Electronic Supplementary Information for

Highly Accelerated Isothermal Nucleic Acid Amplifications by Butanol Dehydration: Simple, More Efficient, and Ultrasensitive

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1. Materials and Instruments

1.1 Materials

DNA, microRNA (miRNA), and PIWI-interacting RNA (piRNA) oligonucleotide strands, Tris-HCl (tri-(hydroxymethyl) amino methane hydrochloride, 10 mM, pH = 7.4) and HEPES (10 mM 4-(2-hydroxyerhyl) piperazine-1-erhanesulfonic acid and 0.0425% NaCl, pH = 7.4) were purchased from Sangon Biotech, China. The proteinase K, RNase A, Triton X-100, and sodium dodecyl sulfate (SDS) were bought from Sigma-Aldrich, USA. Benzenemethanesulfonyl fluoride (PMSF) was provided by Hangzhou Fude Biological Technology Co., China. Phosphate-buffered saline (PBS, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl and 2.68 mM KCl, pH = 7.4), Dulbecco's modified Eagle's medium (DEME), fetal bovine serum (FBS) and penicillin/streptomycin were bought from Gibico, USA. Breast cancer cell line MCF-7 were supplied by cell library of School of Pharmaceutical Sciences, Southern Medical University.

1.2 Instruments

Fluorescence spectra were recorded on a Waters Prep 150 Fluorescence spectrophotometer (Waters Prep 150, Waters, USA). Fluorescence intensities generated by real sample of plasma and exosomes was measured by a multifunctional microporous plate detector (Infinite M1000 Pro, Tecan, Switzerland). Exosomes were isolated utilizing differential centrifugation by high-speed centrifuge (3H16RI, Hunan Hexi Instrument Equipment Co. Ltd.，China) and Beckman ultracentrifuge (Optima XPN-100, Beckman Coulter Life Sciences Inc., USA). Particle size and the concentration of exosomes were analyzed by Flow NanoAnalyzer (NanoFCM, U30, China). Native polyacrylamide gel electrophoresis was run by the gel electrophoresis system (Bio-Rad Mini-PROTEAN®，Bio-Rad, USA) and imaged by multifunctional gel imaging system (Clinx Science Instruments, Clinx, China).

2. Tables of the nucleic acids in the three types of isothermal amplification reactions

Table S1. Nucleic acids in the catalytic hairpin assembly (CHA) reactions

Name	Sequences (5'-3')
$H0_{20365}$	AAACCGTTACCATTACAGTGATCAGAGTACTAACCACTATA CGATCACGGCCCTGATCACTG
$H0_{21}$	AAACCGTTACCATTACAGTGATTCAACATCAGTCTGATAA GCTATCACTGTA
$H0_{39}$	AAACCGTTACCATTACAGTGATCAAGCTGATTTACACCCG GTGAATCACTGTAATG
H1	ATCACTGT(**Cy3**)AATGGTAACGGTTTGGATTCCGAGGCATA CCGTTACCATTACA
H2	AACGGTATGCCTCGGAATCCAAACCGTTACCATT(**Cy5**)ACA GGTTTGGATTCCGAGGC
piR-20365	GGCCGUGAUCGUAUAGUGGUUAGUACUCUG
miR-21	UAGCUUAUCAGACUGAUGUUGA
Cel-miR-39	UCACCGGGUGTAAAUCAGCUUG
miR-1246	AAUGGAUUUUUGGAGCAGG
piR-651	AGAGAGGGGCCCGUGCCUUGGAAAGCGUC
piR-16926	CGGAAGCGUGCUGGGCCCAUAACCCAGA
DNA I	TAGCTTATCAGACTGATGTTGC
DNA II	TAGCTTATCAGACTGATGTTTC

Note: $H0_x$ stands for H0 probe for detecting piR-x or miR-x, for example, $H0_{20365}$ is H0 for detecting piR-20365. Underlined red bases are different from bases at the corresponding positions in miR-21.

Table S2. Nucleic acids in the hybridization chain reaction (HCR)

Name	Sequences (5'-3')
HP1	CCGTCCTTTCCCGACCAGATCTTTGAGTCAGATCTGGTCGG GAA-**Cy3**
HP2	AGATCTGGTCGGGAAAGGACGGT**(Cy5)**TCCCGACCAGATC TGACTCAA
DNA1	AGATCTGGTCGGGAAAGGACGG

Table S3. Nucleic acids in the Split DNAzyme-based amplification reaction

Name	Sequences (5'-3')
Split-A	CTCTTCTCCGAGCGCCGAAGCACTTCTT
Split-B	CTACACCCCAAAATGCCGGTCGAAATAGTC
substrate	**FAM**-CCACAAGACTAT/**rA**/GGAAGAGATGTGG-**BHQ1**
miR-373	GAAGUGCUUCGAUUUUGGGGUGU
M1-373	GAAGUGCUUCGUUUUUGGGGUGU
M1'-373	GAAGUGCUUCGAAUUUUGGGGUGU
miR-1246	AAUGGAUUUUUGGAGCAGG
miR-21	UAGCUUAUCAGACUGAUGUUGA

Note: Underlined red bases are different from bases at the corresponding positions in miR-373.

rA = Adenine ribonucleotide

3. Experimental Methods

3.1 CHA detection

3.1.1 CHA detection of piR-20365 and miR-21 in buffer

Normal method: Mixtures of 30 nM $H0_{20365}$ /$H0_{21}$, 50 nM H1, 100 nM H2, and piR-20365/miR-21 (**Table S1**) of different concentrations in 100 μL PBS buffer containing 400 mM NaCl were incubated at 45 °C in the dark. After 3 h, fluorescence intensities were recorded under excitation wavelength of 540 nm, the maximum excitation wavelength for Cy3. Spectra of *FA/FD* (fluorescence intensity at each emitting wavelength divided by that at 564 nm, the maximum emission wavelength of Cy3) and the standard curve of *FA/FD* values at 660 nm against target concentrations were plotted.

Accelerating method: The mixture abovementioned in a 2 mL EP tube was added with 1100 μL n-butanol and vortexed for several seconds. Then the tube was put into 45 °C metal bath immediately for 5 min incubation and subsequently immersed in ice water to terminate the CHA reaction. Afterwards, 100 μL PBS buffer was added into the tube for a vortex-mixing followed by a centrifugation at 6000 rpm for 8-9 seconds to stratify the butanol and water layer. The lower water phase was pipetted out and supplemented with PBS buffer to 100 μL to scan the fluorescence spectrum.

For selectivity study, 1 nM piR-20365 or miR-21 was respectively substituted by 1nM other piRNAs, miRNAs, and DNAs listed in Table S1 to perform the accelerated CHA detection and the responding signals were compared.

3.1.2 CHA detection of piR-20365, miR-21, and *Cel*-39 in exosomes

Normal method: Exosomes of different concentrations extracted from MCF-7 cell culture were incubated in solution containing 0.5 % Triton X-100, 0.5 % SDS and 84.5 μg mL^{-1} proteinase K at 37 °C for 1 h to rupture the membrane of the vesicles and release the piRNAs/miRNAs from their binding proteins. The treated samples were then added with 30 nM H0, 50 nM H1, 100 nM H2, and 400 mM NaCl in final concentrations and incubated for 3 h at 45 °C. The fluorescence signals were finally measured by a multifunctional microporous plate detector at the emission wavelength of 564 nm (*FD*) and 666 nm (*FA*) respectively under the excitation wavelength of 540 nm. Signal/background of each sample was calculated as its *FA/FD* value divided by *FA/FD* of the blank.

Accelerating method: The accelerating procedure to the pretreated exosomes abovementioned was the same as described in **3.1.1**, except that the CHA reaction time was 10 min.

3.1.3 CHA detection of piR-20365, miR-21, and *Cel*-39 in plasma exosomes

Normal method: The procedure refers to our previous report (Zhang, L. M. etc., Anal. Chim. Acta. 2022, 1192, 339382). Briefly, plasma sample of 100 μL from each breast cancer patient or healthy control was sequentially treated by proteinase K (65μg mL^{-1}) at 37 °C for 30 min to disrupt proteins and the circulating RNA-protein complex, protease inhibitor PMSF (5 mM) at room temperature for 20 min to stop excess proteinase K, RNase A (4 U mL^{-1}) at 37 °C for 30 min to degrade the released RNA, and SDS (0.2%) to inhibit excess RNase A. After the treatment, 1% Triton X-100 and proteinase K (84.5 μg mL^{-1}) were added into the plasma samples for 40 min incubation at 37 °C to rupture the exosomes membrane and release piRNAs from their binding piwi-proteins. The mixture was centrifugated at 2500 × g for 5 minutes and the supernatant was added with 30 nM H0, 50 nM H1, 100 nM H2, and 400 mM NaCl to incubate for 3 h at 45 °C. The fluorescence signals were measured as described in **3.1.2** and the signal/background values of the samples were used to construct the scatter plots.

Accelerated method: Plasma sample of 100 μL from each breast cancer patient or healthy control was treated with 200 μL acetonitrile to precipitate the free proteins and free circulating RNA-protein complexes. The mixture was centrifugated at 15000 rpm for 5 minutes and the precipitation was washed 2 times with 50 μl of PBS. The supernatant combined was incubated with mixture of 0.5 % Triton X-100, 0.5 % SDS and 84.5 μg mL^{-1} proteinase K at 37 °C for 1 h. Finally, CHA probes and 1000 μL butanol was added in the treated plasma samples to execute accelerating reactions as described in **3.1.1** for 10 min.

3.2 HCR detection of DNA1

Normal method: Mixtures of 100 nM HP1, 100 nM HP2, and DNA1 (**Table S2**) of different concentrations in 100 μL PBS buffer containing 400 mM NaCl were incubated

at 45 °C for 3 h in the dark. Fluorescence intensities were subsequently scanned from 555 to 700 nm under excitation wavelength of 540 nm. Spectra of *FA/FD* and standard curve of *FA/FD* values at 660 nm against target concentrations were plotted.

Accelerated method: Mixtures abovementioned were executed reactions as the same accelerating procedure as described in **3.1.1** except that the HCR reaction time was 10 min.

3.3. DNAzyme-based amplification detection of miR-373

Normal method: A 100 uL reaction solution was prepared which contains 10 μL of reaction buffer (250 mM HEPES, 1.5 M NaCl), 10 μL of 10 mM Zn^{2+}, 20 μL of 1 μM substrate (S), 10 μL of 1 μM split A probe, 10 μL of 1 μM split B probe, miR-373 at varying concentrations and DEPC water. The reaction was performed at 20 °C for 3 h. Fluorescence spectra was scanned at room temperature from 500 to 580 nm under the excitation wavelength of 480 nm. The standard curve of peak values at 520 nm against $lgC_{miR\text{-}373}$ was plotted.

Accelerating method: The mixture abovementioned in a 2 mL EP tube was added with 1100 μL n-butanol and vortexed for 6-8 seconds. Then the tube was put into 20 °C water bath immediately for 20 min incubation in the dark and subsequently immersed in ice water to terminate the reaction. Afterwards, 50 μL ultrapure water was added into the tube for a 5 s vortex-mixing followed by a centrifugation at 2000 rpm for 5-6 s to stratify the butanol and water layer. The lower water phase was pipetted out and supplemented with ultrapure water to 100 μL to scan the fluorescence spectrum.

For selectivity study, 100 pM miR-373 was respectively substituted by 100 pM other four RNAs listed in **Table S3** to perform the accelerated DNAzyme-based amplification detection and the responding signals were compared.

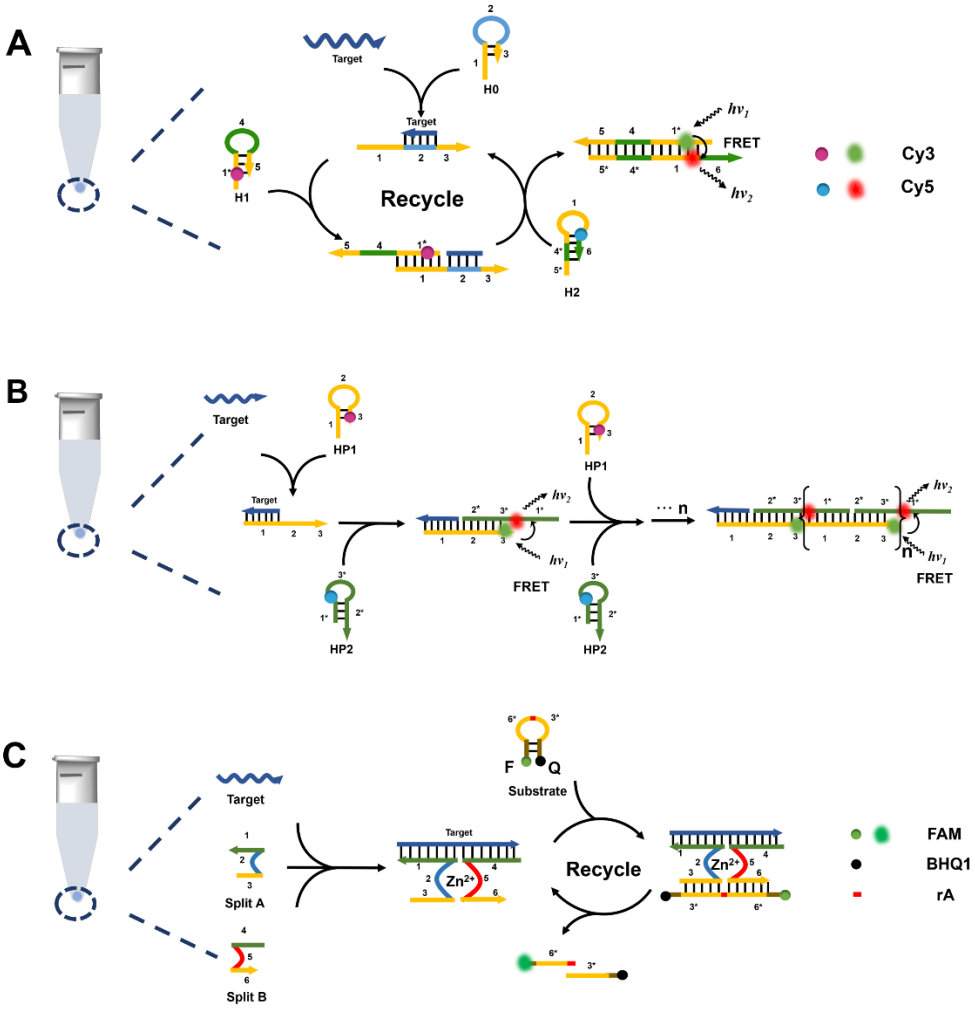
3.4 Native polyacrylamide gel electrophoresis (PAGE) test

Nondenaturing 10% polyacrylamide gel electrophoresis was performed to verify the amplification of the accelerated CHA system. Seven reaction systems including H1, H2, H1 + H2, $H0_{20365}$ + H1 + H2, piR-20365 + $H0_{20365}$ + H1 + H2, $H0_{21}$ + H1 + H2, and

miR-21 + $H0_{21}$ + H1 + H2 were first subjected to accelerated reactions according to the procedure described in **3.1.1**. The initial concentration of H1 is 50 nM and H2 100 nM. Both $H0_{20365}$ and $H0_{21}$ are 30 nM, and both piR-20365 and miR-21 are 10 nM. After 5 min incubation, the lower phases were pipetted out to run the electrophoresis in a 0.5× TBE running buffer (44.5 mM Tris Base, 44.5 mM boric acid, 1 mM EDTA) for 1 h at a voltage of 300 V. Next, the gel was stained with 0.9 % NaCl containing 1× 4S GelRed for 20 min and photographed by a multifunctional gel imaging system.

The PAGE analysis for characterizing the amplification of the accelerated HCR was performed by the similar method and under the same electrophoresis conditions except that both the concentrations of HP1 and HP2 were 100 nM.

4. Scheme illustration of the CHA, HCR, and DNAzyme-based amplification in the RNA analysis



Scheme S1. Schematic illustration of RNA detections by (A) catalytic hairpin assembly (CHA), (B) hybridization chain reaction (HCR), and (C) DNAzyme-based amplification.

5. Results

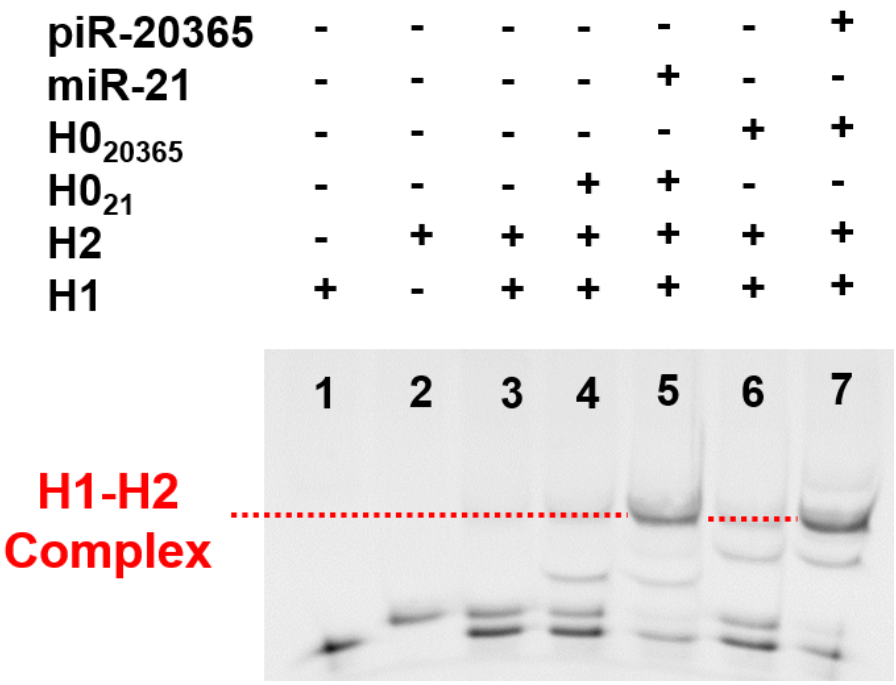


Fig. S1. Native PAGE analysis of the accelerated CHA system after 5 min reaction. Lane 1: H1; 2: H2; 3: H1 + H2; 4: $H0_{21}$ + H1 + H2; 5: miR-21 + $H0_{21}$ + H1 + H2; 6: $H0_{20365}$ + H1 + H2; 7: piR-20365 + $H0_{20365}$ + H1 + H2. The initial concentrations are: H1 = 50 nM, H2 = 100 nM, $H0_{20365}$ = $H0_{21}$ = 30 nM, and piR-20365 = miR-21 = 10 nM with 400 mM NaCl in all the systems.

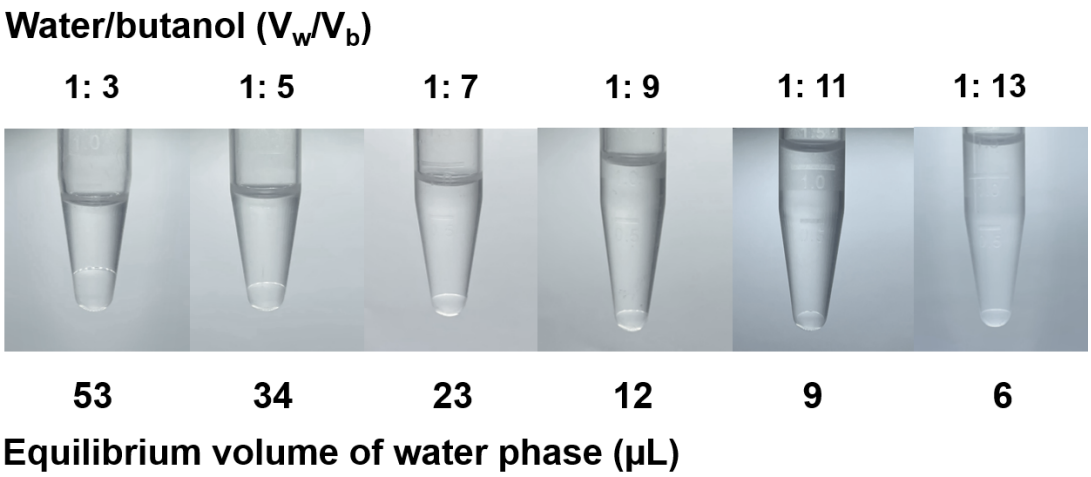


Fig. S2. Photos of the phase equilibrium after 100 μL aqueous solutions of the CHA system mix varying ratios of butanol.

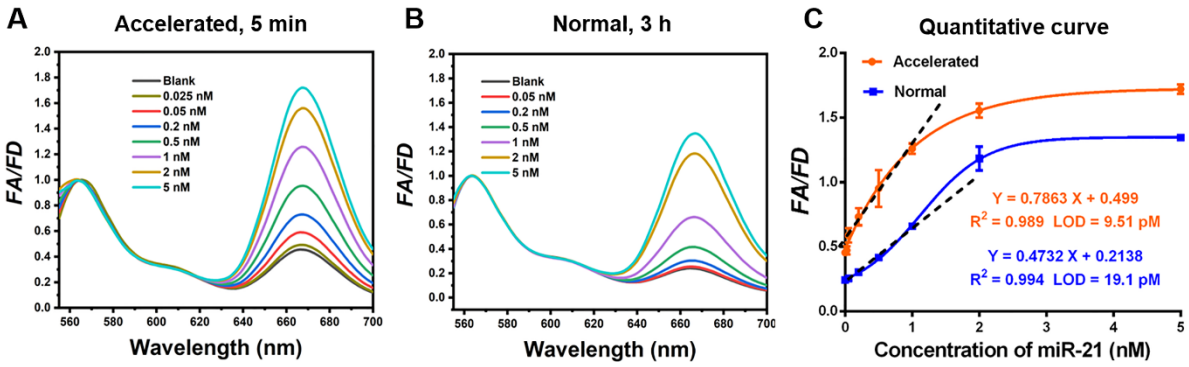


Fig. S3. Comparison of fluorescence spectra and quantitative signal curves of CHA analysis to miR-21 under normal and accelerated methods. (A) FRET spectra of accelerated system. (B) FRET spectra of normal system. (C) Quantitative curves of the normal and the accelerated systems.

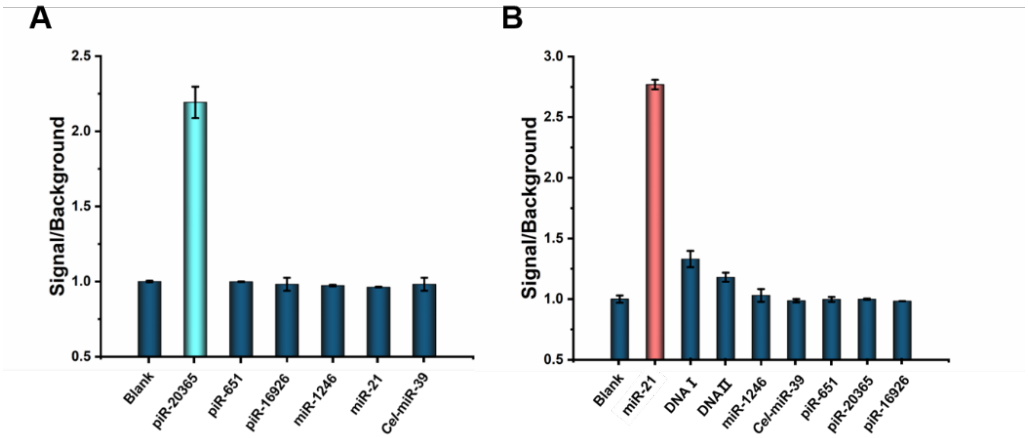


Fig. S4. Selectivity of accelerated CHA reaction in detecting (A) piR-20365 and (B) miR-21. All the concentrations of the RNAs are 1 nM.

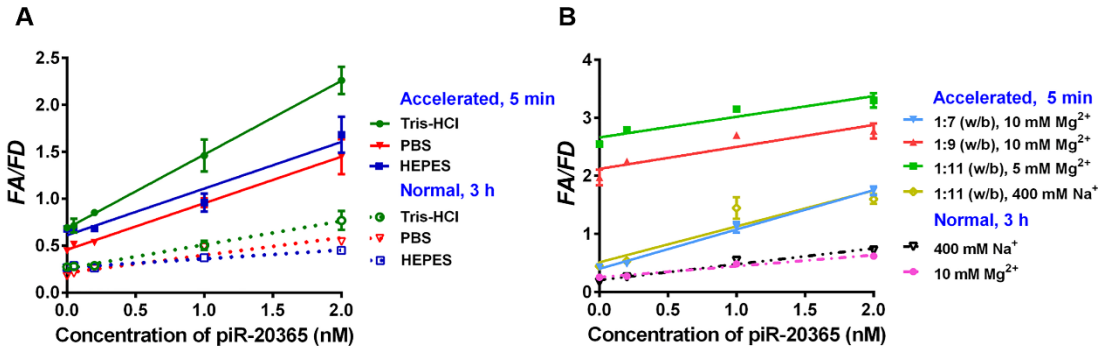


Fig. S5. Sensitivities of accelerated CHA in (A) different buffers and (B) PBS buffers including different metal ions.

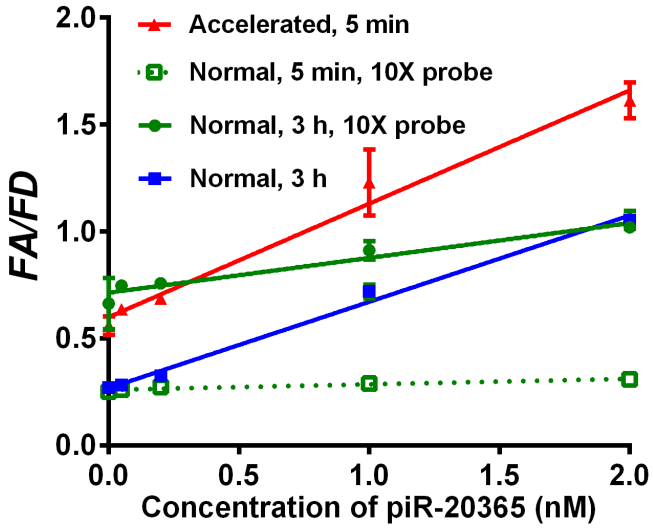


Fig. S6. Sensitivities of accelerated CHA system (H0 = 30 nM, H1 = 50 nM, H2 = 100 nM), normal system (H0 = 30 nM, H1 = 50 nM, H2 = 100 nM), and normal systems with the probes of 10-fold concentrations (H0 = 300 nM, H1 = 500 nM, H2 = 1000 nM).

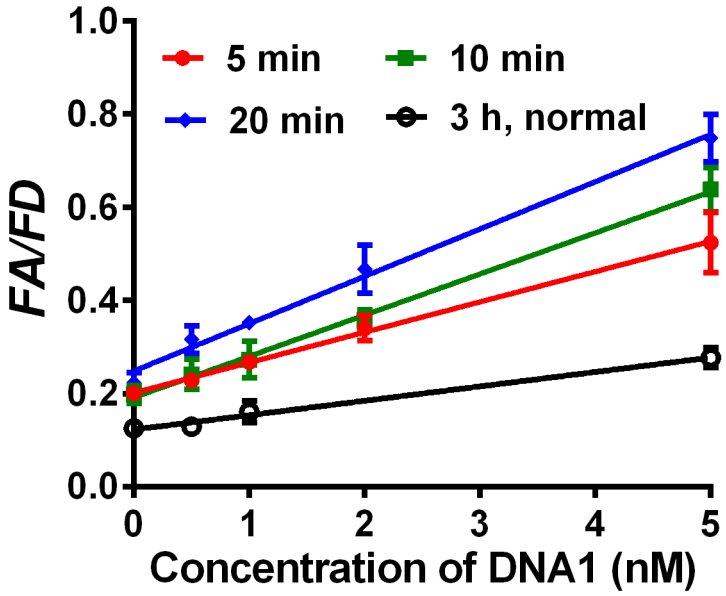


Fig. S7. Sensitivities of accelerated HCR after different reaction time.

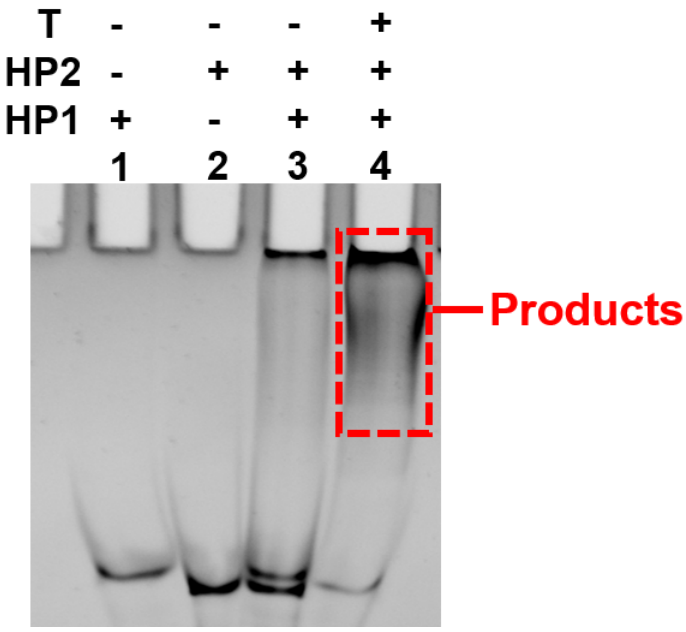


Fig. S8. PAGE analysis of the accelerated HCR system after 20 min reaction. Lane 1: HP1; 2: HP2; 3: HP1 + HP2; 4: Target + HP1 + HP2. The initial concentrations are: HP1 = HP2 = 100 nM, Target = 10 nM with 400 mM NaCl in all the systems.

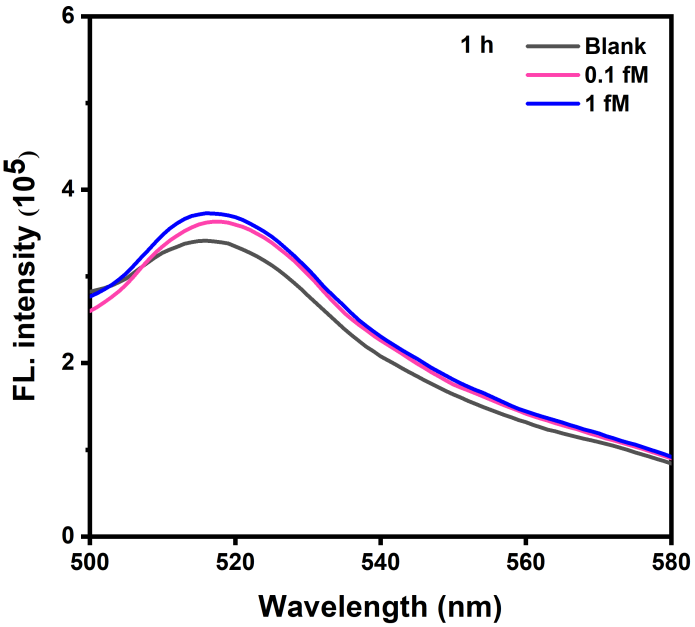


Fig. S9. Spectra of the accelerated DNAzyme-based amplification responding to miR-373 after 1 h incubation.

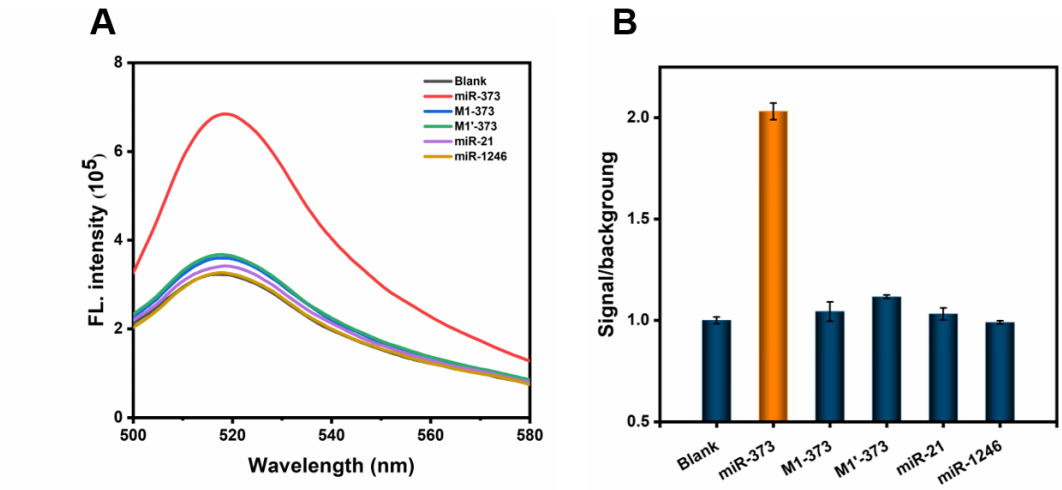


Fig. S10. Selectivity of the accelerated DNAzyme-based amplification analysis to miR-373. Measurements were carried out after 20 min of reaction. (A) Spectra in the presence of target miR-373 or other mismatched targets. (B) Fluorescence signals normalized by the blank at the 520 nm in the presence of different targets. All the tested samples were at concentrations of 100 pM.