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Supporting Information for

Primer exchange reaction-coupled transcription isothermal amplification as a sensitive biomolecular assay

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Materials and methods

Materials

All oligonucleotides (**Table S1**) were purchased from Bionics (Seoul, Republic of Korea), except the cholesterol-modified oligonucleotides (T7 promoter and hairpin), which were synthesized by Bioneer (Daejeon, Republic of Korea) and Integrated DNA Technologies (Coralville, IA, USA), respectively. T7 RNA polymerase (T7 RNAP) and RNase inhibitors were purchased from Enzynomics (Daejeon, Republic of Korea). In addition, dATP, dTTP, dCTP, rNTP, *Bst* 2.0 DNA polymerase (*Bst* 2.0 DNAP), and 10X RNAPol Reaction Buffer were purchased from New England Biolabs (Ipswich, MA, USA). TO1-3PEG-Biotin Fluorophore (TO1-biotin) was purchased from Applied Biological Materials (Richmond, BC, Canada). Pierce™ protein-free blocking buffer and 5-dodecanoylaminofluorescein (DAF) were obtained from Thermo Fisher scientific (Waltham, MA, USA).

Native polyacrylamide gel electrophoresis

To confirm that the short promoter (7-mer) was elongated into a long promoter (15-mer) during the PER step, gel electrophoresis was performed using a 15% polyacrylamide gel. Specifically, the sample solution (35 μL) was prepared with 1X RNAPol reaction buffer, 400 nM short promoter, 200 nM hairpin, 40 μM dNTP (N= A, T, C), 0.11 units/μL *Bst* 2.0 DNAP, and Diethyl pyrocarbonate (DEPC) -treated water. After incubation at 37 °C for 3 h, the samples were heated to 80 °C for 20 min to inactivate the *Bst* 2.0 DNAP, which was then loaded onto a 15% polyacrylamide gel and run in 1X TBE buffer at 140 V for 50 min. After staining the gel with GreenStar Nucleic Acid Staining Solution (Bioneer, Daejeon, Republic of Korea), gel images were acquired using a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA).

Transcription isothermal amplification

For transcription isothermal amplification, a sample solution (40 μL) was prepared with 1X RNAPol reaction buffer, 10 nM Mango template, 100 pM long promoter (15-mer), 400 nM To1-biotin, 0.5 mM rNTP, 0.8 units/μL RNase inhibitor, 1 unit/μL T7 RNAP, and DEPC-treated water. After incubation at 37 ℃ for 30 min, the samples were transferred to 384-black-well plates (SPL Life Sciences, Pocheon, Republic of Korea), and fluorescence signals were

measured at excitation and emission wavelengths of 507 and 547 nm,[1] respectively, using a microplate reader (SpectraMax iD5 Multi-Mode Microplate Reader; Molecular Devices, San Jose, CA, USA).

PER-Trap system

First, a sample solution (35 μL) for the PER step was prepared with 1X RNAPol reaction buffer, 400 nM short promoter, various concentrations of hairpin, 100 nM cleaner G, 40 μM dNTP (N= A, T, C), 0.85 units/μL *Bst* 2.0 DNAP, and DEPC-treated water. Cleaner G, containing a template of C base that its 3′ end could extend, was used to clean up a small amount of dGTP contamination in the sample solution before PER.[2,3] After incubation at 37 ℃ for 3 h, the samples were heated at 80 ℃ for 20 min to inactivate the *Bst* 2.0 DNAP. Next, the sample solution of transcription isothermal amplification (5 μL) consisting of 1X RNAPol reaction buffer, 80 nM Mango template, 3.2 μM To1-biotin, 4 mM rNTP, 6.4 units/μL RNase inhibitor, 8 units/μL T7 RNAP, and DEPC-treated water was applied to the sample solution (35 μL) for PER step, which was then incubated at 37 ℃ for 30 min. Finally, the samples were transferred to a 384-black-well plate, and fluorescence signals were measured using a microplate reader with excitation and emission wavelengths of 507 and 547 nm, respectively.

Cell culture

SW620 cells (human colorectal cancer) were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) and maintained at a low passage number (< 6^{th}). The SW620 cells were cultured in Dulbecco's modified Eagle's medium (Welgene, Gyeongsan, Republic of Korea) supplemented with 10% v/v fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and 1% v/v penicillin-streptomycin (Welgene). A humidified atmosphere with 5% CO_2 at 37 °C was maintained during cell culture.

Exosome isolation

After culturing to 80% confluence, the cells were washed with Dulbecco's phosphate-buffered saline. Cells were incubated in conditioned medium containing 5% v/v exosome-depleted FBS (Gibco; Thermo Fisher Scientific) and 1% v/v penicillin-streptomycin in an incubator with a humidified atmosphere (5% CO_2 at 37 °C) for an additional 48 h. To remove dead cells, cellular debris, microvesicles, and apoptotic bodies, the conditioned media were collected

and centrifuged at 300 × g for 5 min, and the supernatant was used in the same way at 2,000 × g for 20 min and 10,000 × g for 30 min to obtain the supernatant. All centrifugations were performed at 4 °C for exosome stability. The obtained supernatants were then filtered using 0.45 mm and 0.2 mm pore-size syringe filters (Sartorius, Göttingen, Germany) to remove particles larger than 200 nm. The obtained filtrate was then filtered using a Tangential Flow Filtration system with a 300-K membrane (TFF; Pall Corporation, New York, NY, USA) for purification and concentration. Finally, the concentrated medium was isolated via size exclusion chromatography (SEC) using a qEV10 nm column in a qEV automated fraction collector (Izon Science, Christchurch, New Zealand). The fraction containing exosomes from SEC was stored at -80 °C until use.

Nanoparticle Tracking Analysis (NTA)

The concentration and size distribution of the SW620 exosomes were measured using ZetaView (PMX130, Germany). Samples were diluted in 1 × PBS (0.22-filtered) for optimal measurements, and each diluted sample was analyzed three times using the same settings for detection. Prior to the sample readings, PS100 nanoparticles (Applied Microspheres B. V., Netherlands) with a known average size of 100 nm were used to calibrate the instrument. Cell quality checks, instrument auto-alignment, and focusing were performed prior to the use of ZetaView for sample measurements. The samples were diluted in 1 × PBS to a final volume of 1 mL. For each measurement, one cycle was performed by scanning 11 cell positions and capturing 60 frames per position under the following settings: focus: autofocus; camera sensitivity for all samples: 80; shutter: 100; scattering intensity: 6.0 (detected automatically); cell temperature: 23 °C. After capturing, the videos were analyzed using the built-in ZetaView Software 8.05.16 SP3 with the following specific analysis parameters: maximum area: 1000; minimum area: 10; minimum brightness: 30; maximum brightness: 255. Hardware: embedded laser: > 30 mW at 488 nm; camera: SCMOS; microscope magnification: x10.

Western blot analysis

SW620 exosomes were concentrated using an Amicon Ultra 0.5 device with a 30 K filter (Merck Millipore, Burlington, MA, USA) following the manufacturer's instructions. The amount of protein in the sample was quantified using a TaKaRa BCA protein assay kit (TaKaRa, Japan) according to the manufacturer's instructions. We added 2.5

μL of 5x sodium dodecyl-sulfate polyacrylamide gel electrophoresis loading buffer (Biosesang, Seongnam, Republic of Korea) to 50 μg/10 μL of sample to make it suitable for gel electrophoresis. Then, it was heated for 10 min 95 °C and resolved in 10% TGX stain-free protein gel (Bio-Rad Laboratories, Hercules, CA, USA) using a Mini-Protean tetra system (Bio-Rad Laboratories) for 20 min at 300 V. According to the manufacturer's instructions, we transferred the resolved proteins to 0.2 μm polyvinylidene fluoride membranes (Bio-Rad Laboratories) in a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). For 1 h, membranes were blocked with 5% bovine serum albumin in TBS-T (0.1%; 1× TBS with 0.1% Tween-20) and then incubated with primary antibodies [mouse anti-CD63 (Santa Cruz, Dallas, TX, USA), rabbit anti-CD9, and rabbit anti-calnexin (ABclonal, Woburn, MA, USA), 1:1000] diluted in Can Get Signal solution 1 (Toyobo, New York, USA) for 1h at room temperature. The membranes were then washed three times with TBS-T (0.1%). They were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies [goat anti-mouse IgG (BioLegend, San Diego, CA, USA), 1:5,000, and goat anti-rabbit IgG (ABclonal, Woburn, MA, USA), 1:10,000] diluted in Can Get Signal solution 2 (Toyobo) at room temperature for 1 h, followed by washing with TBS-T (0.1%) three times. Proteins in the membrane were visualized using the Clarity Western ECL substrate (Bio-Rad Laboratories) on a ChemiDoc Imaging System (Bio-Rad Laboratories).

TEM analysis

TEM was used to confirm the morphology and size of SW620 exosomes. Negative staining was performed via glow discharge for 10 s using a glow discharge coater (SMC12R-Plus; Semian, Daejeon, Republic of Korea) to render the carbon-formvar film-coated grid hydrophilic. A drop of exosome sample, which was previously concentrated to a protein concentration of 1000 μg/mL using Amicon 30-k filter, was applied, and the remaining solution was removed using filter paper. Then, a drop of 1% uranyl acetate was added, and the remaining solution was removed using a filter paper. The grid was covered with a culture dish and dried at room temperature for 10 min. TEM images were obtained using an HT7800 instrument (Hitachi, Japan) at an accelerating voltage of 100 kV.

Exosome detection with the PER-Trap system

Various concentrations of SW620 exosomes were added to 100 μL of Maxi-binding immunoplate (SPL Life Sciences, Pocheon, Republic of Korea) and incubated overnight at 4 ℃. After exosome incubation, the plate was washed three times with washing buffer (1X PBS containing 0.1% Tween 20), and then 300 μL of Pierce™ protein-free blocking buffer was added to prevent non-specific binding and incubated at 37 ℃ for 1 h. After blocking, the plate was washed three times with washing buffer, followed by 100 μL of cholesterol-modified hairpin 100 nM stock and incubated at 37 °C for 1 h. After washing the plate three times with washing buffer, the sample solution for the PER step [70 μL, 1X RNAPol reaction buffer, 400 nM short promoter, 100 nM cleaner G, 40 μM dNTP (N= A, T, C), and 0.85 units/μL *Bst* 2.0 DNAP] was added, which was incubated at 37 ℃ for 3 h. Then, 35 μL was transferred to a tube and incubated at 80 ℃ for 20 min to inactivate *Bst* 2.0 DNAP, where the sample solution (5 μL, 1X RNAPol reaction buffer, 80 nM Mango template, 3.2 μM To1-biotin, 4 mM rNTP, 6.4 units/μL RNase inhibitor, and 8 units/μL T7 RNAP) was added for the second transcription isothermal amplification. After incubation at 37 ℃ for 30 min, the samples were transferred to a 384-black-well plate, and fluorescence signals were measured using a microplate reader with excitation and emission wavelengths of 507 and 547 nm, respectively.

Exosome detection with transcription only

The exosome seeding, blocking, and washing steps were the same as those in the above PER-Trap system but with black maxi-binding immunoplates (SPL Life Sciences, Pocheon, Republic of Korea). After blocking, the plate was washed three times with washing buffer, followed by 100 μL of cholesterol-modified T7 Promoter 100 nM stock, and incubated at 37 °C for 1 h. The incubated plate was washed three times with washing buffer, followed by transcription reaction solution (40 μL, 1X RNAPol reaction buffer, 400 nM To1-biotin, 0.5 mM rNTP, 0.8 units/μL RNase inhibitor, and 1 unit/μL T7 RNAP) and incubated at 37 ℃ for 30 min. After incubation, the plate was read using a microplate reader to obtain fluorescence signals at excitation and emission wavelengths of 507 and 547 nm, respectively.

Exosome detection with DAF staining

The exosome seeding, blocking, and washing steps were the same as those in the above PER-Trap system but with black maxi-binding immunoplates (SPL Life Sciences, Pocheon, Republic of Korea). Then, 100 μL of DAF 10 μM stock was added to the plate and incubated at 37 ℃ for 30 min. After incubation, the plate was washed three times with washing buffer, 100 μL of PBS was added, and the fluorescence signal was obtained by setting the excitation and emission wavelengths to 485 and 525 nm, respectively, using a microplate reader.

Table S1. DNA oligonucleotides used in this work.

Probe name	Sequence (5' to 3')	Modification
Cleaner G	CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCC ACTTTCG	
Short promoter (7-mer)	TAATACG	
Hairpin	ACTCACTAGGGCCTTTTGGCCCTAGTGAGTCGTATT AAAAAAAA	
Mango template	TAATACGACTCACTAGTACGACAACTACCCCATACC AAACCTTCCTTCGTACCCCTATAGTGAGTCGTATTA	
Long promoter (15-mer)	TAATACGACTCACTA	
Cholesterol hairpin	ACTCACTAGGGCCTTTTGGCCCTAGTGAGTCGTATT A AAAAAAAAAAAAAAA-3' Cholesterol	3' Cholesterol
Cholesterol promoter	5' Cholesterol-AAAAAAAAAAAAAAATAATACGACTC ACTA	5' Cholesterol

*The underline represents the stem of the hairpin

Table S2. Comparisons with various reported strategies for exosome detection.

Detection method	Limit of Detection (particles/μL)	Reference
Fluorescence (G-quadruplex)	3.4×10^5	[4]
Fluorescence (Copper oxide nanoparticles)	4.8×10^4	[5]
Electrochemistry (Paper-based device)	7.1×10^5	[6]
Electrochemistry (Gold nanoparticles)	4.5×10^6	[7]
Fluorescence (Light-up RNA aptamer)	1.0×10^4	This work

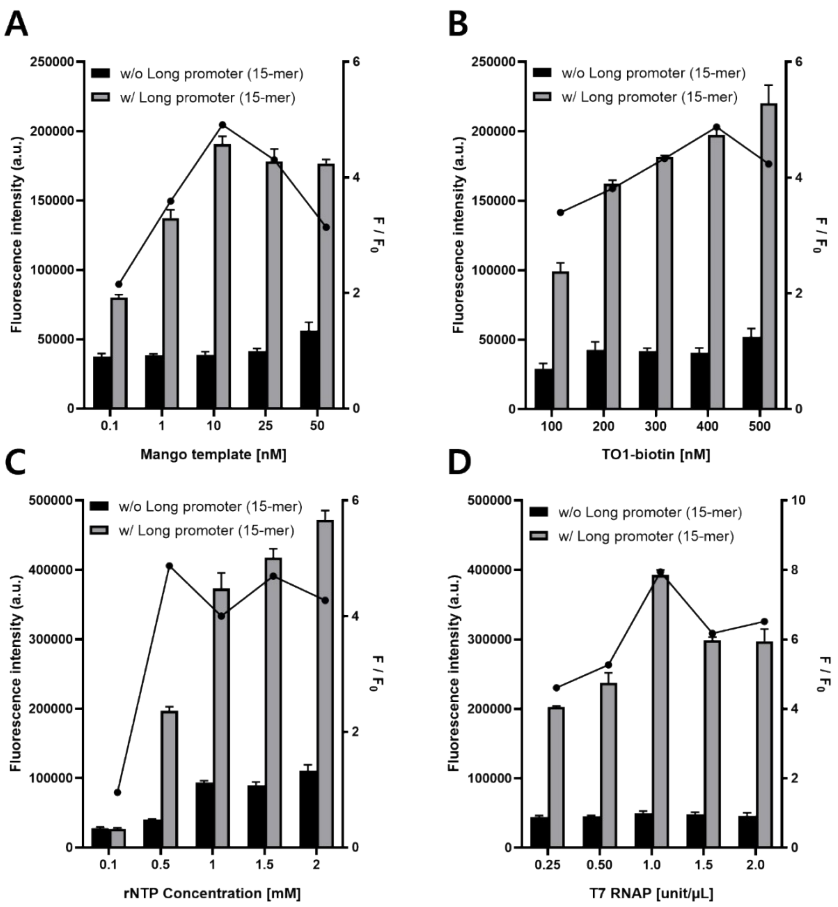

Figure S1. Optimization of transcription isothermal amplification. **(A)** Mango template concentration. **(B)** TO1-biotin concentration. **(C)** RNTP concentration. **(D)** T7 RNAP concentration. The conditions for all samples are the same except for the factor optimization. The concentration of T7 RNAP was 0.25 units/μL in (A), (B), and (C). Black and grey bars show the fluorescence intensities in the absence (F_0) and presence (F) of the long promoter (15-mer) (100 pM), respectively, and the line shows the signal-to-noise ratio (F/F_0). Error bars represent the standard deviation from three independent experiments.

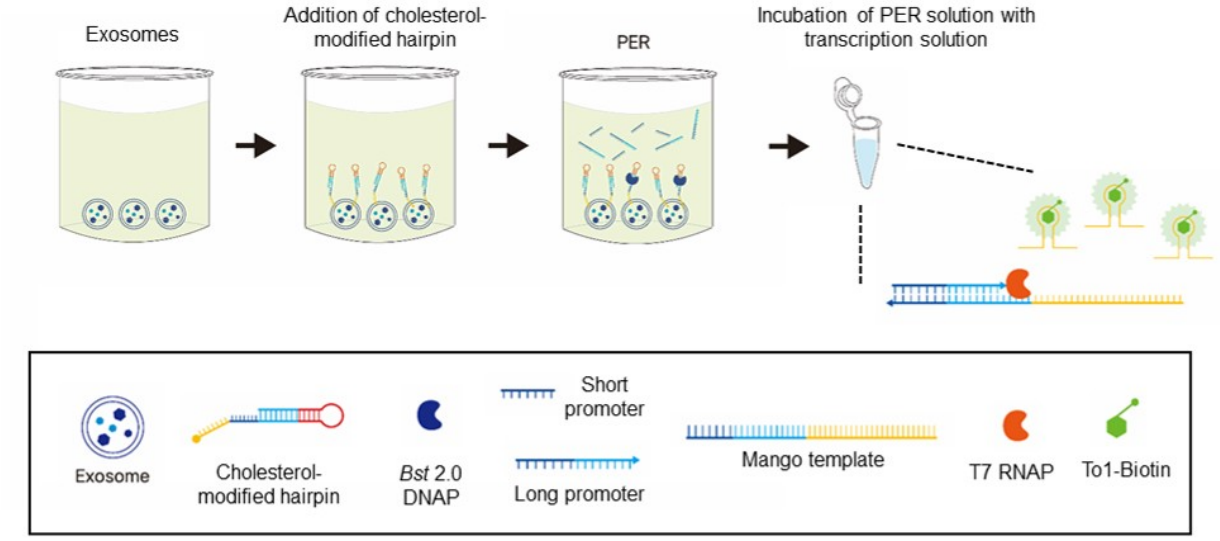

Figure S2. Schematic illustration showing exosome detection with PER-Trap.

Reference

1 T. Yoon, J. Shin, H. J. Choi and K. S. Park, *Biosens. Bioelectron.*, 2022, **208**, 114221.

2 X. Li, X. Li, X. Cheng, X. Bian, B. Shen, X. Ding and S. Ding, *ACS Sens.*, 2022, **7**, 3571–3579.

3 J. Y. Kishi, T. E. Schaus, N. Gopalkrishnan, F. Xuan and P. Yin, *Nat. Chem.*, 2018, **10**, 155-164.

4 Chen, J., Meng, H. M., An, Y., Geng, X., Zhao, K., Qu, L., & Li, Z., *Talanta*, 2020, **209**, 120510.

5 He, F., Wang, J., Yin, B. C., & Ye, B. C., *Anal. Chem.*, 2018, **90**, 8072-8079.

6 Kasetsirikul, S., Tran, K. T., Clack, K., Soda, N., Shiddiky, M. J., & Nguyen, N. T. *Analyst*, 2022, **147**, 3732-3740.

7 Oliveira-Rodríguez, M., Serrano-Pertierra, E., García, A. C., López-Martín, S., Yañez-Mo, M., Cernuda-Morollón, E., & Blanco-López, M., *Biosens. Bioelectron.*, 2017, **87**, 38-45.