Supporting Information

Simultaneous Discrimination of Single-Base Mismatch and Full Match Using a Label-Free Single-Molecule Strategy

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1. Sequence information of molecular beacons and targets

Table S1. Detailed sequence information for all oligonucleotides used in the nanopore study

Name	Sequence	ΔG (kcal·mol^{-1})	Tm (℃)
MB3 (MB)	5'-GAGTCCCGCCTGTGACATGCATTCTCAAAAAA-3'	-1.12	40.3
MB5	5'-GCGAGTCCCGCCTGTGACATGCATTCTCGCAAAAAA-3'	-4.56	59.3
MB7	5'-GCGCGAGTCCCGCCTGTGACATGCATTCTCGCGCAAAAAA-3'	-9.28	75.0
MB0	5'-TCCCGCCTGTGACATGCATTAAAAAAAAA-3'		
T	5'-AATGCATGTCACAGGCGGGA -3'		
1mT	5'-AATGCATGTAACAGGCGGGA -3'		
2mT	5'-AATGCAAGTCACACGCGGGA -3		

Note: Underline denotes base pairs in the stem. Red denotes mismatched bases.

2. Molecular configuration and characteristic current signature of molecular beacons and hybrids

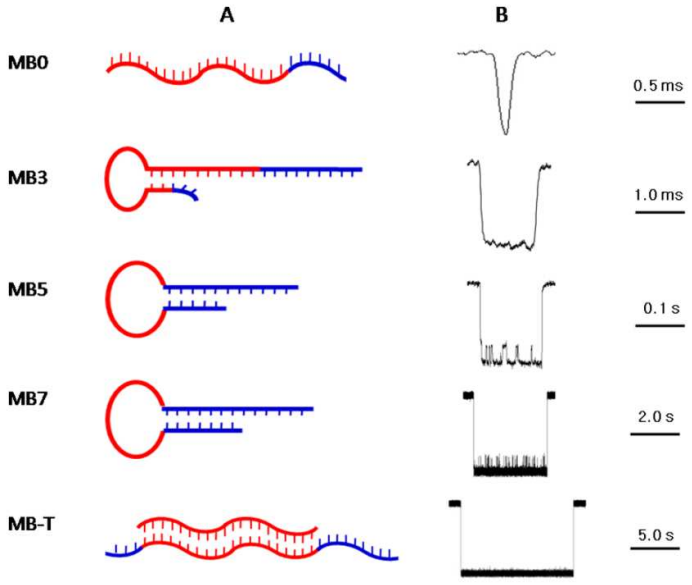

Figure S1. Molecular configuration(A) and characteristic current signature(B) of MB0, MB3, MB5, MB7 and MB-T in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

3. Target DNA translocation through the pore

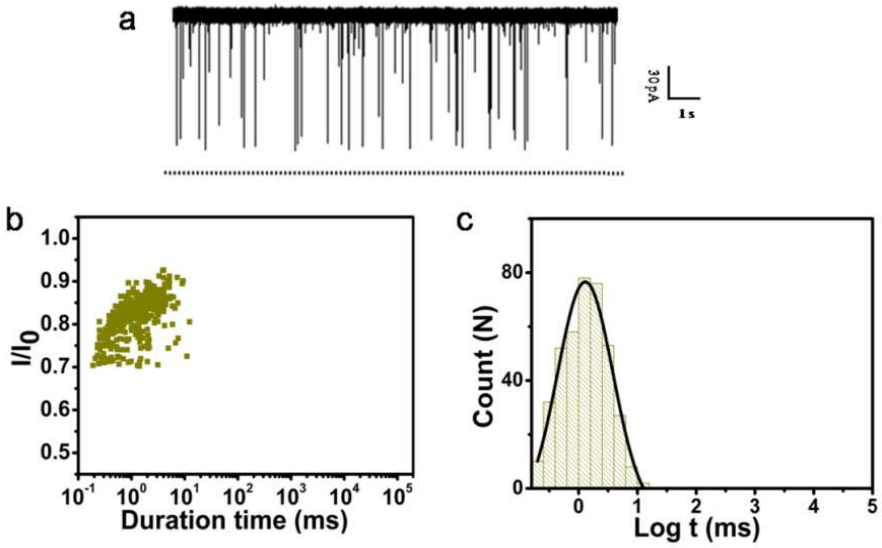

Figure S2. Translocation of the target DNA in the α-HL protein nanopore. (a) Representative traces of the target DNA added into the *cis* side. Dashed lines represent the level of zero current. (b) The scatter plot of events blockage amplitude vs duration time. (c) The histogram of duration time (N=396). The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

4. Single-channel current traces of molecular beacons and hybrids

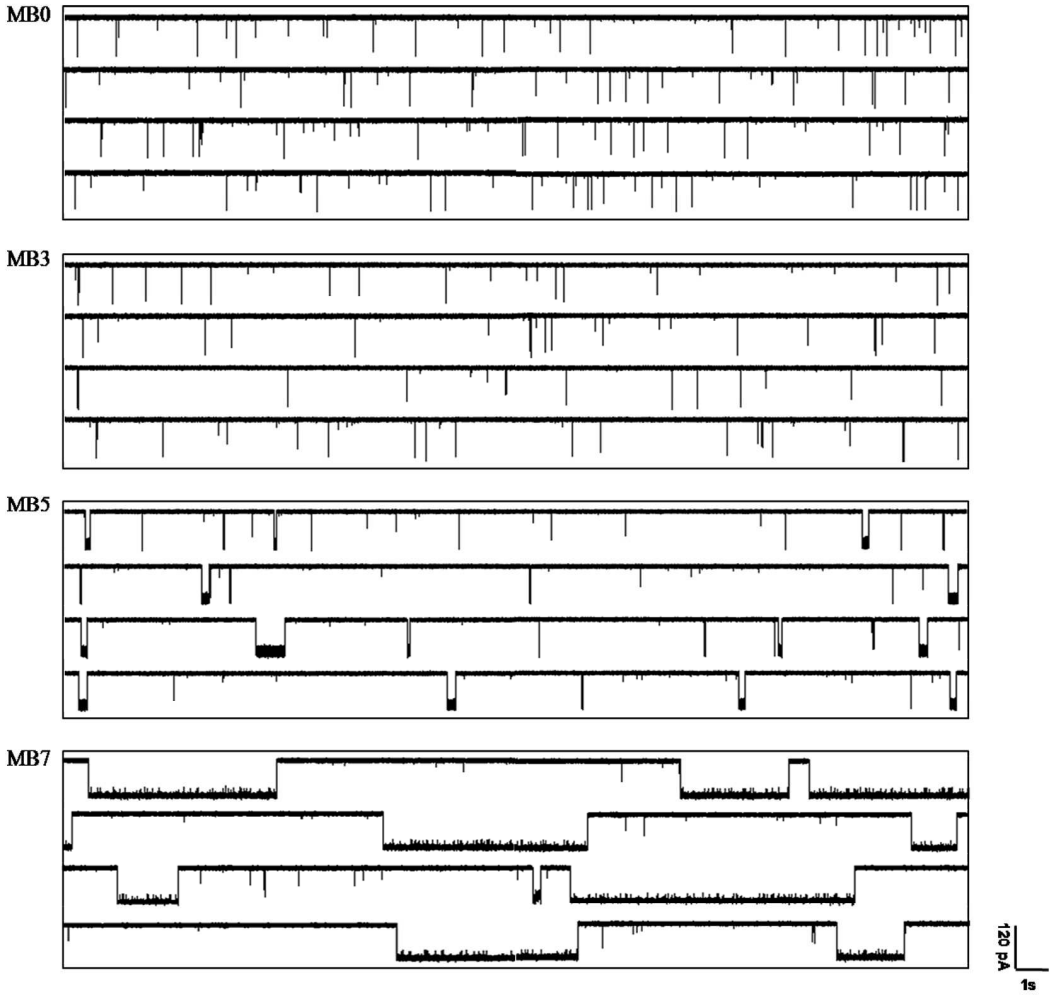

Figure S3. Single-channel current traces of MB0, MB3, MB5 and MB7. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

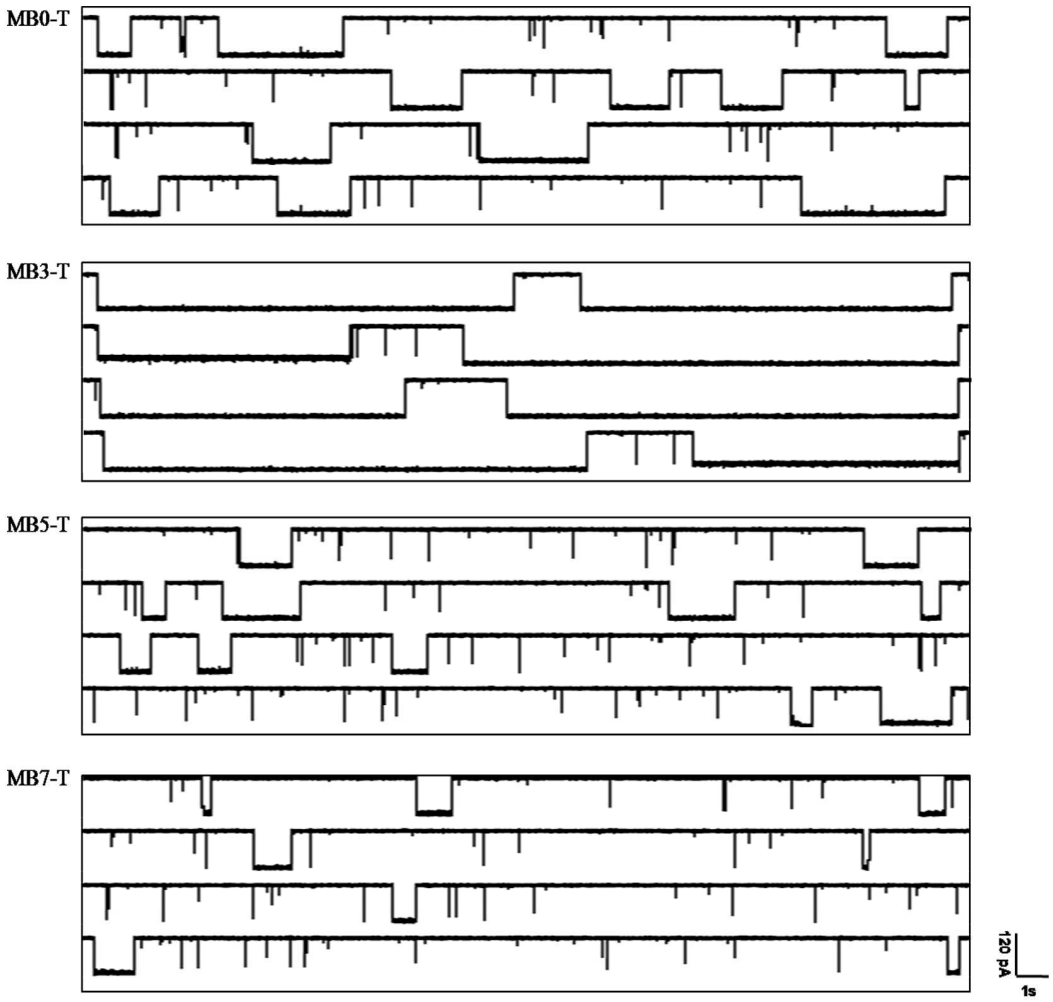

Figure S4. Single-channel current traces of MB0-T, MB3-T, MB5-T and MB7-T hybrids. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

5. Effect of transmembrane voltage on the duration time of molecular beacons and hybrids

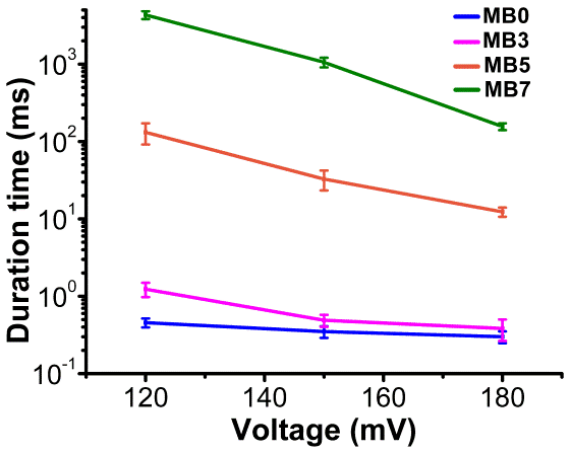

Figure S5. The histogram of duration time for MBs in the α-HL nanopore in the absence of target at various applied potentials. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution.

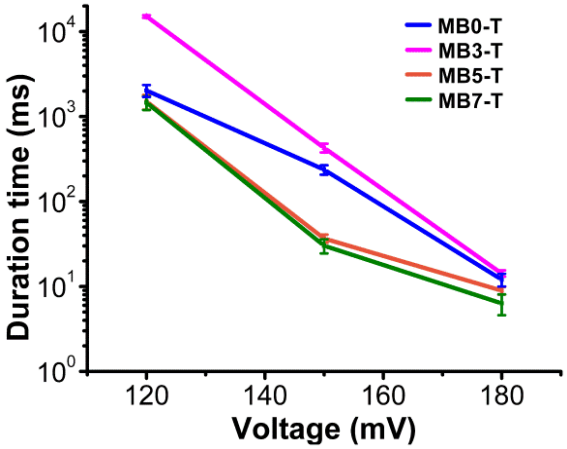

Figure S6. The histogram of duration time for the MB-T hybrids in the α-HL nanopore in the presence of target at various applied potentials. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution.

6. Nanopore thermodynamic analytical method

The thermodynamic analysis is on the basis of the following binary complex formation mode:

$MB_1 \rightleftharpoons MB_2$ (Scheme 1)

$MB_1 + T \rightleftharpoons MB\text{-}T$ (Scheme 2)

$MB_2 + T \rightleftharpoons MB\text{-}T$ (Scheme 3)

where MB, T and MB-T refers to molecular beacon, target DNA and molecular beacon-target duplex, respectively. "MB_1" represents hairpin-like molecular beacon; "MB_2" represents single-strand molecular beacon. The equilibrium dissociation constant K_1 of Scheme 1 is determined by:

$$K_1 = \frac{[MB_2]}{[MB_1]} = \frac{[MB_2]_0}{[MB_1]_0} \qquad (1)$$

where $[MB_1]_0$ and $[MB_2]_0$ are concentrations of hairpin-like molecular beacon and single-strand molecular beacon before the target is introduced in the solution, respectively; $[MB_1]$ and $[MB_2]$ is the concentration of hairpin-like molecular beacon and single-strand molecular beacon after the reaction reaches equilibrium state. The conversion ratio of single-strand molecular beacon to hairpin-like molecular beacon at equilibrium state is defined as k and calculated on the basis of the following equation:

$$k = \frac{N_{MB_1}}{N_{total}} = \frac{N_{MB_1}}{N_{MB_1} + N_{MB_2}} = \frac{[MB_1]_0}{[MB_1]_0 + [MB_2]_0} \qquad (2)$$

where N_{MB1} and N_{MB2} are the number of current events of hairpin-like molecular beacon ($t_{off} > 1$ ms) and single-strand molecular beacon ($t_{off} < 1$ ms) before the target is introduced in the solution, respectively. N_{total} is the number of total events of molecular beacons. So the equilibrium dissociation constant K_1 of Scheme 1 can be described by:

$$K_1 = \frac{1-k}{k} \qquad (3)$$

Because the initial concentration C_0 of molecular beacons is determined by:

$$C_0 = [MB]_0 = [MB_1]_0 + [MB_2]_0 \qquad (4)$$

$$[MB\text{-}T]_1 + [MB\text{-}T]_2 = [MB\text{-}T] \qquad (5)$$

where $[MB\text{-}T]_1$ and $[MB\text{-}T]_2$ are the concentration of duplex for scheme 2 and scheme 3, respectively, after the reaction reaches equilibrium state.

From Eq.1 and Eq.3,

$$[MB_1]_0 = kC_0 \qquad (6) \qquad [MB_2]_0 = (1-k)C_0$$

$$\frac{[MB_1]_0}{[MB_2]_0} = \frac{[MB_1]}{[MB_2]} = \frac{1}{K_1} = \frac{k}{1-k}$$

$$\frac{[MB_1]_0-[MB_1]}{[MB_2]_0-[MB_2]}=\frac{[MB\text{-}T]_1}{[MB\text{-}T]_2}=\frac{k}{1-k}$$

$$[MB\text{-}T]_1=k[MB\text{-}T] \quad (7)$$

It is reported that the translocation frequency f ($f=1/\tau_{on}$) for analytes is directly proportional to its concentration.[1] Then, the binding ratio of MB_1 at equilibrium state is defined as Φ and calculated on the basis of the following equation:

$$\Phi=\frac{[MB\text{-}T]_1}{[MB_1]_0}=\frac{k[MB\text{-}T]}{[MB_1]_0}=k\frac{f_e}{f_0} \quad (8)$$

f_e is the event frequency of MB-target after the reaction reaches equilibrium state, and f_0 is the event frequency of hairpin-like molecular beacon before target is introduced in the solution. The average inter-event interval τ_{on} can be directly obtained by data analysis of the current traces (Figure S7). Event number in each test is not less than 200.

Accordingly, the equilibrium constant K_a of target hybridizing with hairpin-like molecular beacons can be calculated using:

$$K_a=\frac{[MB\text{-}T]}{[MB_1][T]}=\frac{[MB\text{-}T]}{([MB_1]_0-[MB\text{-}T]_1)([T]_0-[MB\text{-}T])}=\frac{\Phi}{(1-\Phi)^2kC_0} \quad (9)$$

Molecular beacon and target have the same initial concentration C_0 of 200nM. The standard Gibbs free energy change can be described by:

$$\Delta G^0=-RT\ln K_a \quad (10)$$

It is noteworthy that the stability of target hybridizing with hairpin molecular beacon was evaluated for MB3, MB5 and MB7. In contrast, the stability of target hybridizing with single-strand molecular beacon was evaluated for MB0. Therefore, when calculating Φ and K_a of target hybridizing with single-strand molecular beacon of MB0, we substituted $[MB\text{-}T]_2$, $[MB_2]_0$ and $[MB_2]$ for $[MB\text{-}T]_1$, $[MB_1]_0$ and $[MB_1]$ in Eq.8 and Eq.9, respectively. Simultaneously, it is necessary to replace k in Eq.8 and Eq.9 with 1-k.

Table S2. The results of the relevant parameters obtained from the nanopore experiments

	f_0 [s^{-1}]	f_e [s^{-1}]	k	Φ	K_a [M^{-1}]	ΔG°[kcal·M^{-1}]
MB0	0.21±0.01	0.18±0.02	0.37±0.10	0.55±0.07	$2.53\pm1.24\times10^7$	-9.94±0.27
MB3	0.09±0.01	0.20±0.03	0.31±0.06	0.70±0.01	$1.35\pm0.30\times10^8$	-10.98±0.13
MB5	0.24±0.09	0.05±0.001	0.95±0.01	0.24±0.09	$2.44\pm1.44\times10^6$	-8.52±0.39
MB7	0.19±0.08	0.03±0.002	0.97±0.01	0.17±0.05	$1.30\pm0.58\times10^6$	-8.19±0.29

Concentration of hybridization were 200 nM MB and 200 nM target. All tests were performed in buffer containing 1 M KCl, 10 mM Tris and 1.0 mM EDTA (pH=8.0) with the transmembrane potential of +120 mV. Error bars are standard deviations from three experiments.

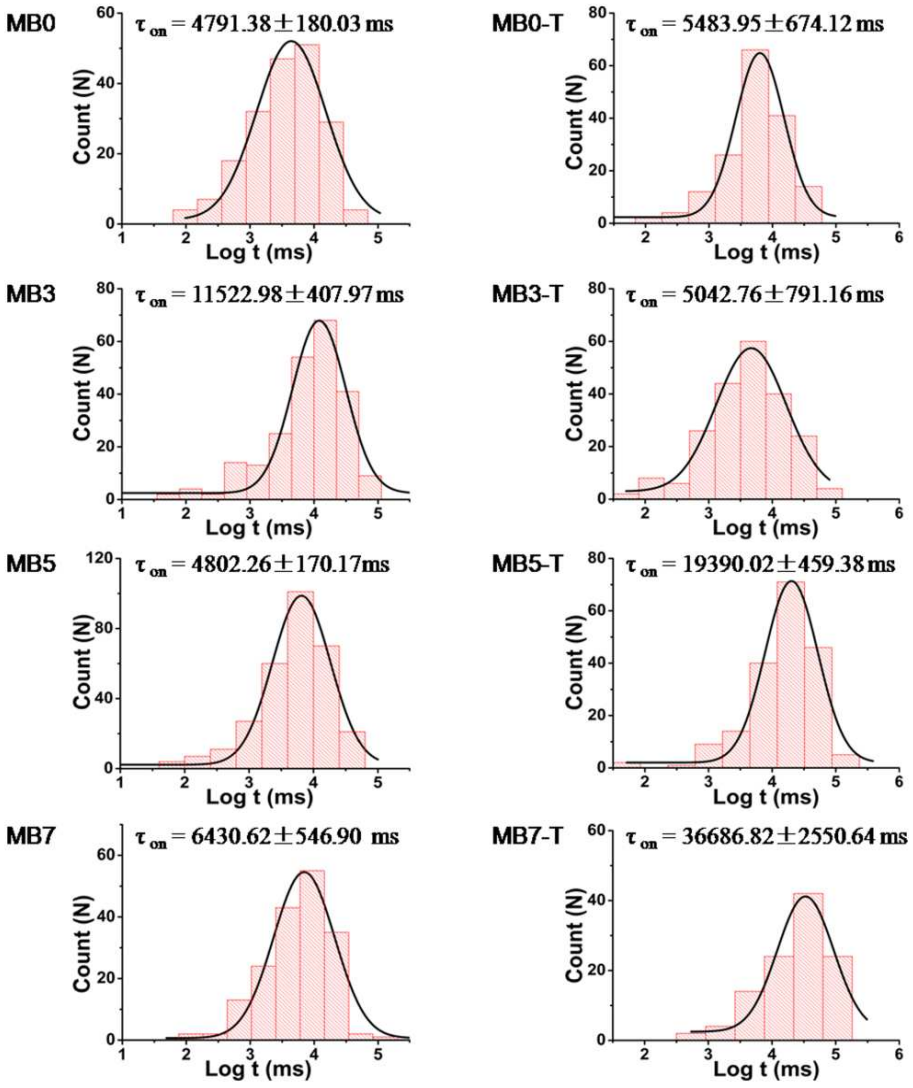

Figure S7. The histogram of inter-event interval τ_{on} of the MB and MB-T passing the α-HL nanopore. The number in each histogram is at least 200. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

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7. Nanopore kinetic analytical method

The kinetic analysis is on the basis of the following binary complex formation mode:

$$\mathrm{MB+T} \underset{k_-}{\overset{k_+}{\rightleftharpoons}} \mathrm{MB\text{-}T} \qquad \text{(Scheme 2)}$$

It is based on the assumptions that scheme 1 is in rapid equilibrium, that is, reaction 1 remains equilibrium state. It is assumed that the concentration of duplex MB-T is x. The hybridization rate r_+ and dissociation rate r_- of Scheme 2 can be described by:

$$r_+ = k_+[\mathrm{MB_1}][\mathrm{T}] = k_+ k(C_0 - x)^2 \qquad (11)$$

$$r_- = k_-[\mathrm{MB\text{-}T}] = k_- x \qquad (12)$$

Where k_+ is the hybridization rate constant and k_- is the dissociation rate constant. When the reaction reaches equilibrium state,

$$k_+[\mathrm{MB_1}][\mathrm{T}] = k_-[\mathrm{MB\text{-}T}] \Rightarrow \quad k_+ = k_- K_a \qquad (13)$$

The overall reaction rate of scheme 2 is:

$$r = \frac{d[\mathrm{MB\text{-}T}]_1}{dt} = \frac{k d x}{dt} = r_+ - r_- = k_- K_a k(C_0 - x)^2 - k_- x \qquad (14)$$

Eq.15 can be obtained by integrating the Eq.14,

$$k_- K_a t = \frac{1}{2m} \ln\left|\frac{x\text{-}n\text{-}m}{x\text{-}n+m}\right| + C \qquad (15) \qquad m = \sqrt{\frac{1}{4k^2 K_a^2} + \frac{C_0}{k K_a}}, \quad n = C_0 + \frac{1}{2k K_a}$$

Where C is constant. When $t=0$, $x=0$. Similarly, it is necessary to replace k with 1-k in the calculation of m and n for MB0.

From Eq.15,

$$2m\, k_- K_a t = \ln\left|\frac{(x\text{-}n\text{-}m)(m\text{-}n)}{(x\text{-}n+m)(m+n)}\right| \qquad (16)$$

The number of translocation events of MB-T during unit time is directly proportional to its concentration.

$$\frac{dN}{dt} = a x, \qquad x = \frac{dN}{a dt}$$

When the cumulative event number of MB-T hybrid translocation versus reaction time performed a linear relationship, the reaction reached equilibrium state. In this state,

$$x = \frac{[\mathrm{MB\text{-}T}]_1}{k} = \frac{\Phi[\mathrm{MB_1}]_0}{k} = \frac{\Phi k[\mathrm{MB}]_0}{k} = \Phi[\mathrm{MB}]_0$$

$$a = \frac{1}{\Phi[\mathrm{MB}]_0}\left(\frac{dN}{dt}\right)_{eq}$$

For convenience, we defined Q as

$$Q = \frac{(x\text{-n-m})(\text{m-n})}{(x\text{-n+m})(\text{m+n})}$$

Thus, k_+ can be obtained from plot of time vs. ln$Q$.

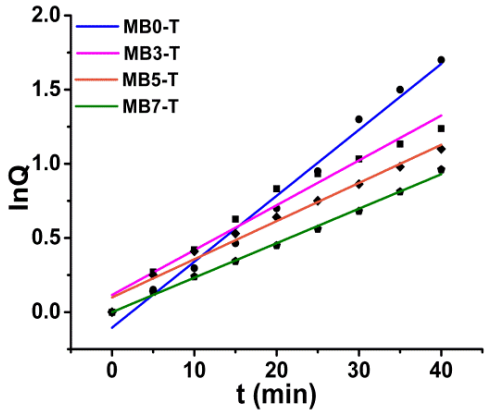

Figure S8. The plot of time (t/min) vs. ln$Q$.

Table S3. The results of the relevant parameters obtained from the nanopore experiments.

	$(dN/dt)_{eq}$	ln$Q$ / t	$k_+[M^{-1} \cdot s^{-1}]$
MB0	2.84	0.045	1.93×10^5
MB3	1.69	0.030	2.14×10^5
MB5	1.21	0.026	3.57×10^4
MB7	0.90	0.023	2.03×10^4

8. Specificity of molecular beacons for recognizing target

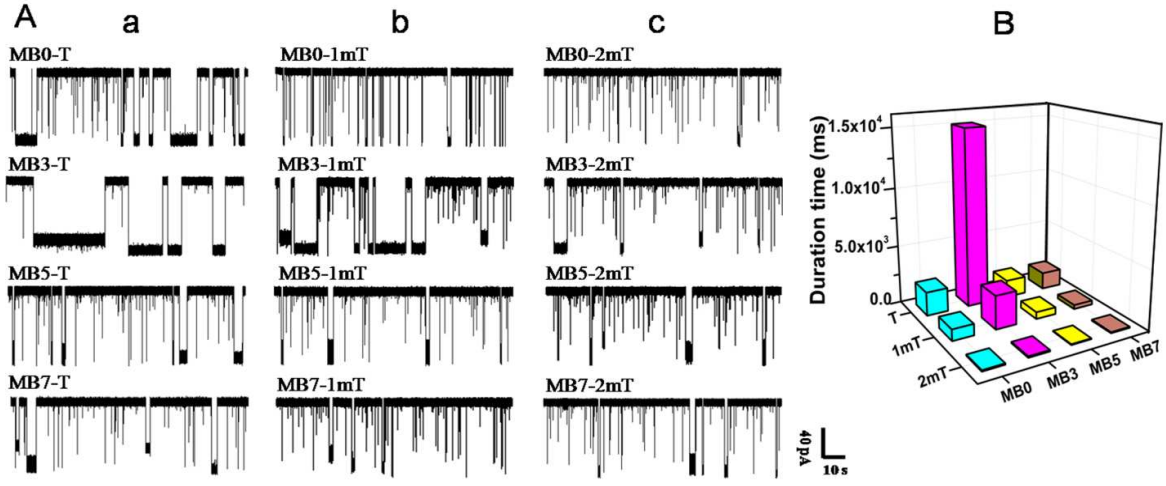

Figure S9. Specificity of MBs for recognizing target. (A) Single-channel current trace of MB-T (a), MB-1mT (b) and MB-2mT (c) passing through the nanopore. (B) Histograms of mean duration time of MB-T, MB-1mT and MB-2mT. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

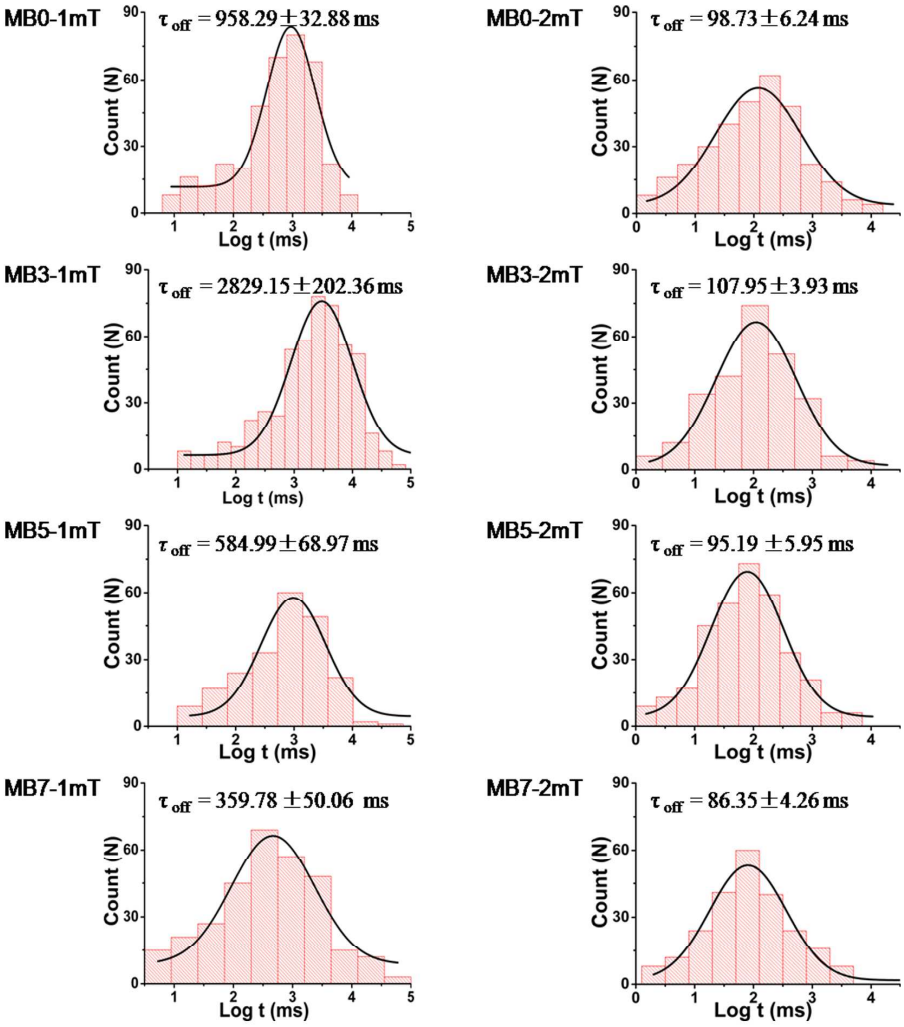

Figure S10. The histogram of duration time of the MB-1mT hybrids with single-base mismatch and MB-2mT hybrids with two-base mismatch passing the *α*-HL nanopore. The number in each histogram is at least 200. The data were recorded in 1.0 M KCl (10 mM Tris and 1.0 mM EDTA, pH 8.0) solution at +120 mV.

References

(1) Zhang, L.; Zhang, K.; Rauf, S.; Dong, D.; Liu, Y.; Li, J. *Anal. Chem.* **2016**, 88, 4533-4540.