap. The extension reaction and invasive reaction was performed at 63 °C for 240 min. The time-course curves were automatically recorded by StepOne® Real-Time PCR System (Applied Biosystems). The reaction rates were calculated based on kinetics of cascade reaction or single-step reaction, respectively. Sequences of oligonucleotides were listed in **Table S2**.

Controllable-extension bridged cascade invasive reaction with hairpin-structured downstream probe

Twenty microliters of a reaction mixture contained 10 mM MOPS (pH 7.5), 0.05% (v/v) NP-40, 0.05% (v/v) Tween-20, 12 μg/mL BSA, 7.5 mM $MgCl_2$, and 6.7 ng/μL *Afu* flap endonuclease prepared in our lab, 50 nM invasive oligo, 600 nM downstream probe, 500 nM FRET probe, 0.005 U of *KlenTaq*® DNA polymerase, 10 μM of dATP, dCTP and dTTP, 1 μL of target. The cascade invasive

reaction was performed at 63 °C for 240 min. The time-course curves were automatically recorded by StepOne® Real-Time PCR System (Applied Biosystems). The reaction rates were calculated based on kinetics of cascade reaction or single-step reaction, respectively. Sequences of oligonucleotides were listed in **Table S3**.

Reaction rate calculation

According to kinetics of the invasive reaction, the reaction rate of the primary invasive reaction can be described as $d[F]/dt= \alpha 1[T]$, therefore, $[F]=\alpha 1[T]t$, where [F] and [T] are the concentrations of the cleaved flap fragment and the target molecule, respectively, α_1 is the cleavage rate, defined as the number of downstream probes cleaved per target molecule per minute, and *t* means the reaction time. Supposed the extension efficiency (E) is constant, the reaction rate of the cascade reaction can be simply described as $d[S]/dt = E\alpha 2[F]= E\alpha 2\alpha 1[T]t$, where [S] represents the generated fluorescence signal, α1, α2 are the cleavage rates of the primary and secondary reaction, respectively. Accordingly, $[S]=E\alpha 2[F]t=1/2E\alpha 2\alpha 1[T]t^2$. Therefore, the reaction rate of secondary invasive reaction can be calculated with the fluorescence intensity divided by reaction time; and the reaction rate of the cascade invasive reaction can be calculated with the fluorescence intensity divided by the quadratic of the reaction time.

DNA logic gates

The reaction mixture was based on the controllable-extension bridged cascade invasive reaction with hairpin-structured downstream probe. In AND gate, 10 pM target DNA and 50 nM invasive oligo were used as inputs; in INHIBIT gate, 10 pM target DNA and 500 μM dGTP were used as inputs; in OR gate, 10 pM of each target DNA were used as inputs. The DNA logic gate was performed at 63 °C for 15 min. The fluorescence signals were automatically recorded by StepOne® Real-Time PCR System (Applied Biosystems).

DNA logic sensor for mutation detection

The reaction mixture was based on the controllable-extension bridged cascade invasive reaction with hairpin-structured downstream probe. The sample DNA after a pre-PCR was used as inputs. The pre-amplification was performed on a 25-μL mixture containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.1 μM of each primer, 1.25 U of Taq DNA Polymerase (Promega, USA), and 2-μL of sample DNA. The PCR program was as follows: 95 °C for 3

min; 25 cycles of 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s. The DNA logic sensor was performed at 63 °C for 15 min. The fluorescence signals were automatically recorded by StepOne® Real-Time PCR System (Applied Biosystems). The relative fluorescence signal was calculated by assigning the plateau signal intensity of positive control (artificially synthesized mutants) as the reference. Sequences of oligonucleotides were listed in Table S4.

Table S1.Sequences of oligonucleotides used in conventional invasive reaction.

Name	Sequence (5'-3')
Invasive probe	ATG TCA CTT CCC CTT GGT TCT CTC C
FRET probe-1	VIC-TCT T (BHQ1) AG CCG GTT TTC CGG CTA AGA CCT CGG CGC G - Spacer C3
Downstream probe	[a]CGC GCC GAG GAT CTG GCC TGG TGC
Target	CTA TTG CAC CAG GCC AGA TGA GAG AAC CAA GGG GAA GTG ACA T

[a] the underline nucleotides are sequences of flap fragment.

Table S2.Sequences of oligonucleotides used in controllable extension enabled invasive reaction.

Name	Sequence (5'-3')
Downstream probe - 0 nt	[a]CGC GCC GAG GAT CTG GCC TGG TGC
Downstream probe - 1 nt	CGC GCC GAG ATC TGG CCT GGT GC
Downstream probe - 2 nt	CGC GCC GAA TCT GGC CTG GTG C
Downstream probe - 3 nt	CGC GCC GAT CTG GCC TGG TGC
Downstream probe - 4 nt	CGC GCC ATC TGG CCT GGT GC
Downstream probe - 5 nt	CGC GCA TCT GGC CTG GTG C
FRET probe-2	VIC-TCT T (BHQ1) AG CCG GTT TTC CGG CTA AGA CCC TGG CGC G - Spacer C3
Flap-1	CGC GCC A
Extended flap	CGC GCCA GGG T

[a] the underline nucleotides are sequences of flap fragment.

Table S3.Sequences of oligonucleotides used in controllable-extension bridged cascade invasive reaction with hairpin-structured downstream probe.

Name	Sequence (5'-3')
Invasive probe	ATG TCA CTT CCC CTT GGT TCT CTC C
Hairpin downstream probe-10 bp	[a]CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCC GGT TTT CCG GCT AAG ATC TGG CCT GGT GC- Spacer C3
Hairpin downstream probe-no gap	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCC GGT TTT CCG GCT AAG ACA CTT AAC AAT CTG GCC TGG TGC
Hairpin downstream probe - 8 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCG TTT TCG CTA AGA TCT GGC CTG GTG C- Spacer C3
Hairpin downstream probe - 6 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TGC GTT TTC GCA GAT CTG GCC TGG TGC- Spacer C3
Hairpin flap - 10 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCC GGT TTT CCG GCT AAG A
Extended hairpin flap - 10 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCC GGT TTT CCG GCT AAG ACA CTT AAC AA
Hairpin flap - 8 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCG TTT TCG CTA AGA
Extended hairpin flap - 8 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCG TTT TCG CTA AGA CAC TTA ACA A
Hairpin flap - 6 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TGC GTT TTC GCA GA
Extended hairpin flap - 6 bp	CAT AGT CCA GGA GGC TTG TTA AGT GTC TGC GTT TTC GCA GAC ACT TAA CAA
FRET probe-3	VIC-AGC CT(BHQ1) C CTG GAC TAT G- Spacer C3
Target	CTA TTG CAC CAG GCC AGA TGA GAG AAC CAA GGG GAA GTG ACA T
MT-a	CTA TTG CAC CAG GCC AGA[b] ***A***GA GAG AAC CAA GGG GAA GTG ACA T
MT-b	CTA TTG CAC CAG GCC AG***T*** TGA GAG AAC CAA GGG GAA GTG ACA T
MT-c	CTA TTG CAC CAG GC***G*** AGA TGA GAG AAC CAA GGG GAA GTG ACA T
MT-d	CTA TTG CAC CAG GCC AGA T***C***A GAG AAC CAA GGG GAA GTG ACA T
MT-e	CTA TTG CAC CAG GCC AGA TG***T*** GAG AAC CAA GGG GAA GTG ACA T
MT-f	CTA TTG CAC CAG GCC AGA TGA GAG AAC CAA ***C***GG GAA GTG ACA T

[a] the underline nucleotides are sequences of flap fragment.

[b] the italic bold nucleotides means the single-base variation.

Table S4.Sequences of oligonucleotides used in DNA logic sensor for mutation detection.

Name	Sequence (5'-3')
Forward primer-858	GGA ACG TAC TGG TGA AAA CAC CGC
Reverse primer-858	TGC ATG GTA TTC TTT CTC TTC CGC ACC
Forward primer-19Del	TGT CAT AGG GAC TCT GGA TCC CAG A
Reverse primer-19Del	GCA GAA ACT CAC ATC GAG GAT TTC CTT GT
Forward primer-790	GGC ATC TGC CTC ACC TCC AC
Reverse primer-790	CCG GAC ATA GTC CAG GAG GCA
Invasive probe-858	CAT GTC AAG ATC ACA GAT TTT GGG CC
Hairpin downstream probe-858	CAT AGT CCA GGA GGC TTG TTA AGT GCC TTA GCG TTT TCG CTA AGG GGC CAA ACT GCT G
Invasive probe-19Del	GAA AGT TAA AAT TCC CGT CGC TAT CAT
Hairpin downstream probe-19Del-1	CAT AGT CCA GGA GGC TTG TTA AGT GTC TTA GCG TTT TCG CTA AGA AAC ATC TCC GAA AGC C
Hairpin downstream probe-19Del-2	CAT AGT CCA GGA GGC TTGTTAAGTGTCTTAGCG TTTTCG CTA AGA GAC ATC TCC GAA AGC
Invasive probe-790	CCG AAG GGC ATG AGC TGC T
Hairpin downstream probe-790	CGT CCG TGG ACC TTG TTA AGT GTC TTA GCG TTT TCG CTA AGA TGA TGA GCT GCA CG
FRET probe-3	VIC-AGC CT(BHQ1) C CTG GAC TAT G- Spacer C3
FRET probe-4	FAM - AGG T(BHQ1) CC ACG GAC G- Spacer C3

[a] the underline nucleotides are sequences of flap fragment.

Table S5.The fluorescence intensities of 37 typical samples by the DNA logic sensor.

Samples	FAM Intensity		VIC Intensity		Output
	Mean	SD	Mean	SD	
NTC	0.0065	0.0001	0.0069	0.0001	0
T1	0.0184	0.0018	0.9702	0.0525	1
T2	0.6806	0.0243	1.0737	0.0316	0
T3	0.0183	0.0006	0.9651	0.0355	1
T4	0.0193	0.0011	0.9909	0.0386	1
T5	0.0173	0.0007	0.6727	0.0315	1
T6	0.0143	0.0009	0.5898	0.0245	1
T7	0.0157	0.0005	0.9594	0.0160	1
T8	0.0182	0.0010	0.9468	0.0224	1
T9	0.0119	0.0021	0.2865	0.0187	1
T10	0.0198	0.0005	0.9575	0.0118	1
T11	0.0071	0.0003	0.0069	0.0001	0
T12	0.0072	0.0002	0.0077	0.0001	0
T13	0.0072	0.0002	0.0098	0.0000	0
T14	0.0103	0.0001	0.3502	0.0133	1
T15	0.0178	0.0009	0.8722	0.0089	1
T16	0.0078	0.0006	0.0136	0.0008	0
T17	0.0072	0.0004	0.0106	0.0005	0
T18	0.0074	0.0005	0.0083	0.0006	0
T19	0.0173	0.0007	0.8798	0.0318	1
T20	0.0083	0.0002	0.0307	0.0013	0
T21	0.0130	0.0005	0.4758	0.0152	1
T22	0.0073	0.0007	0.0080	0.0006	0
T23	0.0192	0.0015	0.8307	0.0139	1
T24	0.0178	0.0010	0.6908	0.0343	1
T25	0.0080	0.0002	0.0129	0.0005	0
T26	0.0074	0.0003	0.0083	0.0002	0
T27	0.0193	0.0009	1.0546	0.0170	1
T28	0.0227	0.0016	1.0654	0.0752	1
T29	0.0073	0.0005	0.0081	0.0005	0
T30	0.0228	0.0013	0.9959	0.0431	1
CF1	0.0079	0.0003	0.0175	0.0009	0
CF2	0.0072	0.0000	0.0156	0.0003	0
CF3	0.0073	0.0006	0.0193	0.0009	0
CF4	0.0075	0.0007	0.0157	0.0008	0
CF5	0.0119	0.0009	0.4301	0.0174	1
CF6	0.0200	0.0008	1.0386	0.0331	1
CF7	0.0202	0.0004	1.0207	0.0399	1

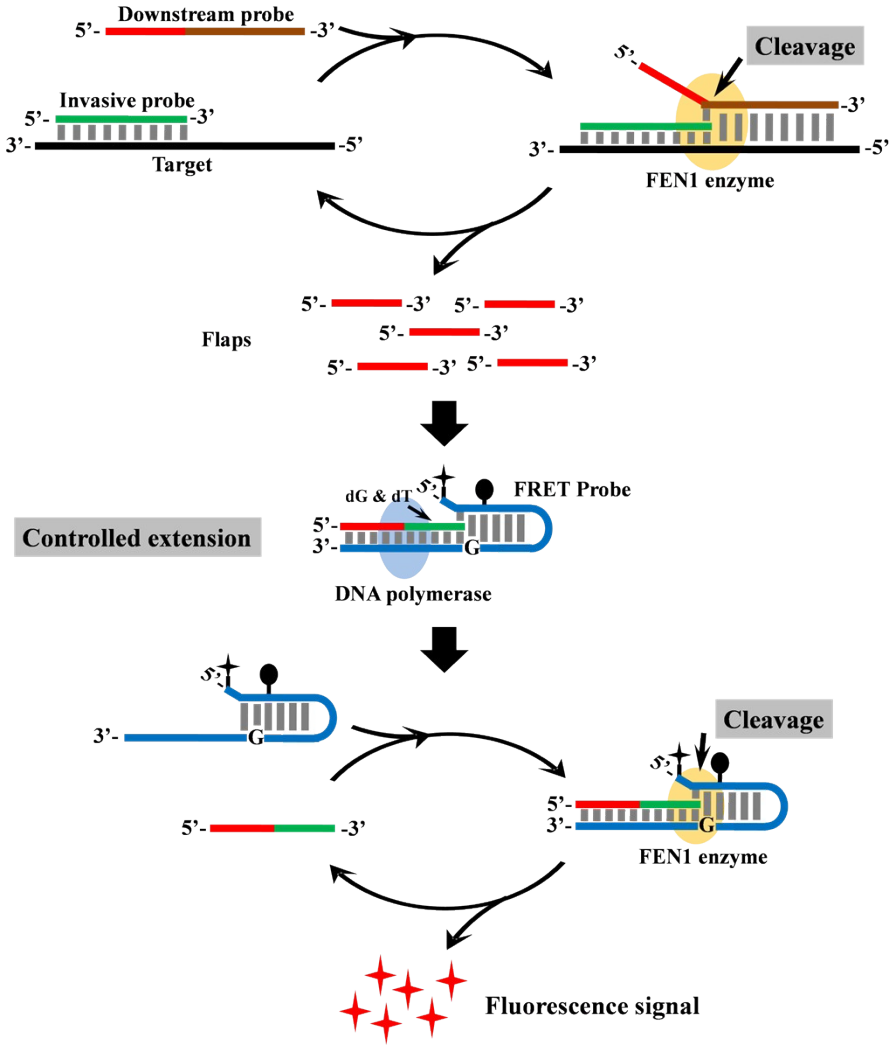


Fig. S1 Schematic illustration of controllable-extension bridged cascade invasive reactions by the conventional single-stranded flaps.

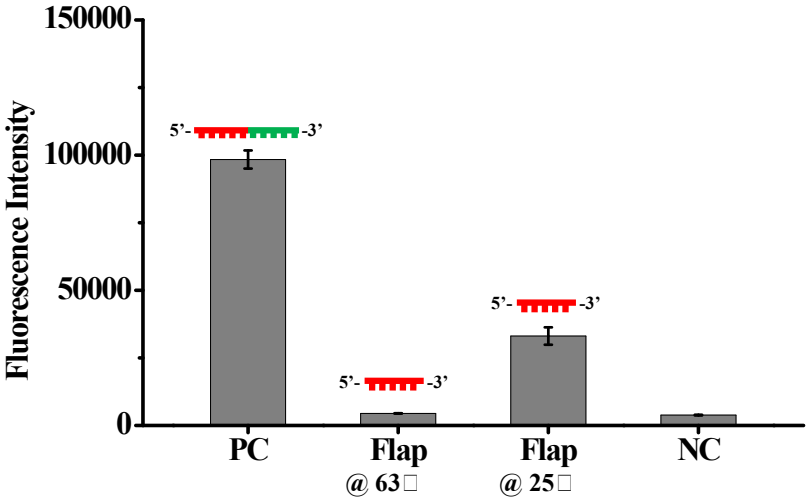


Fig. S2 Fluorescence signals of controllable-extension based second invasive reaction by conventional single-stranded flaps fragments at different temperatures. PC: positive control, signal from 50 pM artificially synthesized extended flaps; NC: negative control, signal from the reaction without flaps. N=3.

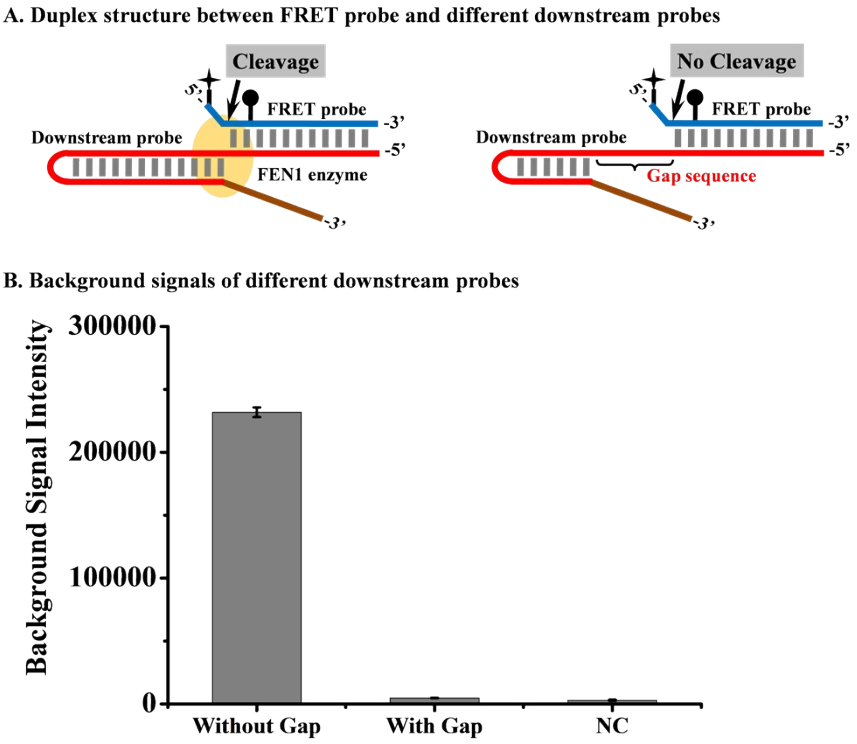


Fig. S3 The background signals from the hybridization of different hairpin-structured downstream probes with FRET probes. A. Duplex structure by FRET probe and different hairpin-structured downstream probes. "X"-structure substrate was formed by the hairpin-structured downstream probe without gap sequence. B. The background signals from different hairpin-structured downstream probes hybridized to FRET probes. NC: negative control, signal from the reaction without downstream probe. N=3.

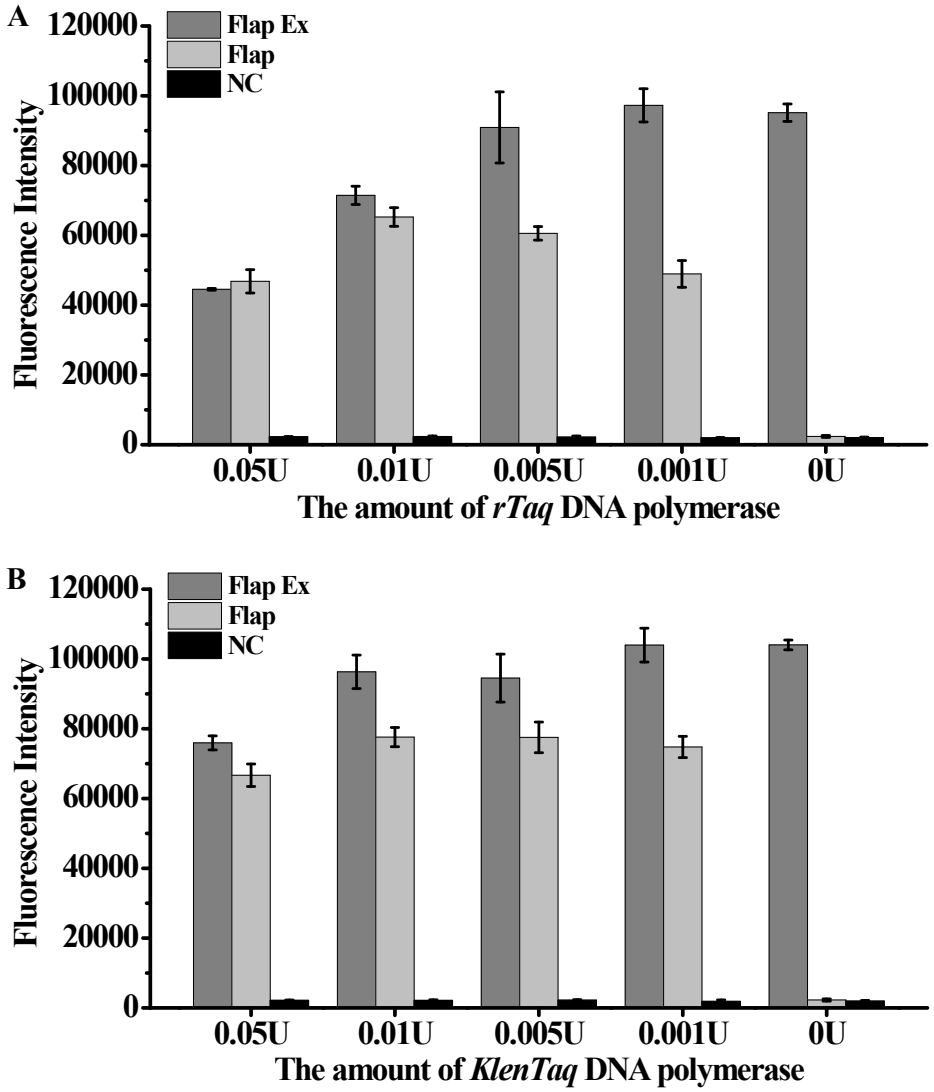


Fig. S4 Fluorescence signals of extension-based secondary invasive reaction using *rTaq* DNA polymerase (A) and *KlenTaq* DNA polymerase (B). Flap Ex: artificially synthesized extended hairpin-structured flaps, Flap: artificially synthesized hairpin-structured flaps, NC: negative control, signal from the reaction without flaps. N=3.

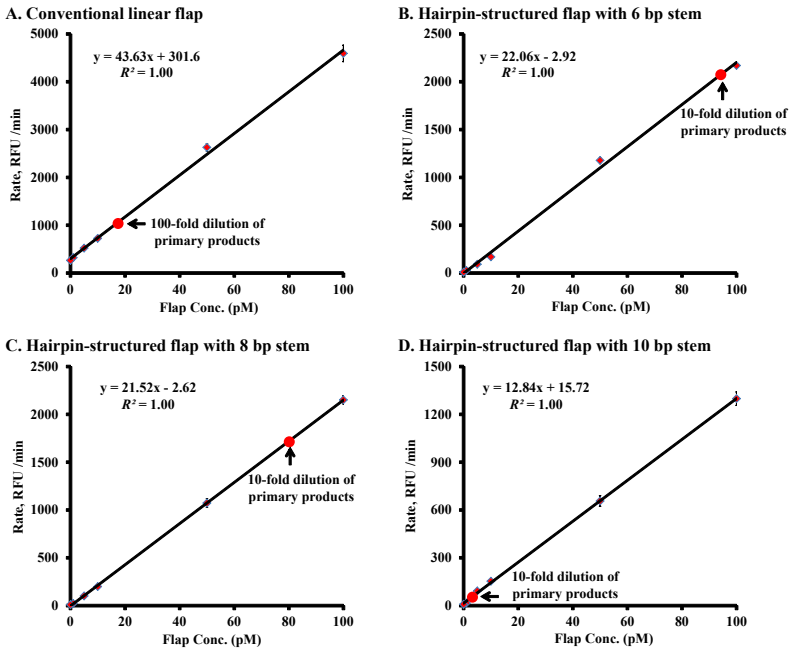


Fig. S5 The standard curves with the concentrations of flap fragment and the corresponding reaction rates. A. reaction with the conventional linear flap. B. reaction with the hairpin-structured flap with 6 bp stem. C. reaction with the hairpin-structured flap with 8 bp stem. D. reaction with the hairpin-structured flap with 10 bp stem. N=3.

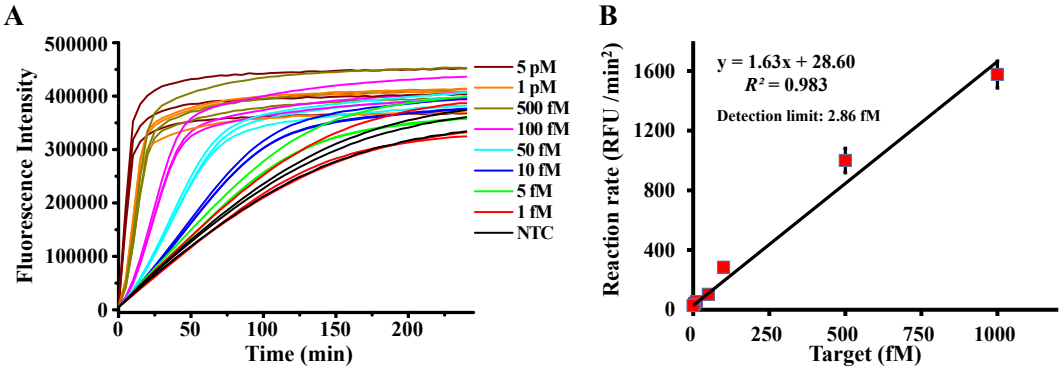


Fig. S6 The sensitivity of conventional cascade invasive reaction for DNA detection. A: The time-course curves for detecting targets with various concentrations. B: The linear relationship between the target concentrations and the initial reaction rates. NTC: no target control, the reaction without target. N=3.

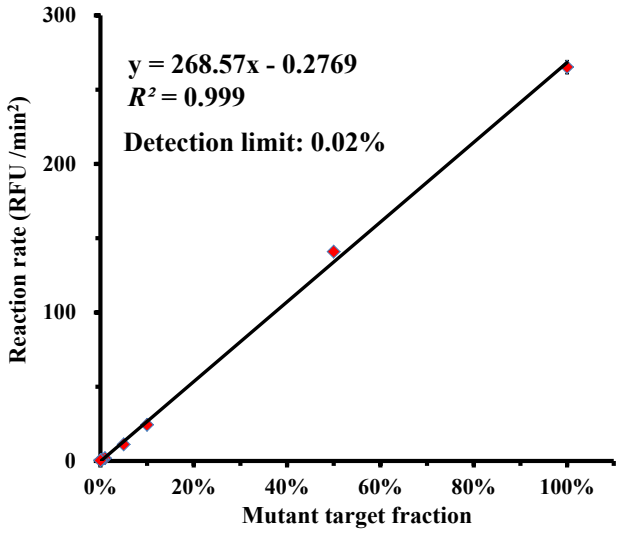


Fig. S7 The linear relationship between the mutant fractions and the initial reaction rates. N=3.

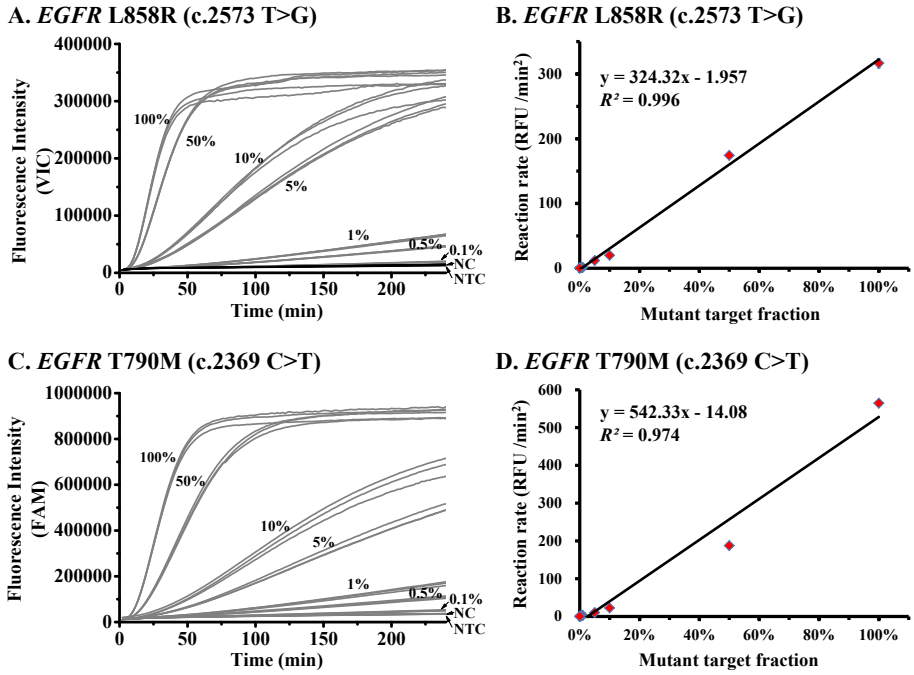
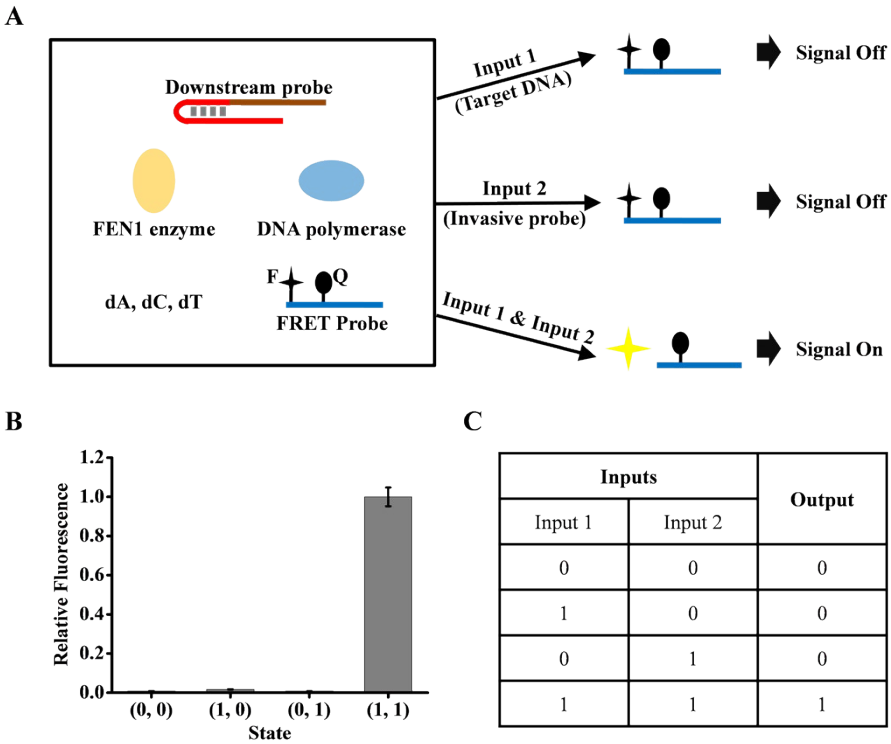
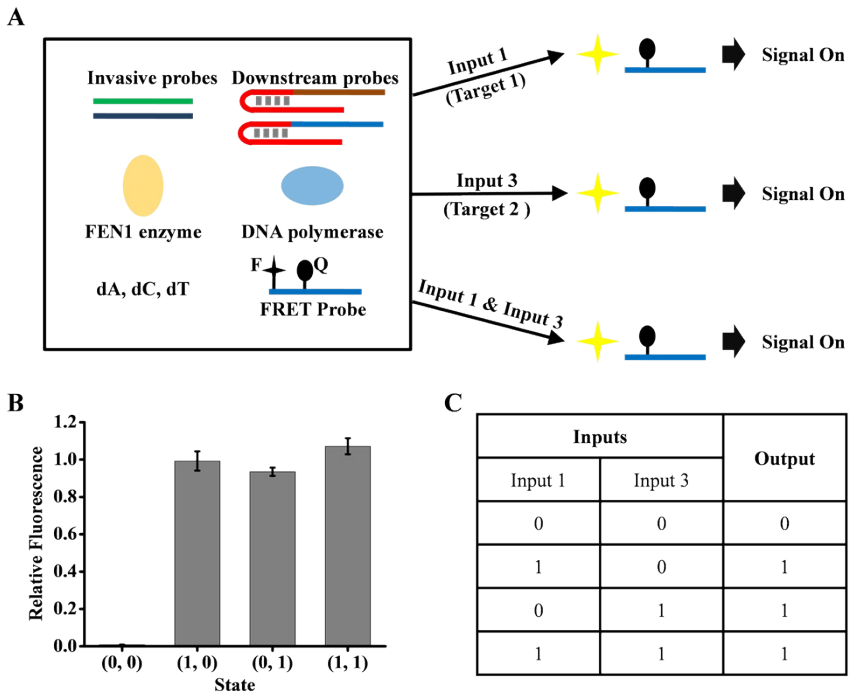


Fig. S8 Analytical specificity of the controllable-extension bridged cascade invasive reaction for *EGFR* mutation detection. A&C. The time-course curves for detecting series of *EGFR* L858R (A) and T790M (C) mutant samples with different fractions in wild-type targets. B&D. The linear relationship between the mutant fractions and the initial reaction rates for *EGFR* L858R (B) and T790M (D) mutation detection. NC: negative control, the reaction with wild-type targets. NTC: no target control, the reaction without target. N=3.



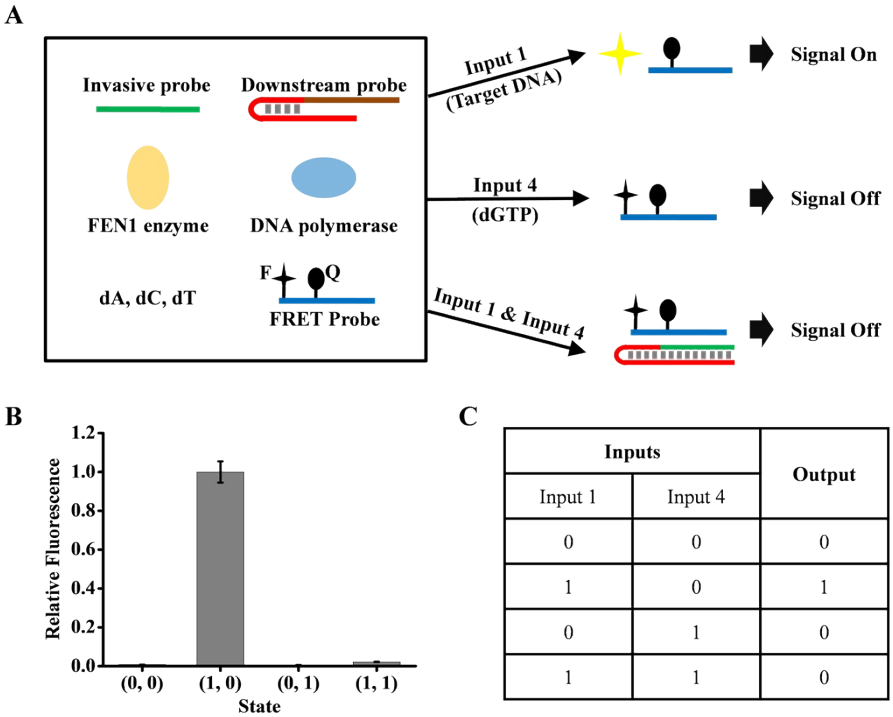
Inputs		Output
Input 1	Input 2	
0	0	0
1	0	0
0	1	0
1	1	1

Fig. S9 Operation of the AND logic gate (A), fluorescence intensities at the different states of inputs (B), and the truth table of the AND logic gate (C).N=3.



Inputs		Output
Input 1	Input 3	
0	0	0
1	0	1
0	1	1
1	1	1

Fig. S10 Operation of the OR logic gate (A), the fluorescence intensities at the different states of inputs (B), and the truth table of the OR logic gate (C).N=3.



Inputs		Output
Input 1	Input 4	
0	0	0
1	0	1
0	1	0
1	1	0

Fig. S11 Operation of the INHIBIT logic gate (A), the fluorescence intensities at the different states of inputs (B), and the truth table of the INHIBIT logic gate (C).N=3.