Supplementary Information for:

Multiplexed discrimination of microRNA single nucleotide variants through triplex molecular beacon sensors

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Materials and Methods

Materials and characterisation. All the DNA and RNA oligomers were purchased from Sangon Biotech (Shanghai). All the chemicals were purchased from Sigma-Aldrich, Alfa Aesar and J&K. Dynabeads™ MyOne™ Streptavidin T1 (10 mg/mL, 7-10×10^9 beads/mL in PBS, pH 7.4 with 0.1% BSA and 0.02% sodium azide as a preservative) was obtained from Invitrogen (Shanghai). Micro Bio-Spin P6 gel columns (Tris buffer) were purchased from Bio-Rad (Hercules, CA). All the columns were pre-equilibrated three times with 80 μL deionised water prior to use. DNA mass spectrometry was analyzed on Thermo-Finnigan LCQ Deca XP Plus. HRMS data of new compounds were obtained using Bruker ultrafleXtreme MALDI-TOF/TOF. Wild type αHL-D8H6 proteins were produced as described previously.[S1]

Buffer preparation. 55.913 g of KCl (99.999%, Sigma-Aldrich), 0.303 g of Tris·HCl (99.0%, Sigma-Aldrich) were dissolved in 230 mL of deionized water (Millipore, MA). 1 M HCl was used to adjust the pH to 5.2. The solution was diluted with deionized water to 250 mL. The final buffer solution consists of 3 M KCl and 10 mM Tris at pH 5.2.

General procedure for the preparation of DNA probes. DNA-Fc probe: 3.3 μL alkyne-containing DNA (100 μM), 1.2 μL deionized water, 2.0 μL azidomethylferrocene (dissolved in acetonitrile, 200 mM), 1.0 μL sodium ascorbate (20 mM), 0.5 μL copper nitrate (20 mM) were added to 2.0 μL HEPES (100 mM, pH 7.4) buffer, with a final volume of 10 μL. The reaction was incubated for 2 h at room temperature, and then 2.0 μL EDTA solution (100 mM) was added to terminate the reaction. DNA-Ad probe: 5.0 μL amino-containing DNA (100 μM), 5.0 μL the *N*-hydroxysuccinimide ester of 1-adamantane carboxylic acid (dissolved in DMSO, 100 mM) were added to 5.0 μL PB buffer (100 mM, pH 9.0). The reaction was incubated for 8 h at 50°C. The DNA product was purified with Micro Bio-spin P6 columns. Next, 10.0 μL CB[7] aqueous solution (5.0 mM) was added to the DNA solution and incubated for 2 h to afford the final DNA probes.

MicroRNA detection experiments.

DNA3 (1.5 μL, 135 μM) and DNA11-Fc (5.7 μL, 35 μM) were added to 12.8 μL incubation buffer (10 mM PB, 20 mM NaCl, 2.5 mM $MgCl_2$, pH 5.2) and incubated overnight at 4°C to

form the triplex sensor. The total volume was 20 μL. Then the triplex sensor was incubated with various concentrations of miRNA-155 (final concentration: 0.005, 0.05, 0.5, 1, 10, 50, 100 and 200 nM) for 2 h at room temperature. Meanwhile, magnetic beads suspension (100 μL, 10 mg/mL) was washed 3 times with 100 μL 1×BW buffer (1 M NaCl, 0.5 mM EDTA, 5 mM Tris, pH 7.5). The resulting miRNA-tMB sensor solution was mixed with magnetic beads in 200 μL buffer (DEPC-treated 0.1 M NaCl) and vortexed for 15 min. The supernatant was collected with the aid of a magnetic separator, and magnetic beads were washed with deionized water. The solutions were combined and ultra-centrifuged with an Amicon Ultra-0.5 centrifugal filter (3 KD). 10 μL CB[7] (5 mM) was added to the concentrated sample and incubated for 2 h. The sample after incubation was ready for single channel recording experiments.

Selectivity experiments for tMB sensors.

DNA11 (129 μM, 1.6 μL), DNA12 (152 μM, 1.3 μL) and DNA13 (156.5 μM, 1.3 μL) were incubated with DNA8-Fc (35 μM, 5.7 μL) in 13.0 μL incubation buffer overnight at 4°C, respectively, to form the triplex molecular beacon sensors. Then the tMB sensors were incubated with *let-7a*, *let-7b*, *let-7c* (final concentration: 200 nM) respectively for 2 h at room temperature. The resulting solution was mixed with magnetic beads suspension in 200 μL buffer (DEPC-treated 0.1 M NaCl) and vortexed for 15 min. The supernatant was collected with the aid of a magnetic separator and magnetic beads were washed with deionized water. The solutions were combined and ultra-centrifuged with an Amicon Ultra-0.5 centrifugal filter (3 KD). 10 μL CB[7] (5 mM) was added to the concentrated sample and incubated for 2 h. The sample after incubation was ready for single channel recording experiments.

Simultaneous detection of microRNAs experiments.

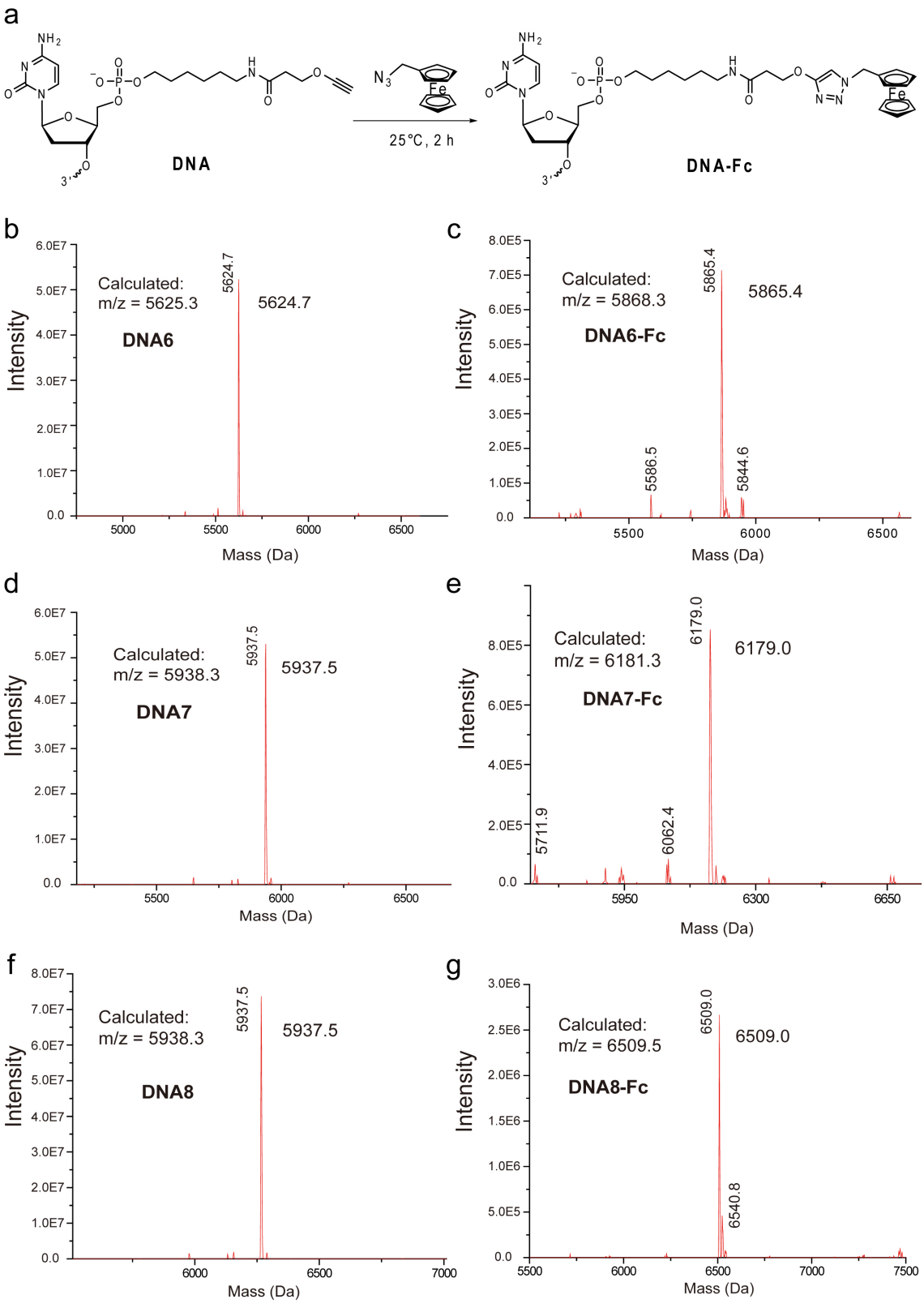
Triplex molecular beacon sensors were formed by incubating DNA11 (129 μM, 1.6 μL) and DNA8-Fc (35 μM, 5.7 μL) in 12.7 μL incubation buffer, DNA12 (152 μM, 1.3 μL) and DNA14-Fc (30 μM, 6.6 μL) in 12.1 μL incubation buffer, DNA13 (157 μM, 1.3 μL) and DNA15-Ad (36 μM, 5.5 μL) in 13.2 μL incubation buffer, respectively. Then the triplex sensors were mixed and then incubated with *let-7a*, *let-7b* and *let-7c* (each final concentration 50 nM) in a mixture for 2 h at room temperature. The resulting solution was mixed with magnetic beads suspension in 200 μL buffer (DEPC-treated 0.1 M NaCl) and vortexed for 15 min. The supernatant was collected with the aid of a magnetic separator and magnetic beads

were washed with deionized water. The solutions were combined and ultra-centrifuged with an Amicon Ultra-0.5 centrifugal filter (3 KD). 10 μL CB[7] (5 mM) was added to the concentrated sample and incubated for 2 h. The sample after incubation was ready for single channel recording experiments.

Single-channel current recording. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine was used to form a synthetic lipid bilayer across an aperture 100-150 μm in diameter in a 25-μm-thick polytetrafluoroethylene film (Goodfellow, Malvern, PA) that divided a planar bilayer chamber into two compartments, *cis* and *trans*. Both compartments contained 1 mL of buffer solution. DNA samples were added to the *cis* compartment, which was connected to ground. The *trans* compartment was connected to the head-stage of the amplifier. All experiments were carried out in 3 M KCl, 10 mM Tris, 10 mM $MgCl_2$, pH 5.2, at 22.5 ± 2 °C, unless otherwise stated. Ionic currents were measured by using Ag/AgCl electrodes with a patch-clamp amplifier (Axopatch 200B; Axon instruments, Foster City, CA), filtered with a low-pass Bessel filter with a corner frequency of 10 kHz and then digitized with a Digidata 1440A A/D converter (Axon Instruments) at a sampling frequency of 100 kHz.

Data analysis. Current traces were analysed with Clampfit 10.2 software (Axon Instruments). Events were detected using the Event Detection feature, and used to construct amplitude and dwell time histograms. Origin 8.5 (Microcal, Northampton, MA) and Clampfit 10.2 were used for histogram construction, curve fitting and graph presentation. Adobe Illustrator CS5 was used for making figures.

Supplementary Figures



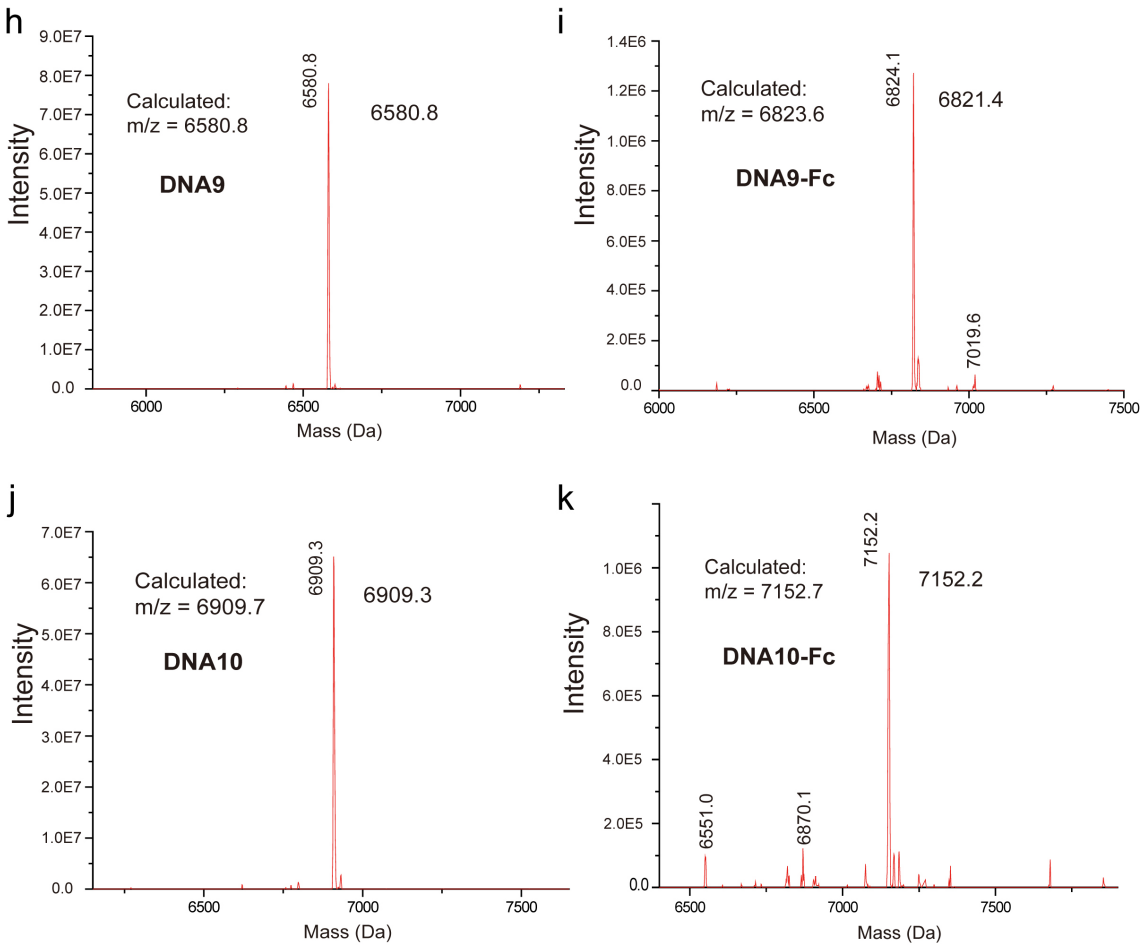


Fig. S1 Modification of DNA6-10 with azidomethylferrocene and mass spectroscopic characterisation. (**a**) Chemical reactions of the modification of DNA6-10 with azidomethylferrocene via "click" chemistry. (**b**) Mass spectroscopic characterization of DNA6. (**c**) Mass spectroscopic characterization of DNA6-Fc. (**d**) Mass spectroscopic characterization of DNA7. (**e**) Mass spectroscopic characterization of DNA7-Fc. (**f**) Mass spectroscopic characterization of DNA8. (**g**) Mass spectroscopic characterization of DNA8-Fc. (**h**) Mass spectroscopic characterization of DNA9. (**i**) Mass spectroscopic characterization of DNA9-Fc. (**j**) Mass spectroscopic characterization of DNA10. (**k**) Mass spectroscopic characterization of DNA10-Fc.

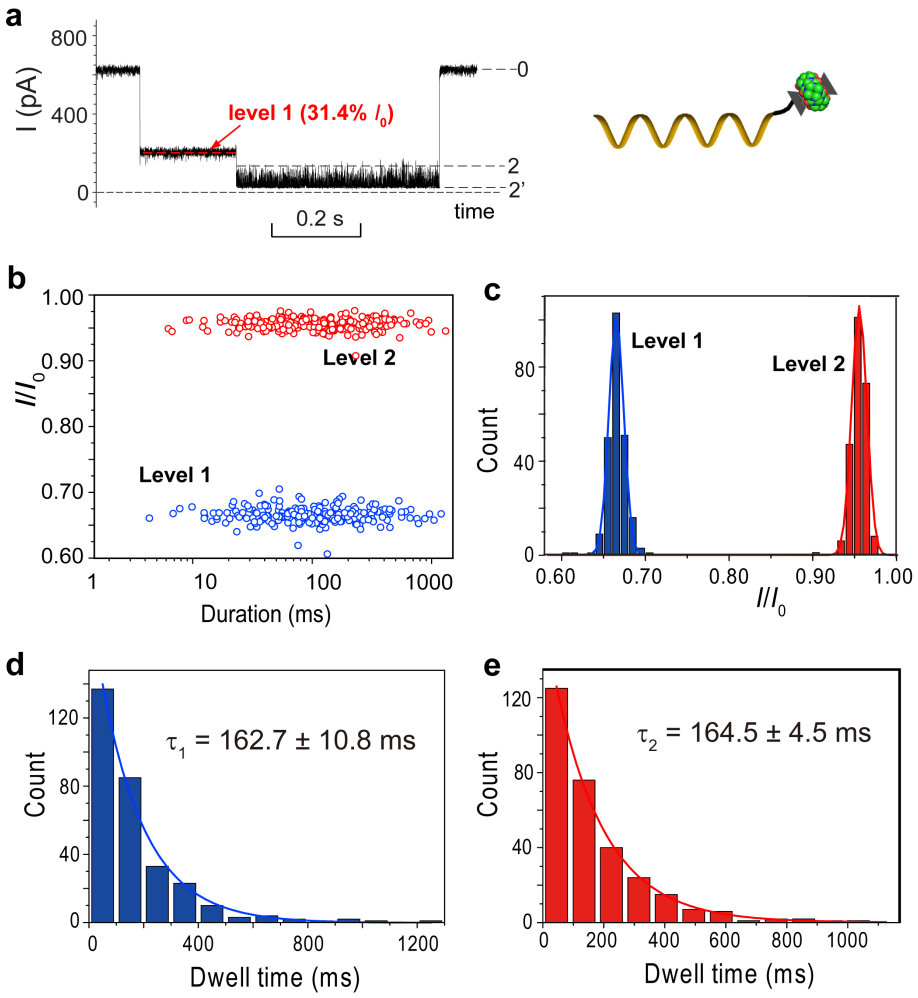


Fig. S2 Statistical analysis of the signature events generated by the translocation of DNA8-Fc⊂CB[7] through αHL. (**a**) A typical signature current event. I_0 stands for open pore current; I_1 and I_2 stand for the current blockade of level 1 and 2. (**b**) Scatter plots showing current blockades versus event durations of levels 1-2 in the signature events. The number of events is ~400. (**c**) Histograms of the current blockades of level 1-2. The solid lines are Gaussian fit to the histograms. (**d-e**) Dwell time histograms of level 1-2. The solid lines are the single exponential fit to the histograms. All data were acquired in the buffer of 3 M KCl, 10 mM tris and 10 mM $MgCl_2$, pH 5.2, with the transmembrane potential held at +200 mV.

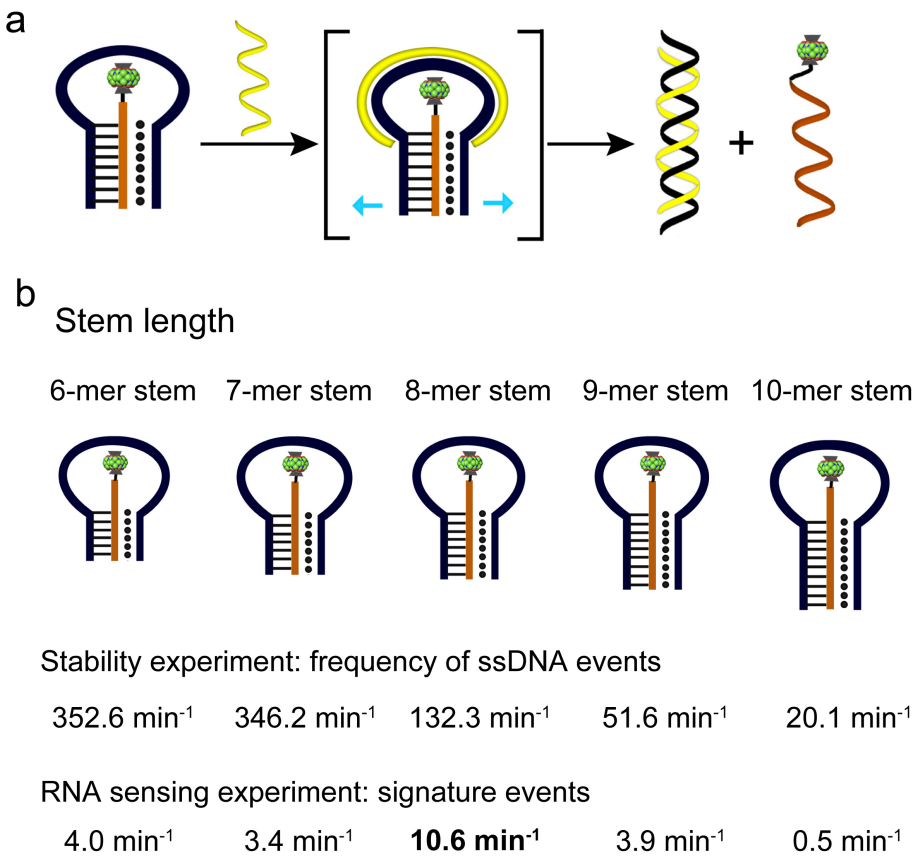


Fig. S3 Determination of the stem length of the tMB sensor. (**a**) Schematic representation of the detection of miRNA with a tMB sensor. (**b**) Condition screening by varying the length of the stem. The frequency of ssDNA events indicates the stability of the sensor structure. The frequency of signature events indicates the efficiency of the tMB sensor for the detection of miRNAs. The combined results showed that 8-mer stem is most appropriate for tMB sensor. All data were acquired in the buffer of 3 M KCl, 10 mM tris and 10 mM $MgCl_2$, pH 5.2, with the transmembrane potential held at +200 mV. Number of individual experiments n = 3.

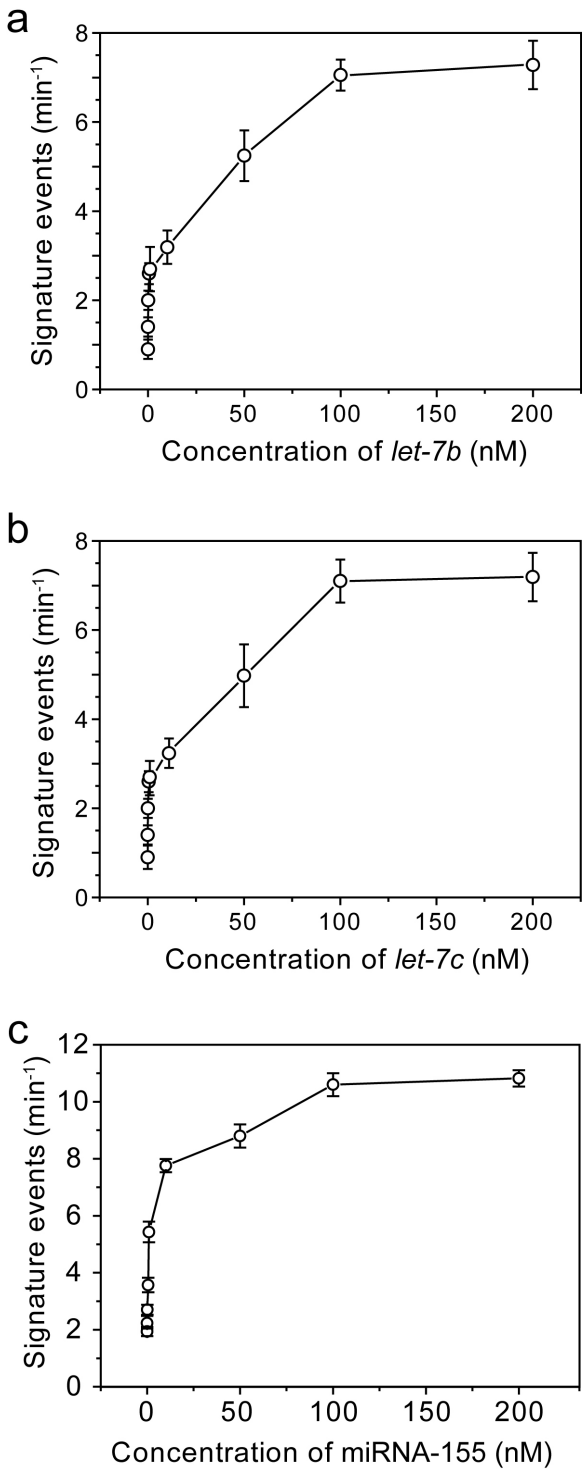


Fig. S4 Quantitative determination of *let-7* and micro-155 miRNAs with tMB sensor. (**a-c**) Correlation of the frequency of signature events (f_{sig}) with the concentration of *let-7b*, *let-7c* and miRNA-155 (0.005, 0.05, 0.5, 1, 10, 50, 100 and 200 nM), respectively. The final concentration of DNA is 200 nM. All data were acquired in the buffer of 3 M KCl, 10 mM tris and 10 mM $MgCl_2$, pH 5.2, with the transmembrane potential held at +200 mV. Number of individual experiments n = 3.

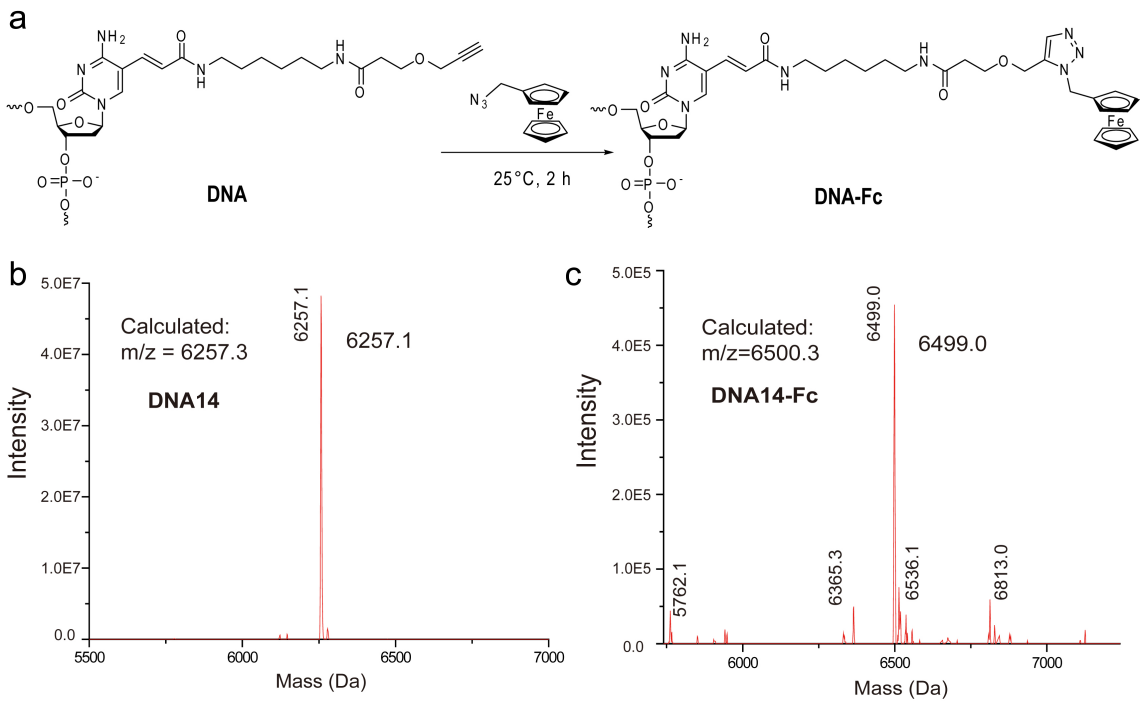


Fig. S5 Modification of DNA14 with azidomethylferrocene and mass spectroscopic characterization. (**a**) Chemical reactions of the modification of DNA14 with azidomethylferrocene via "click" chemistry. (**b**) Mass spectroscopic characterization of DNA14. (**c**) Mass spectroscopic characterization of DNA14-Fc.

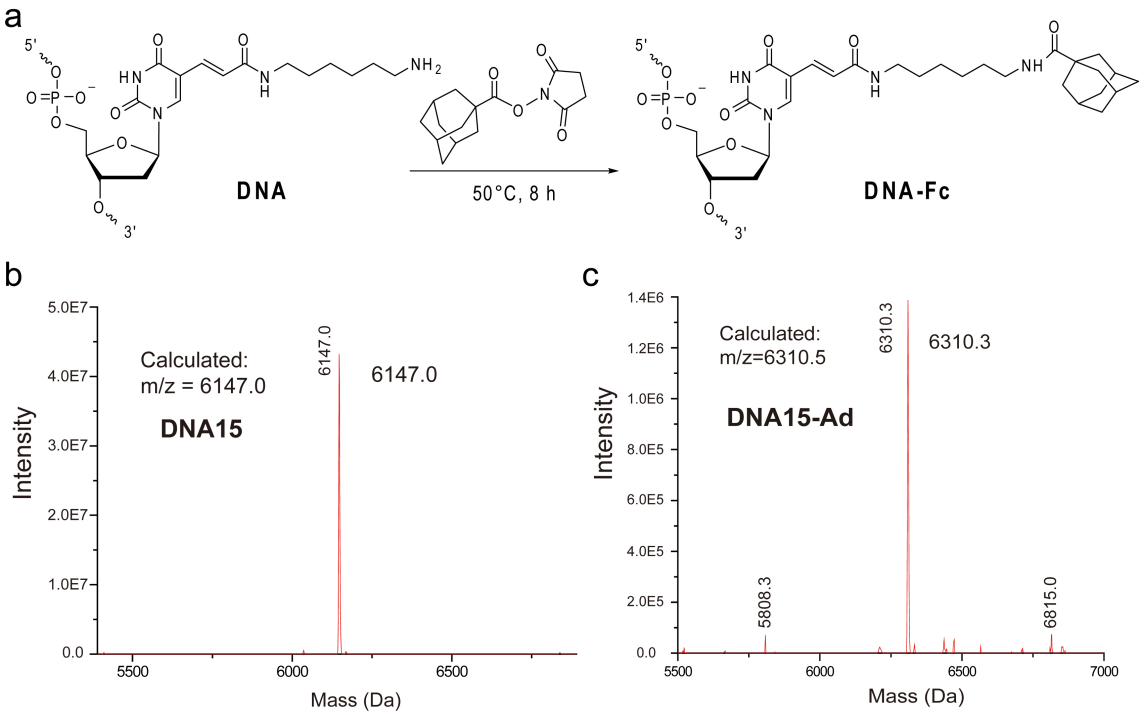


Fig. S6 Modification of DNA15 with Ad-NHS and mass spectroscopic characterization. (**a**) Chemical reactions of the modification of DNA15 with Ad-NHS via amidation reaction. (**b**) Mass spectroscopic characterization of DNA15. (**c**) Mass spectroscopic characterization of DNA15-Ad.

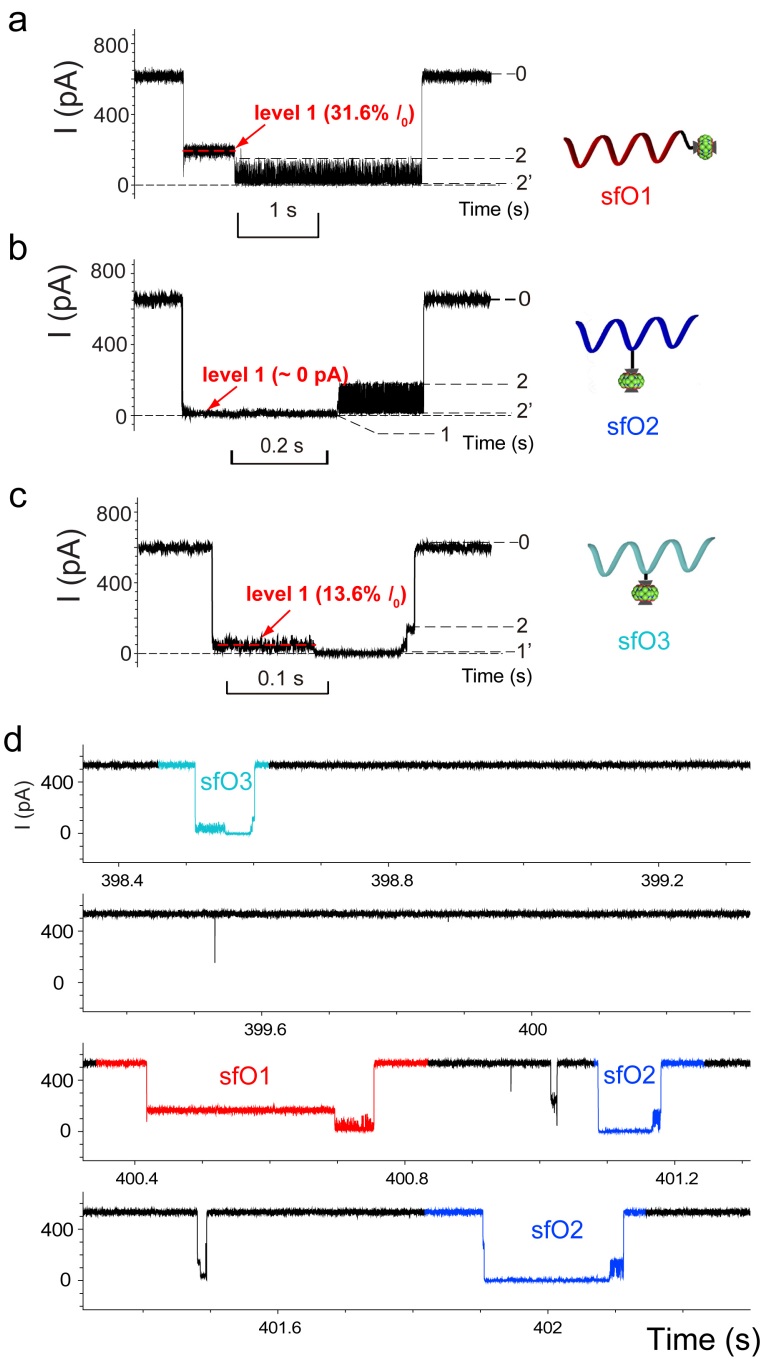


Fig. S7 Current signatures used in simultaneous detection of the mixture of *let-7* miRNAs. (**a-c**) The current signatures generated by the translocation of sfO1-3 through αHL nanopore. (**d**) A typical current trace acquired during the sensing of the mixture of *let-7* miRNAs. The current signals generated by sfO1-3 are distinctly different. All data were acquired in the buffer of 3 M KCl, 10 mM tris and 10 mM $MgCl_2$, pH 5.2, with the transmembrane potential held at +200 mV.

Supplementary Table

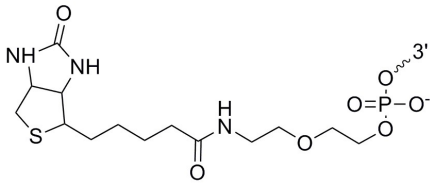
Table S1. Sequences of miRNA targets and their DNA probes in this study.

Name	Sequence	Modification
miRNA-155	5’-UUAAUGCUAAUCGUGAUAGGGG-3’	none
let-7a	5’-UGAGGUAGUAGGUUGUAU**A**GUU-3’	none
let-7b	5’-UGAGGUAGUAGGUUGU**G**U**G**GUU-3’	none
let-7c	5’-UGAGGUAGUAGGUUGUAU**G**GUU-3’	none
let-7d	5’-UGAGGUAGU**U**GGUUGUAU**G**GUU-3’	none
let-7e	5’-UGAGGUAGUAGGUUGU**U**U**A**GUU-3’	none
let-7f	5’-UGAGGUAGUAG**A**UUGUAU**A**GUU-3’	none
let-7g	5’-UGAGGUAGUAG**U**UUGUAU**A**GUU-3’	none
DNA1	**5’-biotin**-AAAAAAAAAATCTCTC**CCCCTAT CACGATTAGCATTAA**CTCTCT-3’	5’-biotin modification
DNA2	**5'-biotin**-AAAAAAAAAATCTCTCT**CCCCTA TCACGATTAGCATTAA**TCTCTCT-3’	5’-biotin modification
DNA3	**5’-biotin**-AAAAAAAAAATCTCTCTC**CCCCT ATCACGATTAGCATTAA**CTCTCTCT-3’	5’-biotin modification
DNA4	**5'-biotin**-AAAAAAAAAATCTCTCTCT**CCCC TATCACGATTAGCATTAA**TCTCTCTCT-3’	5’-biotin modification
DNA5	**5'-biotin**-AAAAAAAAAATCTCTCTCTCCC**C CTATCACGATTAGCATTAA**CTCTCTCTCT -3’	5’-biotin modification
DNA6	**5’**-CCCCCCCCCCCCGAGAGA-3’	5’-alkyne modification
DNA7	**5’**-CCCCCCCCCCCCAGAGAGA-3’	5’-alkyne modification
DNA8	**5’**-CCCCCCCCCCCCGAGAGAGA-3’	5’-alkyne modification
DNA9	**5’**-CCCCCCCCCCCCAGAGAGAGA-3’	5’-alkyne modification
DNA10	**5’**-CCCCCCCCCCCCGAGAGAGAGA-3’	5’-alkyne modification
P_{7a}	**5’-biotin**-AAAAAAAAAATCTCTCTC**AACTA TACAACCTACTACCTCA**CTCTCTCT-3’	5’-biotin modification
P_{7b}	**5’-biotin**-AAAAAAAAAATCTCTCTC**AACCA CACAACCTACTACCTCA**CTCTCTCT-3’	5’-biotin modification
P_{7c}	**5’-biotin**-AAAAAAAAAATCTCTCTC**AACCA TACAACCTACTACCTCA**CTCTCTCT-3’	5’-biotin modification

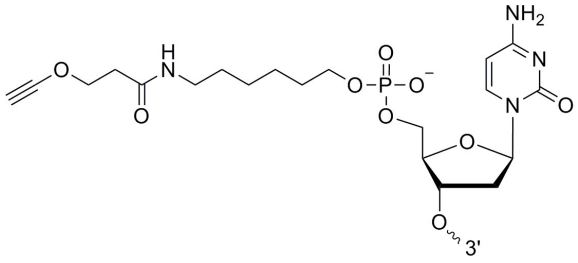
P_{7d}	**5'-biotin**-AAAAAAAAAATCTCTCTC**AACCA TACAACCAACTACCTCA**CTCTCTCT-3'	5'-biotin modification
P_{7e}	**5'-biotin**-AAAAAAAAAATCTCTCTC**AACTA AACAACCTACTACCTCA**CTCTCTCT-3'	5'-biotin modification
P_{7f}	**5'-biotin**-AAAAAAAAAATCTCTCTC**AACTA TACAATCTACTACCTCA**CTCTCTCT-3'	5'-biotin modification
P_{7g}	**5'-biotin**-AAAAAAAAAATCTCTCTC**AACTA TACAAACTACTACCTCA**CTCTCTCT-3'	5'-biotin modification
DNA11	5'-CCCCCCCCCT*CCGAGAGAGA-3'	T*: alkyne-modified thymine
DNA12	5'-CCCCCCCCCT#CCGAGAGAGA-3'	T#: amine-modified thymine

Note: all the loop sequences are in bold; all the stem sequences are underlined; the modified site on DNA is marked in red.

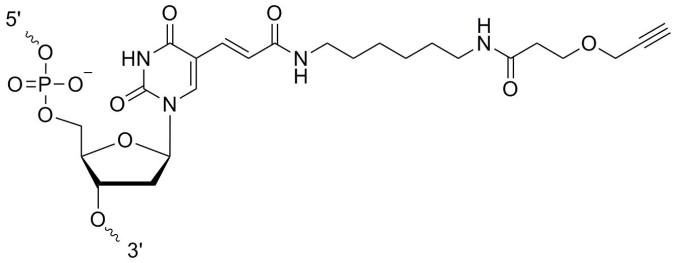
5'-biotin modification



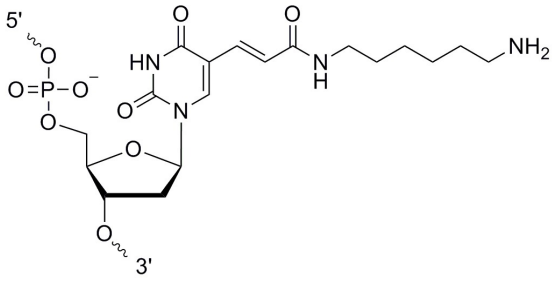
5'-alkyne modification



T*—alkyne-modified thymine:



T#—amine-modified thymine:



Supplementary reference

S1. S. Wen, T. Zeng, L. Liu, K. Zhao, Y. Zhao, X. Liu, H.-C. Wu, *J. Am. Chem. Soc.*, 2011, **133**, 18312-18317.