Supporting Information for:

Systematic comparison between toehold exchange and toehold displacement: a exploration for high specific and sensitive DNA detection

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Table of Contents

Text S1. The valuation of DF based on experimental results

In a simplistic view of the strand displacement reaction based on "toehold displacement" or "toehold exchange", both of them can be described as a reversible reaction as follow: A + B ⇌C + D, where A is the target or SNVs, B is the probe, C is the waste, D is the fluorescent product. The discrimination factor (DF) which represents the discrimination ability can be defined as the signal of target to the signal of SNVs.

$$DF = \frac{\chi_{Target}}{\chi_{SNV}} = \frac{[D_{Target}]/[B_{probe}]}{[D_{SNV}]/[B_{probe}]} \approx \frac{RFU_{Target} - RFU_{background}}{RFU_{SNV} - RFU_{background}}$$

(χ is the hybridization yield, D is the concentration of fluorescence product, B is the initial concentration of probe1, RFU is the related fluorescence intensity.)

Table S1. Experimental DF of short target at equilibrium

	20mM Tris-HCl		**1×PBS**		**1×TAE**	
	DF_{dis}	**DF_{exch}**	**DF_{dis}**	**DF_{exch}**	**DF_{dis}**	**DF_{exch}**
SNV1	1.20	6.02	1.04	3.17	1.30	7.78
SNV2	1.31	5.63	<1.31	4.27	1.63	7.36
SNV3	1.24	7.50	<1.26	4.11	1.60	6.67
SNV4	1.13	5.29	1.03	3.58	1.08	7.16

Table S2. Experimental DF of long target at equilibrium

	20mM Tris-HCl		**1×PBS**		**1×TAE**	
	DF_{dis}	**DF_{exch}**	**DF_{dis}**	**DF_{exch}**	**DF_{dis}**	**DF_{exch}**
SNV1	1.01	5.10	<1.57	5.35	1.20	5.33
SNV2	<1.60	6.12	<4.74	5.59	<2.11	5.12
SNV3	1.16	4.25	<4.12	4.56	1.57	4.57
SNV4	1.05	5.37	1.37	4.73	1.47	6.74

Text S2. The evaluation of DF based on thermodynamic equation

When the equilibrium of the reaction A + B ⇌C + D is achieved, the equilibrium constant K_{eq} = **Error!**. The Gibbs energy change (ΔG) is a thermodynamic parameter that decides the occurrence and productivity of a reaction. The relationship between ΔG and the equilibrium constant is: $\Delta G = -RT\ln(Keq) = (\Delta G_C+\Delta G_D)-(\Delta G_A+\Delta G_B)$. The discrimination factor (DF) which reprecents the discrimination ability is defined as: $DF= \frac{\chi_{Target}}{\chi_{SNV}} = \frac{[D_{Target}]}{[D_{SNV}]}$

$\Delta G = -RT\ln(K_{eq})$

$K_{eq}=\frac{[C][D]}{[A][B]} = \frac{[D]^2}{[A][B]} = \frac{[D]^2}{(1-[D])^2}$

$DF=\frac{[D_{Target}]}{[D_{SNV}]}$

(ΔG is the Gibbs energy change, T is the Kelvin temperature, R is the gas constant, Keq is the equilibrium rate constant of reaction)

Table S3. The theoretical DF of toehold exchange at equilibrium

	$\Delta G_{reaction}$(kcal mol^{-1})	K_{eq}	[D]	DF
Target	-0.44	2.11	$5.92\cdot10^{-1}$	—
SNV1	3.35	$3.43\cdot10^{-3}$	$5.53\cdot10^{-2}$	10.71
SNV2	5.27	$1.32\cdot10^{-4}$	$1.14\cdot10^{-2}$	52.04
SNV3	5.07	$1.86\cdot10^{-4}$	$1.34\cdot10^{-2}$	44.02
SNV4	3.18	$4.57\cdot10^{-3}$	$6.33\cdot10^{-2}$	9.35

Table S4. The theoretical DF of toehold displacement at equilibrium

	$\Delta G_{reaction}$(kcal mol^{-1})	K_{eq}	[D]	DF
Target	-8.59	$2.09 \cdot 10^{6}$	$9.99 \cdot 10^{-1}$	—
SNV1	-4.96	$4.47 \cdot 10^{3}$	$9.85 \cdot 10^{-1}$	1.01
SNV2	-2.88	$1.32 \cdot 10^{2}$	$9.20 \cdot 10^{-1}$	1.09
SNV3	-3.08	$1.85 \cdot 10^{2}$	$9.31 \cdot 10^{-1}$	1.07
SNV4	-5.13	$5.96 \cdot 10^{3}$	$9.87 \cdot 10^{-1}$	1.01

Text S3. The interaction between the fluorophore and the quencher

The interaction between the FAM fluorophore and the Dabcyl quencher also contributes to the thermodynamic stability of the reaction. Consequently, our experiments in **Fig. 2** and **Fig. S2** have demonstrated the separation ΔG (ΔG_{sep}) of the probe1 duplex whose strands modified with FAM fluorophore and Dabcyl quencher respectively. In principle, the ΔG_{sep} of the separation of the fluorescence and quencher is equal to the $-\Delta G_{gath}$ of gather of the fluorescence and quencher, which can be measured by the ΔG_{rea} of the designed X+ YZ ⇌Y + XZ, where X is the strand modified with FAM fluorophore, Y is the strand without modification, Z is the the strand modified with Dabcyl quencher.[1] Fig S2 shows the relationship between the fluorescence intensities and the concentration of fluorophore modified strand. Based on **Fig. S2**, Fluorescence intensity can be converted to fluorophore concentration according to y =4.854x -4.996 (R^2 =0.9992). Where x denotes the concentration of fluorophore and y denotes the fluorescence intensity (RFU).

y (nM) =4.8584x -4.9956 (R^2 =0.9992)

The concentration of FAM after adding buffer: x1 =92.86 nM

The concentration of FAM after adding probe4 (YZ duplex): x2 =27.32 nM

The efficiency of quenching: (92.86-27.32)/92.86=70%

X+ YZ ⇌Y + XZ,

$$K_{eq} = \frac{[Y]^2}{(1-[Y])^2}, \qquad \Delta G_{gath} = \Delta G_{rea} = -RT\ln(K_{eq}) = -0.995 \text{ Kcal mol}^{-1}$$

So the ΔG_{sep} = 0.995 Kcal mol^{-1}

Text S4. The Combination of "toehold exchange" with blocking probe

1. As to preparing the 200 nM probe1/probe1(2) (containing 200 nM blocking probe/blocking probe(2)), we firstly constructed the 400 nM probe1/probe1(2) and the 400 nM blocking probe/blocking probe(2) in Tris–HCl (pH 8.0, containing 1 M Na+) buffer. The two solutions were then mixed together. The blocking probe sequences were list in Scheme S2.

2. For the experiments , first, 100 μL 200 nM probe1 (containing 200 nM blocking probe) was injected into the cuvette, then 100 μL 200 nM target/SNVs were injected to the cuvette.

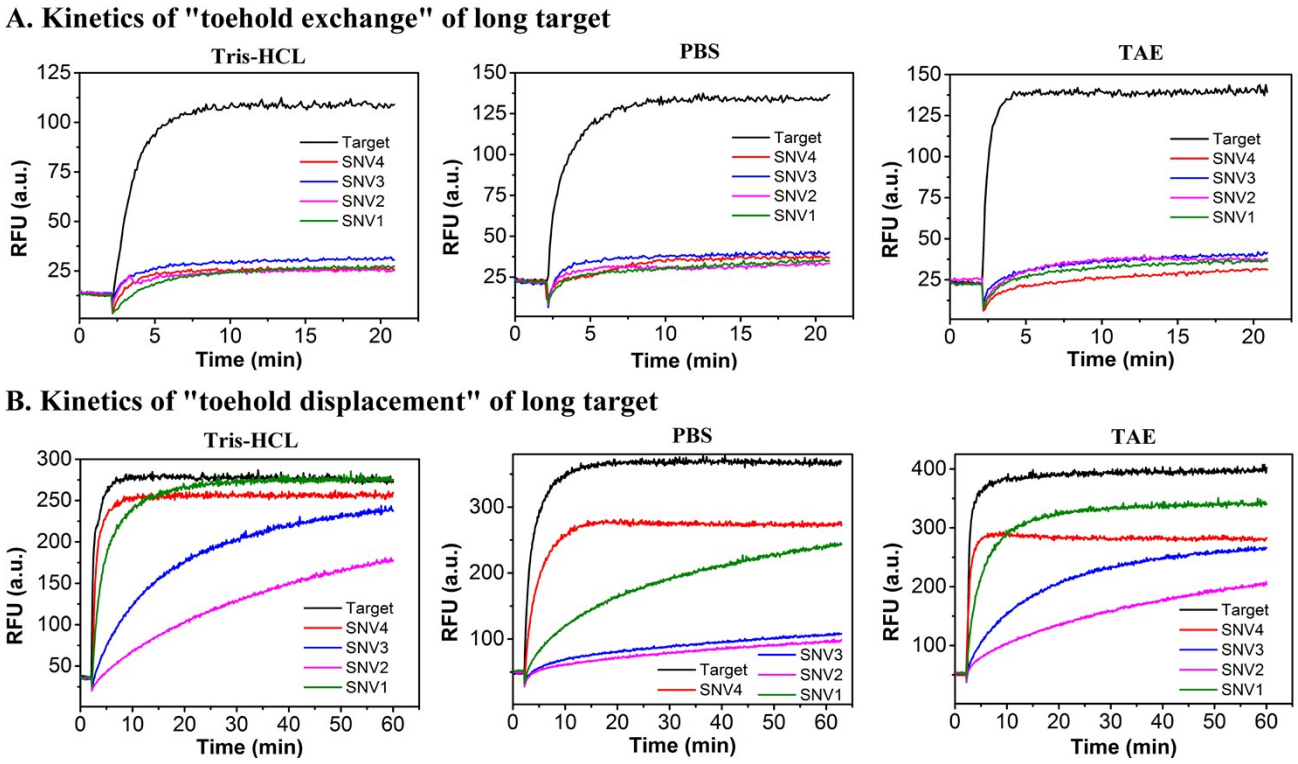

Fig. S1. Kinetics of long target based on "toehold exchange" and "toehold displacement". (A) Kinetics of "toehold exchange". (B) Kinetics of "toehold displacement". (Experiments were performed in three different buffer: 20 mM Tris-HCl with 1 M Na^{+}, 1× PBS, 1× TAE with 12.5 mM Mg^{2+} at room temperature)

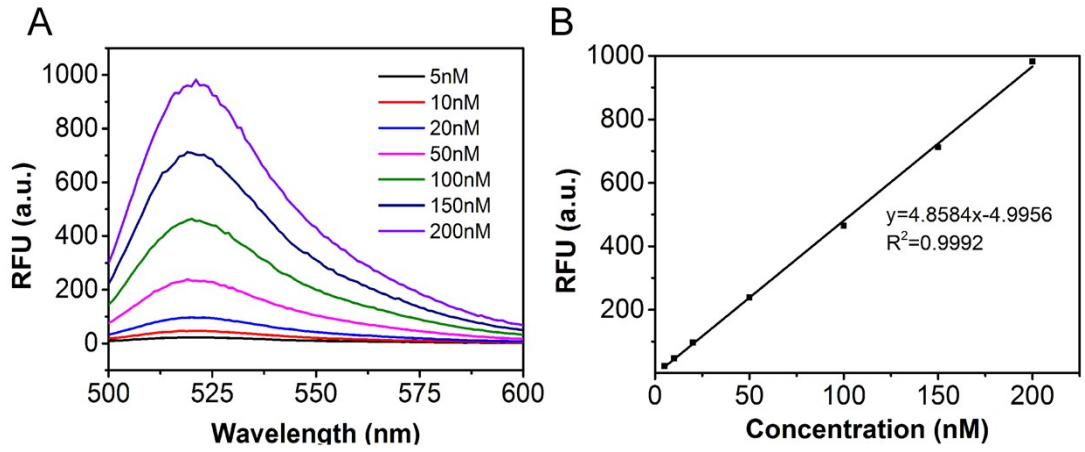

Fig. S2. The relationship between the arbitrary unit of fluorescence intensity (RFU) and the concentration of FAM fluorophore. (A) The fluorescence intensity responses to the concentration of FAM fluorophore (from 5nM to 200nM). (B) The linear relationship between the fluorescence intensity and the concentration of FAM fluorophore. (Excitation wavelength is 480 nm, emission wavelength is 500 nm to 600 nm, excitation slit is 10 nm, emission slit is 10 nm.)

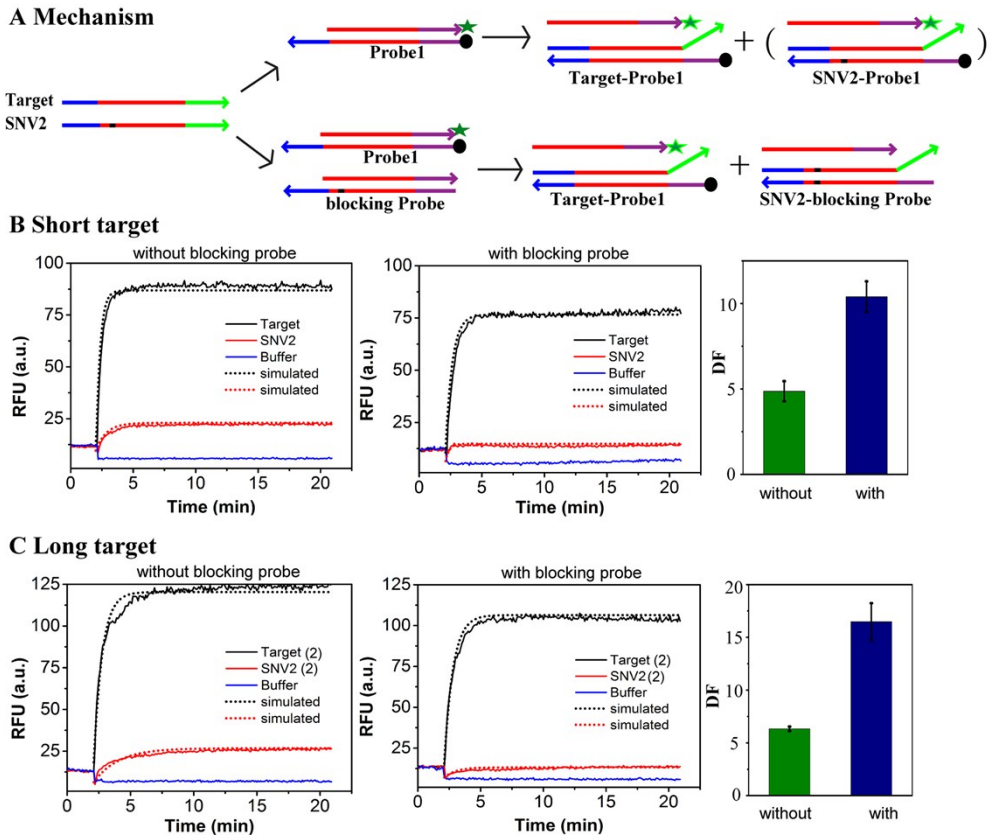

Fig. S3. Combination of "toehold exchange" with blocking probe. (A) The mechanism of blocking. (B) The performance with and without blocking probe to short target detection. (C) The performance with and without blocking probe to long target detection. (The simulation curve (dotted line) was correlated with the first-order reaction).

Scheme S1.

Sequence Name	Oligo Sequence(5'-3')
Target (short target)	caaggtaggtaggtaggtaggcaag
SNV1	caagataggtaggtaggtaggcaag
SNV2	caaggtagataggtaggtaggcaag
SNV3	caaggtaggtagataggtaggcaag
SNV4	caaggtaggtaggtagataggcaag
probe1-FAM (X)	aggtaggtaggtatctgtc-FAM
probe1-Dabcyl (Z)	Dabcyl-gacagatacctacctacctaccttg
probe2-FAM	aggtaggtaggta-FAM
probe2-Dabcyl	Dabcyl-tacctacctacctaccttg
probe3a	aggtaggtaggta
probe3b	gacagatacctacctacct
Y	aggtaggtaggtatctgtc
Catalyst (C)	caaggtaggtaggtaggta
Target(2) (long target)	caaggtcaggtcaggtcaggtcaggcaag
SNV1(2)	caagatcaggtcaggtcagtgtcaggcaag
SNV2(2)	caaggtcagatcaggtcaggtcaggcaag
SNV3(2)	caaggtcaggtcagatcaggtcaggcaag
SNV4(2)	caaggtcaggtcaggtcagatcaggcaag
probe1-FAM(2)	caggtcaggtcaggtcatctgtc-FAM
probe1-Dabcyl(2)	Dabcyl-gacagatgacctgacctgacctgaccttg
probe2-FAM(2)	caggtcaggtcaggtca-FAM
probe2-Dabcyl(2)	Dabcyl-tgacctgacctgacctgaccttg
probe3a(2)	caggtcaggtcaggtca (for toehold=6)

probe3b(2)	gacagatgacctgacctgacctg
prob3a(2)-1	caggtcaggtcaggtcatc (for toehold=4)
prob3a(2)-2	caggtcaggtcaggtcat (for toehold=5)
prob3a(2)-3	caggtcaggtcaggtc (for toehold=7)
prob3a(2)-4	caggtcaggtcaggt (for toehold=8)
prob3a(2)-5	caggtcaggtcagg (for toehold=9)
prob3a-Dabcyl(2)	Dabcyl-caggtcaggtcaggtc
prob3b-FAM(2)	gacagatgacctgacctgacctg-FAM

Scheme S2.

Sequence Name	**Oligo Sequence(5'-3')**
For short target: blocking probe a	agataggtaggtatctgtc
blocking probe b	gacagatacctacctatctaccttg
For long target: blocking probe a(2)	cagatcaggtcaggtcatctgtc
blocking probe b(2)	gacagatgacctgacctgatctgaccttg