Supporting Information

Urinary Exosomal mRNA Detection using Novel Isothermal Gene Amplification Method based on Three-way Junction

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Experimental Section

Materials and Reagents

All DNA oligonucleotides purified by high-performance liquid chromatography (HPLC) were purchased from Genotech Co. (Daejeon, Korea), and all RNA oligonucleotides purified by RNase-free HPLC were purchased from Integrated DNA Technologies, Inc (Coralville, IA, USA). The sequence information is shown in Table S1. Bst 2.0 WarmStarter DNA polymerase (M0538S), Deoxynucleotide (dNTP) Solution Mix (N0447S), ThermoPol reaction buffer (B9004S), and NEBuffer 3.1 (B7203S) were purchased from New England Biolabs Inc. (Beverly, MA, USA). Nt.BstNBI (R097S) was purchased from Enzynomics (Daejeon, Korea). The miRNeasy Mini Kit (217004) was purchased from Qiagen (Hilden, Germany). Exo-spin buffer (EX06-250) was purchased from Cell Guidance Systems Ltd (Cambridge, UK). Diethyl pyrocarbonate (DEPC)-treated water (95284), ThT (T3516), and SYBR Green I (S7563) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gel-Red Nucleic Acid Stain (41003) was purchased from Biotium (Fremont, CA, USA).

RNA Detection by Isothermal G-quadruplex Amplification Reaction based on 3WJ Probes

The reaction buffer was prepared as Part A and Part B. Part A consisted of 3WJ-template, 3WJ-primer, F-template, and DEPC-water. Part B consisted of Bst 2.0 WarmStarter DNA polymerase, Nt.BstNBI nicking endonuclease, dNTPs, ThermoPol reaction buffer, NEBuffer 3.1, DEPC-treated water, and ThT. For the detection of RNA, 2 μL of the target RNA sample was added to 8 μL of Part A solution and immediately mixed with 10 μL of Part B solution. The reaction was conducted at 55 ℃ in a final volume of 20 μL containing 1× ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8), 0.5× NEBuffer 3.1 (50 mM NaCl, 25 mM Tris-HCl, 5 mM $MgCl_2$, 50 μg/mL

BSA, pH 7.9), 3WJ-template (3 nM), 3WJ-primer (3 nM), F-template (30 nM), dNTP (250 μM), Bst DNA polymerase (1U), Nt.BstNBI nicking endonuclease (1.6U), and ThT (25 μM). The fluorescence intensity was monitored at intervals of 1 min by a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, CA, USA) with the FAM filter set.

Gel Electrophoresis

First, Part A solution was prepared in different combinations using target RNA, 3WJ-template, 3WJ-primer, and F-template. Part B consisted of Bst 2.0 WarmStarter DNA polymerase, Nt.BstNBI nicking endonuclease, dNTPs, ThermoPol reaction buffer, NEBuffer 3.1, and DEPC-treated water. The prepared Part A solution was added to the Part B solution, and the mixed solution was incubated at 55 ℃ for 0, 10, and 20 min. Second, Part B was prepared with different enzyme combinations and incubated at 55 ℃ for 20 min after mixing with the Part A solution consisting of target RNA, 3WJ-template, 3WJ-primer, and F-template. The final concentration in each solution was 1× ThermoPol reaction buffer, 0.5× NEBuffer 3.1, dNTP (250 μM), Bst DNA polymerase (1.6U), Nt.BstNBI nicking endonuclease (2U), target RNA (50 nM), 3WJ-template (50 nM), 3WJ-primer (50 nM), and F-template (500 nM). Then, 20 μL of each reaction product was resolved on a 10% polyacrylamide gel using 1× TBE as the running buffer at a constant voltage of 90 V for 100 min. After Gel-Red staining, gels were scanned using a UV transilluminator (ATTO Corporation, Tokyo, Japan).

Extraction of Total RNA from Cells and Exosomes

HCC1954 and HCC1143 cells were cultured in RPMI-1640 medium (11835-030, Thermo Scientific, USA) containing 10% fetal bovine serum (26140-079, Thermo Scientific, USA) and 5% antibiotics (15240-062, Thermo Scientific, USA) in a humidified incubator at 37 ℃. Total RNA from cells was extracted using the miRNeasy Mini Kit (Qiagen). For the extraction of RNA from exosomes, the cell growth medium was replaced with fetal bovine serum-free

medium and incubated for 24 h before the medium was harvested. The harvested medium was centrifuged at 300 ×g for 10 min to remove cells, and the supernatants were additionally filtered through a 0.2 μm membrane filter. Two volumes of cell-free supernatant were mixed with 1 volume of Exo-spin™ buffer and incubated overnight at 4 ℃. The mixture was centrifuged at 16,000 ×g for 1 h. The supernatants were discarded, and the pellet was redissolved in 700 μL of Qiazol (Qiagen). Exosomal RNA was extracted using the miRNeasy Mini Kit. The quality and quantity of extracted RNA were identified with a Nanodrop (Thermo Scientific, USA). The extracted RNA samples were stored at -80 ℃.

Animal Experiments

All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee, Yonsei University Health System. Six-week-old athymic Balb/c nude mice (Orient Bio, Seongnam-Si, Korea) were used for tumor experiments. The mice were retained in microisolator cages under sterile conditions and observed for at least 1 week before study initiation to ensure proper health. Temperature, lighting, and humidity were controlled centrally. The mice were anesthetized with a solution of Zoletil (30 mg/kg; Virbac Korea, Seoul, Korea) and xylazine (10 mg/kg; Bayer Korea, Seoul, Korea) delivered intraperitoneally. The breast cancer xenograft model was developed by the injection of HCC1954 cells (6×10^6 cells/mouse) into the mammary fat pad of female mice (total 16 mouse model). The tumor volume was calculated using the formula volume = [largest diameter × (smallest diameter)]^2/2. The mice were sacrificed when the tumor volume reached 1,000 mm or became ulcerated. The urine of breast cancer mice was collected to a volume of 800 μL, and the collected urine was centrifuged at 300 ×g for 10 min. The supernatant was centrifuged again at 16,000 ×g for 30 min. The exosomes in urine were isolated using the Exo-spin^{TM} Exosome Purification system (Cell Guidance Systems, St. Louis, MO) according to the manufacturer's instructions.

qRT-PCR

The extracted RNA from HCC1143 and HCC1954 cells was transcribed to cDNA using a TOPscript cDNA Synthesis Kit (Enzynomics, Daejeon, Korea) following the manufacturer's protocol. Each cDNA aliquot was used as a template for real-time PCR with TOPreal qPCR 2× PreMIX (Enzynomics, Daejeon, Korea). The amplification reaction was performed in a 20 μL total volume under the following sequential conditions: initial denaturation at 95 ℃ for 15 min, followed by 50 cycles of 95 ℃ for 10 s, 60 ℃ for 15 s, and 72 ℃ for 20 s. The target gene expression level was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

Western Blot Analysis

Exosomes were lysed in RIPA buffer and boiled (at 98 ℃ for 10 min). Protein concentration was measured using a BCA Protein Assay kit (ThermoFisher Scientific, Waltham, USA). Lysates were subjected to SDS/PAGE (10% gel) and the proteins were transferred on to a polyvinylidene fluoride membrane (Life Technologies, Grand Island, NY, USA). Subsequent to immunoblotting with Anti-CD63 (cat. no. Ab134045; 1:500; Abcam, Cambridge, MA, USA), the reaction product was revealed with an Amersham ECL Western Blotting system (GE Healthcare Life Sciences, Chalfont, UK).

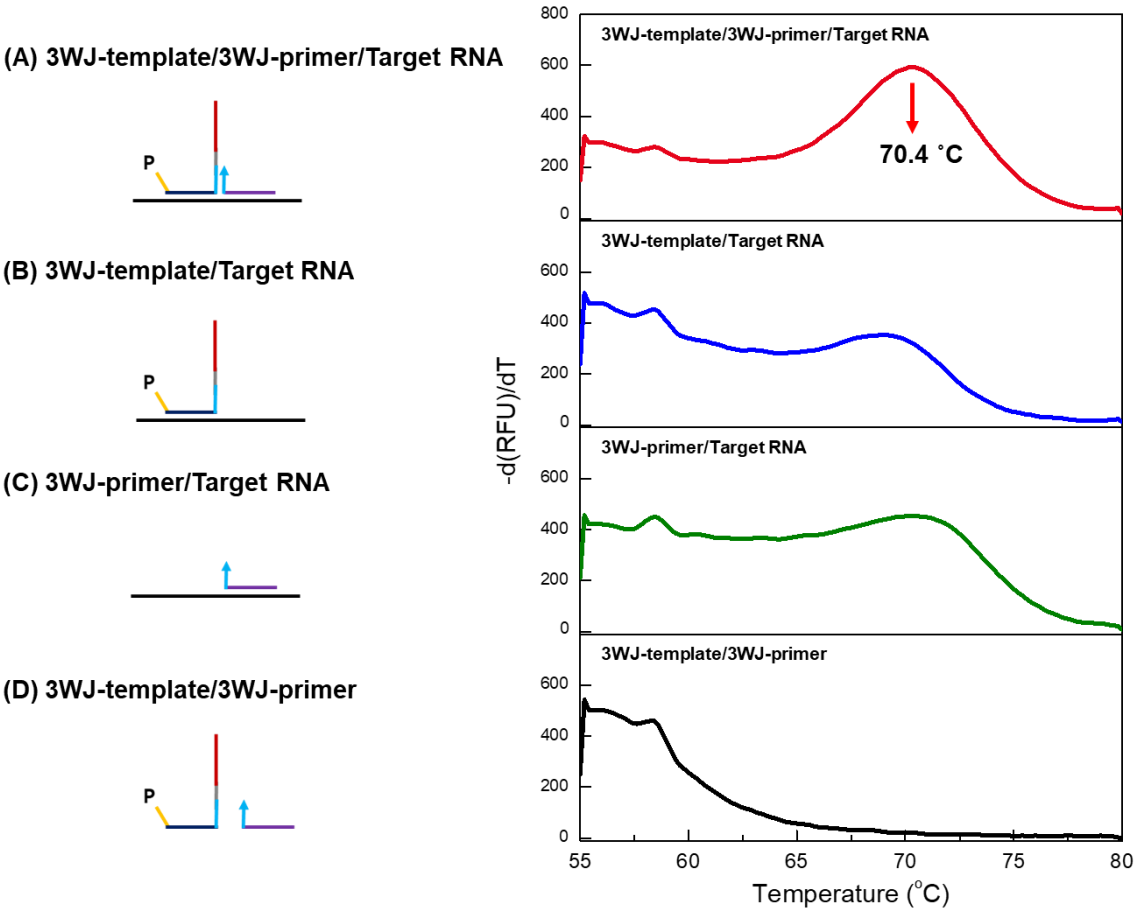

Figure S1. Melting temperature analyses with (A) 3WJ-template, 3WJ-primer, and target RNA, (B) 3WJ-template and target RNA, (C) 3WJ-primer and target RNA, and (D) 3WJ-template and 3WJ-primer. The final concentrations of target RNA, 3WJ-template, and 3WJ-primer were 100 nM. The reaction was conducted in a final volume of 20 µL containing 1× ThermoPol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8), 0.5× NEBuffer 3.1 (50 mM NaCl, 25 mM Tris-HCl, 5 mM $MgCl_2$, 50 µg/mL BSA, pH 7.9), and 1× SYBR Green I. The temperature was increased from 55 to 80 ℃ at 0.2 ℃/10 s. The first negative derivative plot [-d(RFU)/dT] was used to determine the melting temperature.

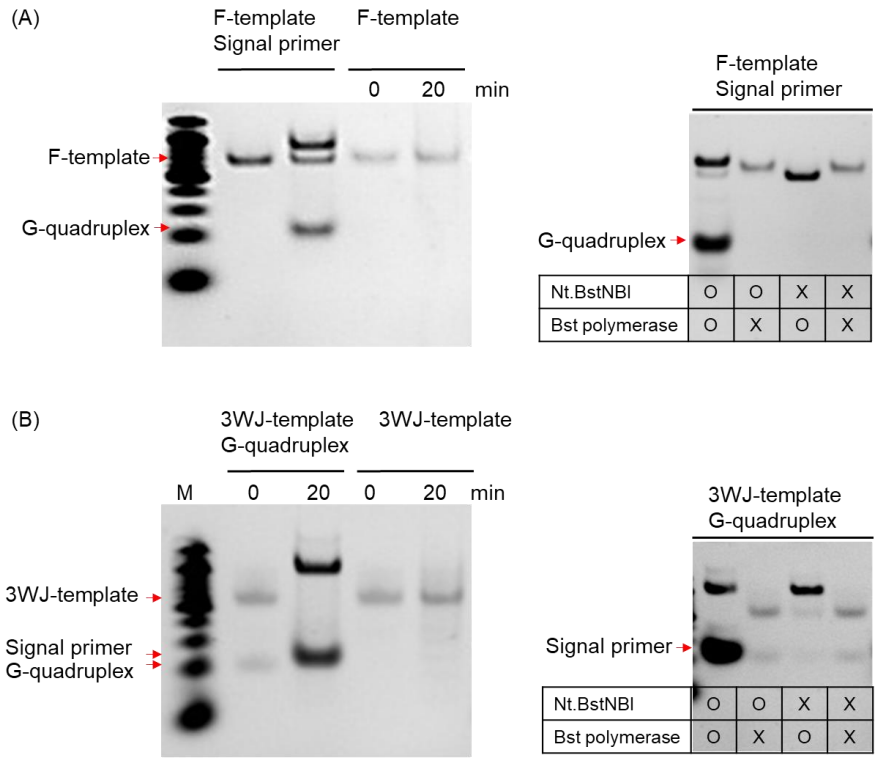

Figure S2. (A) Polyacrylamide gel analysis of the Cycle 2 reaction. The F-template was mixed with or without the signal primer, and the reaction was conducted with Bst DNA polymerase and Nt.BstNBI nicking endonuclease for 0 and 20 min (left). The F-template was mixed with the signal primer, and the reaction was conducted with different combinations of Bst DNA polymerase and Nt.BstNBI nicking endonuclease for 20 min (right). (B) Polyacrylamide gel analysis of the Cycle 3 reaction. The 3WJ-template was mixed with or without a G-quadruplex, and the reaction was conducted with Bst DNA polymerase and Nt.BstNBI nicking endonuclease for 0 and 20 min (left). The 3WJ-template was mixed with G-quadruplex, and the reaction was conducted with different combinations of Bst DNA polymerase and Nt.BstNBI nicking endonuclease for 20 min (right). Lane M is a 10- to 150-bp oligonucleotide marker. [F-template] = 100 nM, [Signal primer] = 50 nM, [3WJ-template] = 50 nM, [3WJ-primer] = 50 nM, [G-quadruplex] = 50 nM, [Bst DNA polymerase] = 1.6 U, and [Nt.BstNBI nicking endonuclease] = 2 U.

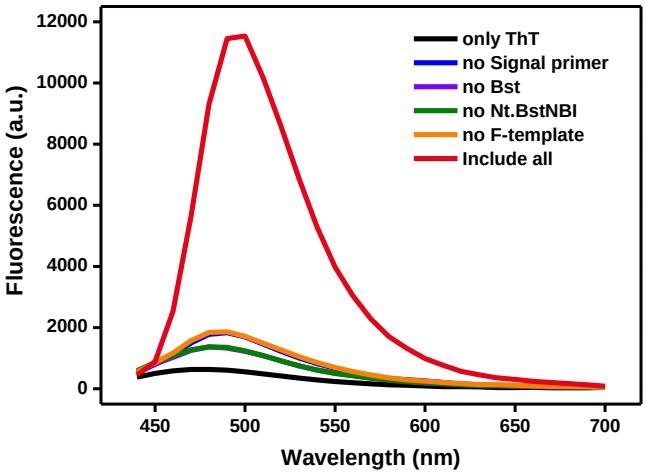

Figure S3. Fluorescence signals of ThT/G-quadruplex after Cycle 2 reaction. [F-template] = 100 nM, [Signal primer] = 50 nM, [Bst DNA polymerase] = 1 U, [Nt.BstNBI nicking endonuclease] = 1.6 U, [Thioflavin T] = 25 μM.

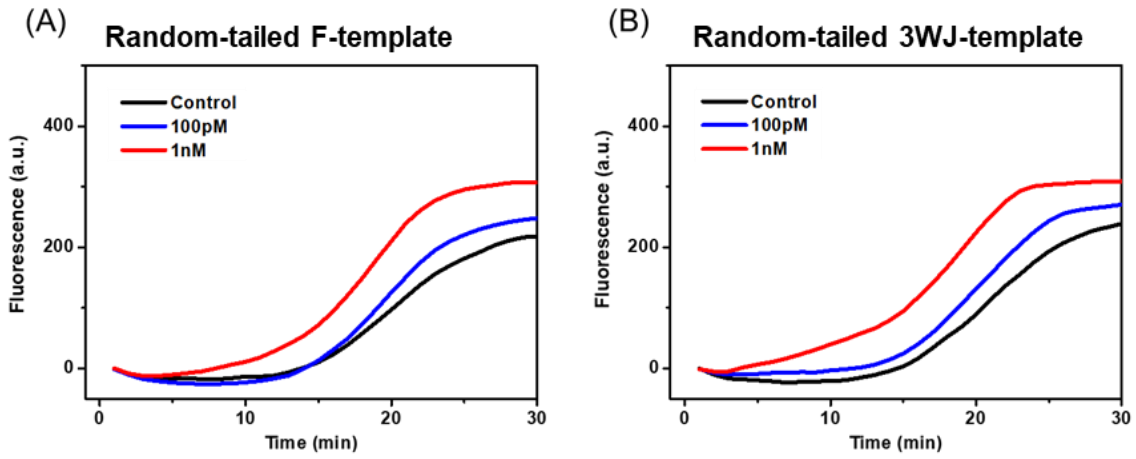

Figure S4. (A) Real-time fluorescence curves obtained during Cycle 2 and 3 reactions with 3WJ-template, random-tailed F-template, and signal primer (0, 100 pM, and 1 nM). In the presence of the random-tailed F-template and normal 3WJ-template, a random-tailed G-quadruplex was produced by the Cycle 2 reaction; however, this random-tailed G-quadruplex could not combine with the 3WJ-template. As a consequence, exponential signal enhancement was not observed. (B) Real-time fluorescence signals obtained during Cycle 2 and 3 reactions with random-tailed 3WJ-template, F-template, and signal primer (0, 100 pM, and 1 nM). In the presence of the normal F-template and random-tailed 3WJ-template, a normal G-quadruplex sequence was generated by Cycle 2, but it could not bind to the random-tailed 3WJ-template, resulting in less fluorescence signal enhancement.

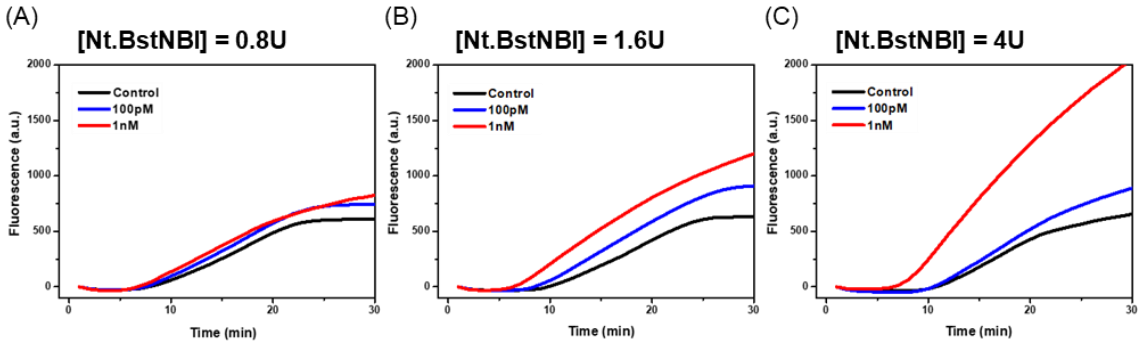

Figure S5. Optimization of Nt.BstNBI nicking endonuclease concentration. Real-time fluorescence curves during the detection of PPP1R1B RNA (0, 100 pM, and 1 nM) at the different concentrations of Nt.BstNBI (0.8, 1.6, and 4 U). When the amount of Nt.BstNBI was 1.6 U, the maximum difference in Cq value was found among the control and the samples. [Bst DNA polymerase] = 1 U, [3WJ-template] = 5 nM, [3WJ-primer] = 5 nM, and [F-template] = 50 nM.

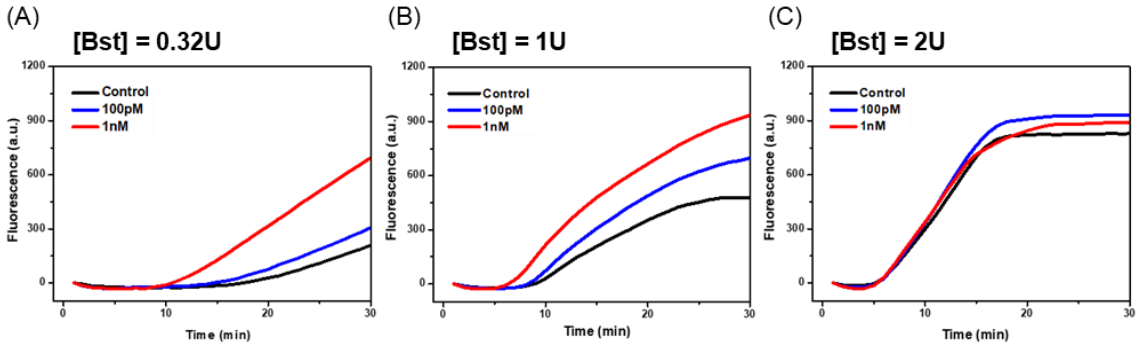

Figure S6. Optimization of Bst DNA polymerase concentration. Real-time fluorescence curves during the detection of PPP1R1B RNA (0, 100 pM, and 1 nM) at the different concentrations of Bst DNA polymerase (0.32, 1, and 2 U). When the amount of Bst polymerase was 0.32 U, the extension rate slowed down and the sensitivity was low. The fluorescence signals of the control and the samples were undistinguishable at 2 U of Bst polymerase condition. [Nt.BstNBI nicking endonuclease] = 1.6 U, [3WJ-template] = 5 nM, [3WJ-primer] = 5 nM, and [F-template] = 50 nM.

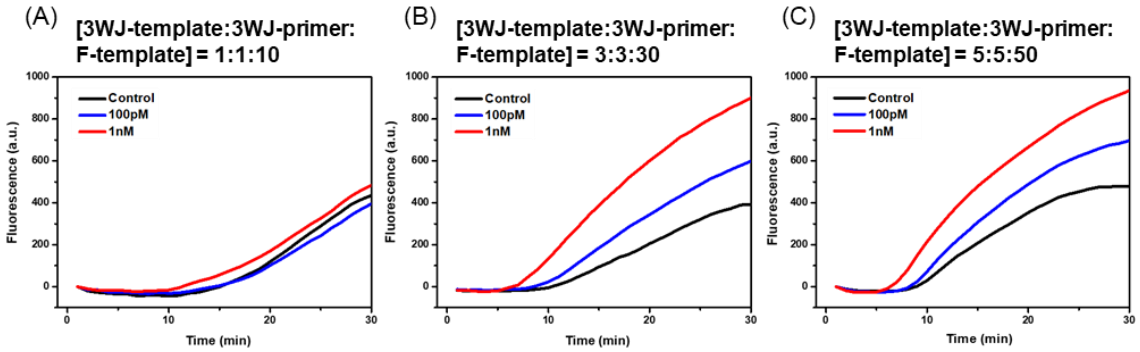

Figure S7. Optimization of 3WJ probes and F-template concentration. Real-time fluorescence curves during the detection of PPP1R1B RNA (0, 100 pM, and 1 nM) at different concentrations of 3WJ-template:3WJ-primer:F-template (1:1:10, 3:3:30, and 5:5:50 nM). [Nt.BstNBI nicking endonuclease] = 1.6 U, [Bst DNA polymerase] = 1 U.

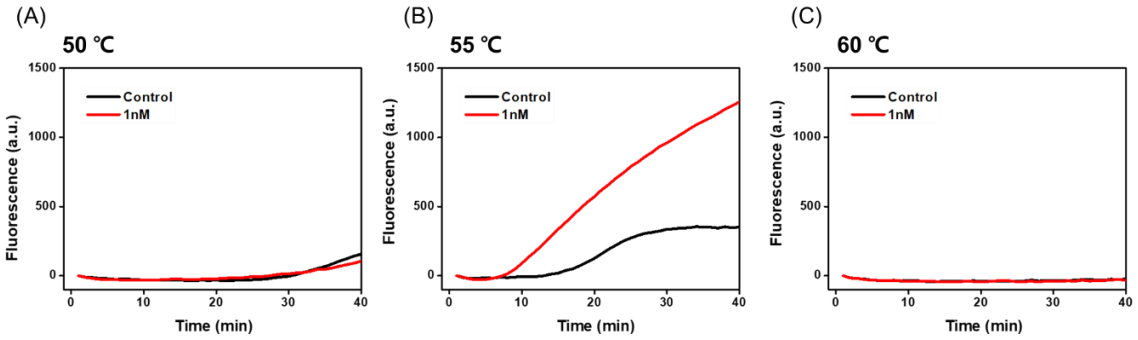

Figure S8. Optimization of reaction temperature. Real-time fluorescence curves during the detection of PPP1R1B RNA (0 and 1 nM) at different temperature conditions (50, 55, and 60 ℃). Reaction stability was sensitive to temperature condition due to the use of enzymes. [Nt.BstNBI nicking endonuclease] = 1.6 U, [Bst DNA polymerase] = 1 U, [3WJ-template] = 3 nM, [3WJ-primer] = 3 nM, and [F-template] = 30 nM.

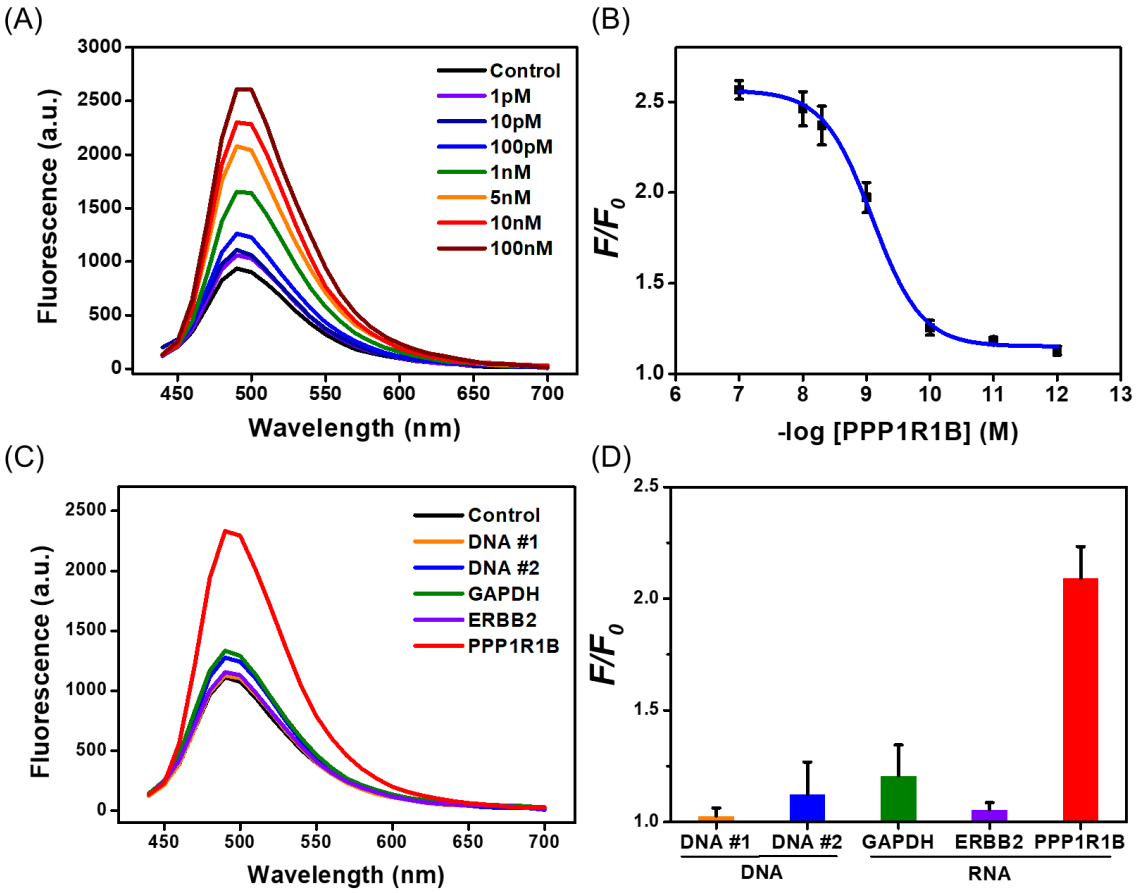

Figure S9. (A) End-point fluorescence spectra and (B) relative fluorescence intensity (F/F_0) after the detection of PPP1R1B RNA (0, 1, 10, 100 pM, 1, 5, 10, 100 nM). F and F_0 represent the fluorescence intensities at 490 nm in the presence and absence of PPP1R1B RNA, respectively. (C) End-point fluorescence spectra and (D) relative fluorescence intensity (F/F_0) after the detection of non-target ssDNAs, and GAPDH, ERBB2, and PPP1R1B RNAs. F and F_0 represent the fluorescence intensities at 490 nm in the presence and absence of nucleic acids, respectively. The concentrations of all nucleic acids were 1 nM. Error bars were estimated from triplicate tests.

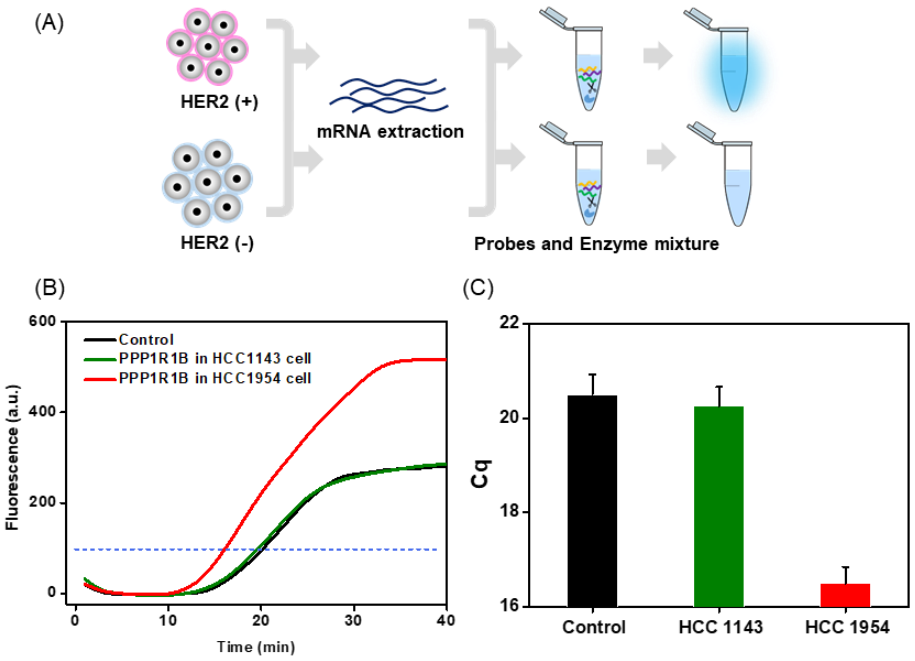

Figure S10. (A) Schematic illustration of PPP1R1B mRNA detection in HCC1143 (HER2 (-)) and HCC1954 (HER2 (+)) cells. (B) Real-time fluorescence curves and (C) corresponding Cq values during the detection of PPP1R1B RNA from the cell extract samples of HCC1954 and HCC1143. Error bars were estimated from triplicate tests.

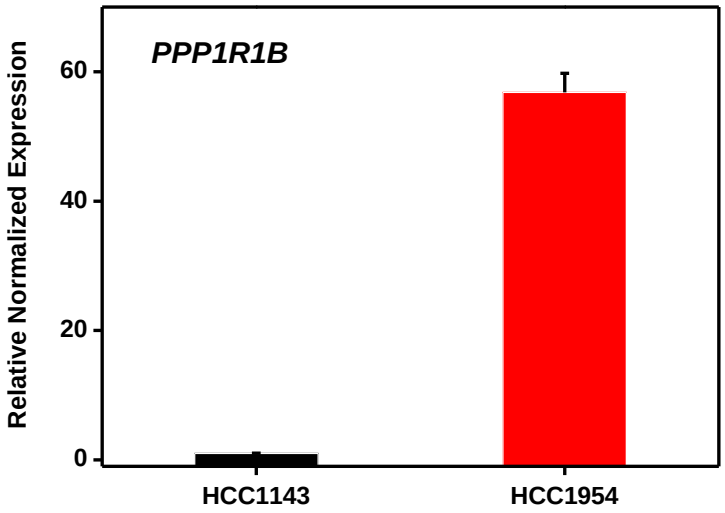

Figure S11. qRT-PCR results for the detection of PPP1R1B RNA from cell extract samples of HCC1954 and HCC1143.

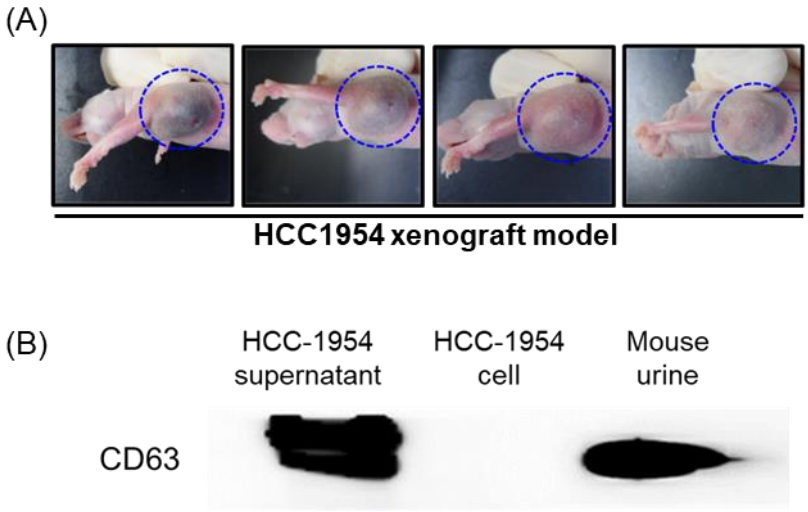

Figure S12. (A) Images of breast cancer xenograft mouse models injected with HCC1954 cells. (B) Detection of CD63 in HCC-1954 supernatant (PC), HCC-1954 cell (NC), and mouse urine. Exosomal marker protein CD63 was mainly detected in HCC-1954 supernatant and mouse urine samples and diminished in HCC-1954 cell.

Table S1. Oligonucleotide sequences

Name	Sequence (5'→3')
Target RNA (PPP1R1B)	UGU GCC UAC ACA CCA CCU UCG CUG AAA GCU GUG CAG CGC AUU GCU GAG UCU C
Non-target RNA (ERBB2)	GUC CAG CCU CGU UGG AAG AGG AAC AGC ACU GGG GAG UCU UUG UGG AUU CUG A
Non-target RNA (GAPDH)	GCU CUC UGC UCC UCC UGU UCG ACA GUC AGC CGC AUC UUC UUU UGC GUC GCC A
Non-target DNA #1	GGT AGG TGC TTG TAC GGA TGC CTG TGT TGG TAC ATC CGT ACA AGC ACC TAC CAA CA
Non-target DNA #2	TCT TCC TGC TTG GTG AAG ATG AAG GGG CTC TTA TCT TCA CCA AGC AGG AAG AGC CC
3WJ-template	AAC TAT ACA ACC TAC TAC CTC A*TC CAG ACT C*TT TTT T**AT TCG AA**A GCG AAG GTG GTG T***GT AGG TTT TT*** - p
3WJ-primer	GCA ATG CGC TGC ACA GCT TTC **TTC GAA T**
Signal primer	TGA GGT AGT AGG TTG TAT AGT T
F-template	GTA GGT TTT TTC CCT CCC TCC CTC CCA G*GG ACG ACT C*TA ACT ATA CAA CCT ACT ACC TCA A – A
G-quadruplex	CTG GGA GGG AGG GAG GGA ***AAA AAC CTA C***
Random-tailed 3WJ template	AAC TAT ACA ACC TAC TAC CTC A*TC CAG ACT C*TT TTT T**AT TCG AAA** GCG AAG GTG GTG TGT AGG NNN NN - p
Random-tailed F- template	NNN NNN NNN NTC CCT CCC TCC CTC CCA G*GG ACG ACT C*TA ACT ATA CAA CCT ACT ACC TCA A - A

*p indicates 3' phosphate modification; A indicates 3' amino(C6) modification.
*Underline indicates signal primer sequences.
*Bold characters indicate hybridization sequences between 3WJ probes.
*Bold and italic characters indicate hybridization sequences between G-quadruplex and 3WJ-Template.
*Italic characters indicate the recognition sequence of Nt.BstNBI.

Table S2. Comparison of different isothermal signal amplification methods.

Methods	Signal	No. of steps	Analysis time (h)	Detection limit	Ref.
Cleavage-mediated IEXPAR	Fluorescence	2	~2	10 fM	(Wang et al. 2017)
PG-RCA	Fluorescence	2	4-5	~15 fM	(Murakami et al. 2012)
Cleavage-based RNA amplification	Colorimetric	3	3-4	10 fM	(Zhao et al. 2013)
PMB-based ISDA	Fluorescence	3	6	10 pM	(Li et al., 2019)
HTDC assay	Fluorescence	2	4	1 pM	(Wang et al., 2018)
Dual-GDP based active signal amplification	Colorimetric	8	7-8	3.2 pM	(Li et al, 2020)
3WJ-based gene amplification	Fluorescence	1	~ 0.5	1.23 pM	This work

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