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Supplementary information

Table S1. Oligonucleotide sequences used in this work.

Title	Sequences (5' to 3')
SP	GGA TCA AAA ACA TCT TTA CAC TrA GTC TTT TTT TTT GA TCC GAG CCG GAC GAA GTG ACA GTG TT
Tracker	CAC TTC TTT TAG TGT AAA GAT GTT AGC TTT TTG ATC CTT GGG GA AAG GC TAG CTACA ACGAA GAAGT G
Substrate	Cy5A CTT CT rAr UT TCC CCT TTT TTT TTT TTT
"1"	GGA TCA AAA ACA TCT TTA CAC T
Target miRNA	UAA CAC UGU CUG GUA AAG AUG G
miRNA-a	UAA CAUUGU CUG GUA AAG AUG G
miRNA-b	UAA CAUUA CUG GUA AAG AUG G
miRNA-c	UAA CAUUA UG GUA AAG AUG G

Supplemented experimental section:

qRT-PCR Protocol

Quantitative reverse transcription PCR (qRT-PCR) was performed using the miRcute Plus miRNA qPCR Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions. Briefly, total RNA was extracted from serum samples using the miRcute Serum/Plasma miRNA Isolation Kit (TIANGEN). Reverse transcription was carried out with 2 μL of RNA sample in a 20 μL reaction mixture containing miRcute Plus miRNA RT Primer and miRcute Plus Enzyme Mix, incubated at 42 °C for 60 min, followed by 95 °C for 3 min. Real-time PCR was performed on a LightCycler 480 II System (Roche) using 2 μL of cDNA product in a 20 μL reaction with miRcute Plus miRNA Premix. The thermal cycling protocol was: 95 °C for 15 min, followed by 40 cycles of 94 °C for 20 s and 60 °C for 34 s. U6 snRNA was used as the endogenous control for normalization. Each sample was analyzed in triplicate.

Preparation of the DNA walker@plate

The DNA walker@plate was prepared by sequential immobilization of the biotinylated tracker strand and the substrate strand onto a streptavidin-coated 96-well plate (Corning, USA). First, 100 μL of the biotinylated tracker strand (100 nM in PBS buffer, pH 7.4) was added to each well and incubated at 37 °C for 2 h to allow immobilization via streptavidin–biotin interaction. The wells were then washed three times with 200 μL of washing buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) to remove unbound probes. Subsequently, 100 μL of the substrate strand (1 μM in PBS buffer) was introduced into each well and incubated at 37 °C for 1 h. The molar ratio of tracker to substrate was 1:10, as optimized in the main text (Fig. 3B). After immobilization, the wells were washed three times with washing buffer to remove nonspecifically adsorbed substrates. The successful assembly of the DNA walker@plate was verified by fluorescence measurements using a FAM-labeled substrate and a Cy5-labeled tracker. The prepared DNA walker@plate was used

immediately for subsequent detection experiments.

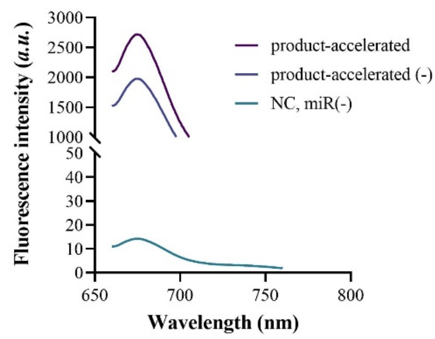

Figure S1. Fluorescence spectrum of the method for target miRNA detection when using substrate with or without product accelerate capability.