Supporting Information for

Ultrasensitive multiplexed miRNA detection based on self-priming hairpin-triggered isothermal cascade reaction

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Contents

1. Materials and methods

1.1. Materials

Oligonucleotides used in the present study were synthesized and purified with polyacrylamide gel electrophoresis (PAGE) by Bioneer® (Daejeon, Korea). The sequences of the oligonucleotides are listed in Table S1. Klenow fragment (3'-5' exo-) DNA polymerase (KF), Nt.AlwI nicking endonuclease (NE), 10X NEBuffer™ 2 (100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 100 mM $MgCl_2$, and 10 mM DTT), and 10X CutSmart® buffer (200 mM Tris-Ac, pH 7.9, 500 mM KAc, 100 mM $Mg(Ac)_2$, and 1 mg/ml BSA) were purchased from New England Biolabs Inc. (Beverly, MA, USA). GO was purchased from Graphene Supermarket (USA). All other chemicals were of analytical grade and used without further purification.

1.2. Procedures for the multiplex miRNA detection

Prior to the assay, 1 μM of different types of SP-HPs in 0.5X NEBuffer™ 2 and 0.5X CutSmart® buffer were heated at 95 ˚C for 5 min and slowly cooled to 25 ˚C (0.1 ˚C/s) to separately generate the stable hairpin structure. The assay solution (36 μL) containing 500 nM of a set of SP-HPs, 300 nM of a set of TATs, 250 μM dNTPs, 1 U/μL KF, 0.375 U/μL NE, 0.5X NEBuffer™ 2, 0.5X CutSmart® buffer, and target miRNAs at varying concentrations was prepared and incubated at 37 °C for 30 min, followed by the addition of 4 μL of GO (20 mg/mL). After the incubation at 25 °C for 5 min, the fluorescence emission spectra for a set of

fluorophores were measured by using Tecan Infinite M200 pro-microplate reader (Mnnedorf, Switzerland): i) 500–600 nm at an excitation wavelength of 470 nm for FAM, ii) 530–650 nm at an excitation wavelength of 490 nm for HEX, and iii) 650–750 nm at an excitation wavelength of 600 nm for Cy5.

1.3. Gel electrophoresis

For PAGE, a 10 μL aliquot of the reaction solution was resolved on 15% polyacrylamide gel in 1X TBE buffer at 120 V for 120 min. After GelRed (Biotium, CA, USA) staining, the gel was scanned using UV transilluminator (Bio-rad, CA, USA).

1.4. Detection of target miRNA in human serum

Target miRNAs at varying concentrations were spiked in diluted human serum (10%). Each sample was then analyzed by the same reaction procedure as described above.

Name	Sequence (5' → 3')[a]
miRNA let-7a	UGA GGU AGU AGG UUG UAU AGU U
miRNA let-7b[b]	UGA GGU AGU AGG UUG UGU GGU U
miRNA let-7c[b]	UGA GGU AGU AGG UUG UAU GGU U
miRNA let-7d[b]	AGA GGU AGU AGG UUG CAU AGU U
miRNA let-7e[b]	UGA GGU AGG AGG UUG UAU AGU U
miRNA-141	UAA CAC UGU CUG GUA AAG AUG G
miRNA-98	UGA GGU AGU AAG UUG UAU UGU U
SHP-7a [c,d]	FAM-CCG ACC TTC CAC CGA GCT AGA TCC CTG GAC GAC TTG AAA **AAC TAT ACA ACC TAC TAC CTC A** *TTT CAA GTC GTC CAG TTG AAA*
TTP-7a	FAM- AAC TAT ACA ACC TAC TAC CTC AGC TAG ATC CCC GAC CTT CCA CCG A
SHP-141[c,d]	HEX-GCG TTC ATG CGG CGT GCT AGA TCC CTG GAC GAC TTG AAA **CCA TCT TTA CCA GAC AGT GTT A** *TTT CAA GTC GTC CAG TTG AAA*
TTP-141	HEX- CCA TCT TTA CCA GAC AGT GTT AGC TAG ATC CGC GTT CAT GCG GCG T
SHP-98 [c,d]	Cy5-GGC GAA ACC GCT GGA GCT AGA TCC CTG GAC GAC TTG AAA **AAC AAT ACA ACT TAC TAC CTC A** *TTT CAA GTC GTC CAG TTG AAA*
TTP-98	Cy5- AAC AAT ACA ACT TAC TAC CTC AGC TAG ATC CGG CGA AAC CGC TGG A

[a] The colors of oligonucleotide sequences correspond to those of the domains depicted in Scheme 1.

[b] Underlined letters indicate the mismatched bases against miRNA let-7a.

[c] Italic letters in SHPs represent the self-priming domain.

[d] Bold letters in SHPs represent the target recognition domain.

Table S1. Oligonucleotide sequences used in this work.

Table S2. Comparison of this method with previous isothermal amplification methods for miRNA detection.

Method	LOD	Linear range	Detection time (hr)	Limittpions	Refer ence
Strand displacement amplification (SDA) - rolling circle amplification (RCA)	150 fM	0.1 pM – 1 nM	1	- Lots of enzymes - Multiple primer sets - Long analysis time - Singleplex	[S1]
Branched rolling circle amplification (BRCA)	25 fM	50 fM – 500 fM	2.6	- Additional ligation step for RCA padlock probe - Low sensitivity - Singleplex	[S2]
Combining hairpin (HP) assisted cascade isothermal amplification	0.7 pM	1 pM – 20 pM	32.5	- Additional step for preparation of DNA-silver nanoclusters - Complicated primer design - Singleplex	[S3]
Helicase-dependent amplification (HAD)	12.8 fM	100 fM – 10 nM	0.5	- Lots of enzymes - Multiple primer sets - Singleplex	[S4]
Toehold-mediated strand displacement reaction (TSDR)	3.2 pM	10 pM – 10 nM	3	- Long analysis time - Low sensitivity - Singleplex	[S5]
self-priming hairpin-triggered cascade reaction	42.63 aM for miRNA let-7a, 13.08 aM for miRNA-141, and 10.14 aM for miRNA-98	100 aM – 1 nM	0.5	–	This work

2. Optimization of self-priming hairpin-triggered cascade reaction in the multiplex detection system

We next optimized key reaction conditions to maximize the efficacy of self-priming hairpin-triggered cascade reaction by comparing fluorescence signal-to-background ratios (F/F_0) under different reaction conditions. Considering that Trigger should stably bind to TTP for efficient self-priming hairpin- triggered cascade reaction, its length was first optimized with target miRNA let-7a and 15-mer of Trigger-7a was the most efficient (Figure S2). Next, the concentrations of SHP per each, TTP per each, KF, NE, and GO, and the reaction time were optimized with miRNA let-7a. The results showed that 500 nM of each SHP, 300 nM of each TTP, 1 U/μL KF, 0.375 U/μL NE, 2 mg/mL GO, and 30 min were optimal, providing the highest F/F_0, which were employed for further experiments (Figures S3-S6).

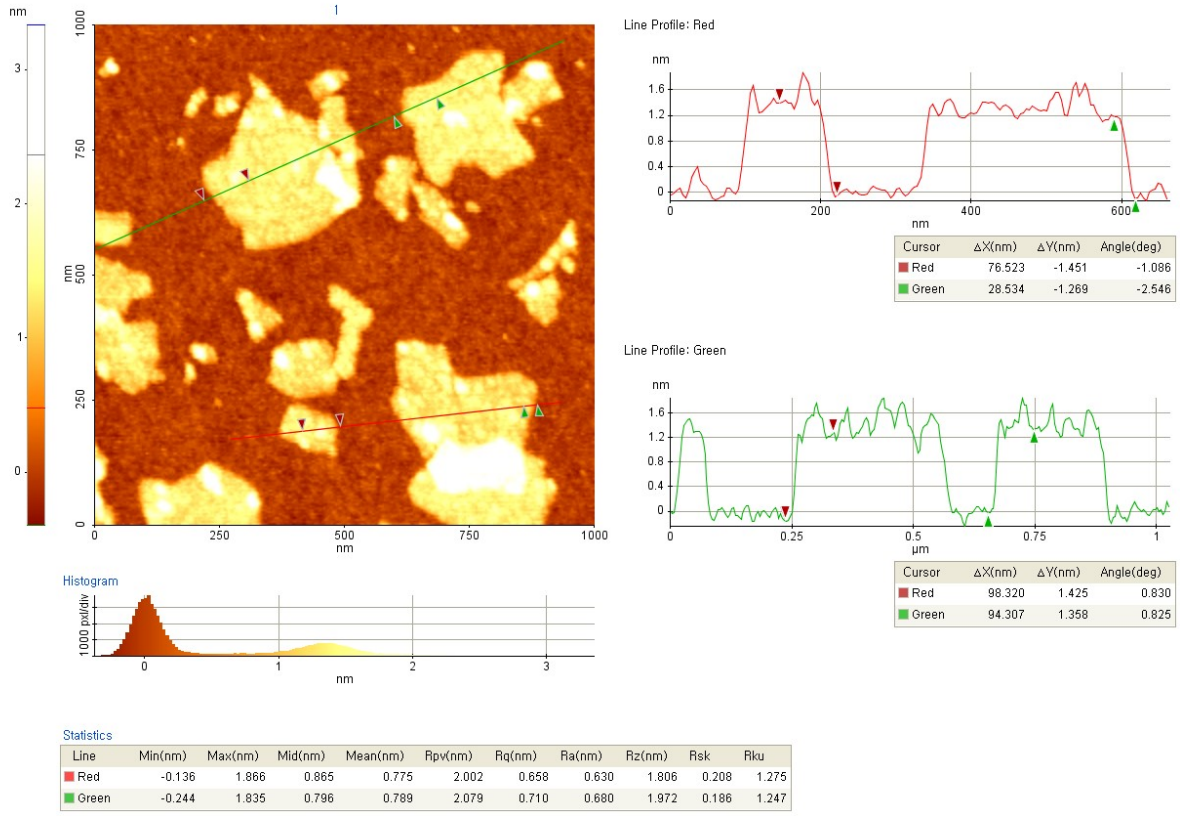

Figure S1. Characterization of GO with atomic force microscopy (AFM). The GO nanosheet is confirmed to be single-layered with a topographic height of 1.2-1.6 nm.

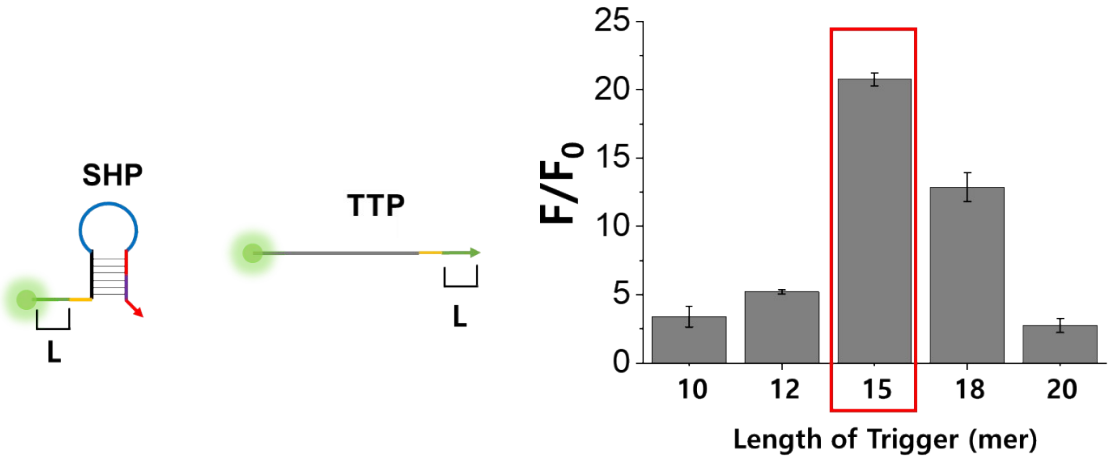

Figure S2. Optimization of the Trigger length. (a) By controlling the part denoted as 'L' in SHP and TTP, the length of Trigger can be changed. (b) Fluorescence signal-to-background ratios (F/F_0) when miRNA let-7a-specific probes with the different lengths of L were applied. F_0 and F are the fluorescence intensities at 520 nm in the absence and presence of target miRNA let-7a, respectively. The final concentrations of miRNA let-7a, each SHP, each TTP, KF, NE, and GO are 1 nM, 500 nM, 500 nM, 1.5 U/μL, 1 U/μL, and 2.5 mg/mL, respectively.

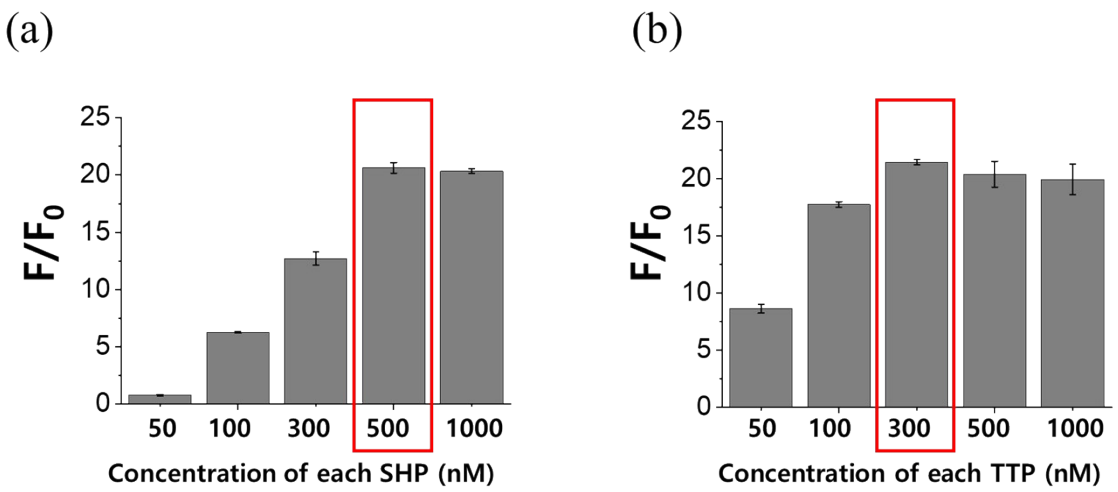

Figure S3. Optimization of the probe concentrations. (a) Fluorescence signal-to-background ratios (F/F_0) in the presence of each SHP (i.e. SHP-7a, SHP-141, and SHP-98) at different concentrations. (b) F/F_0 in the presence of each TTP (i.e. TTP-7a, TTP-141, and TTP-98) at different concentrations. The final concentrations of miRNA let-7a, KF, NE, GO are 1 nM, 1.5 U/μL, 1 U/μL, and 2.5 mg/mL, respectively.

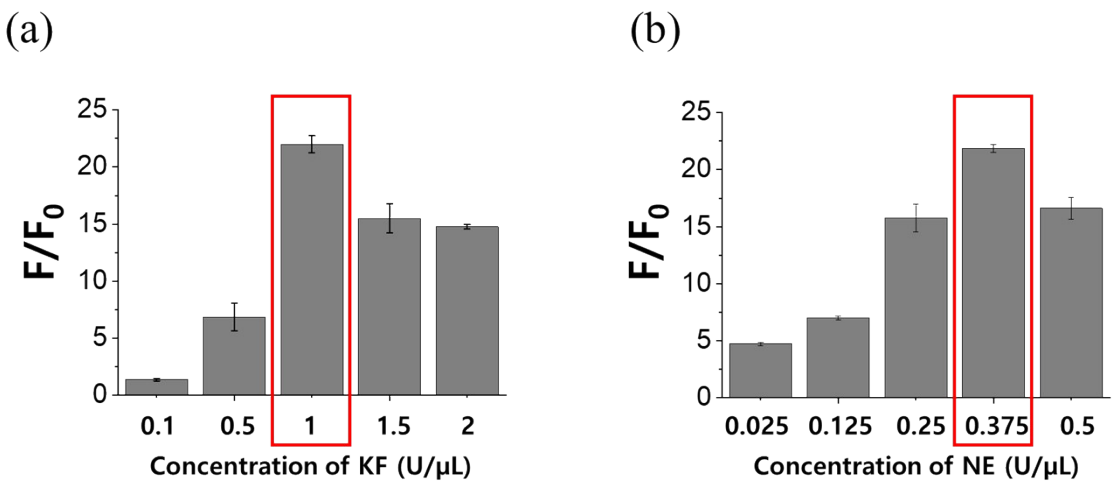

Figure S4. Optimization of the enzyme concentrations. (a) Fluorescence signal-to-background ratios (F/F_0) in the presence of KF at different concentrations. (b) F/F_0 in the presence of NE at different concentrations. The final concentrations of miRNA let-7a, each SHP, each TTP, and GO are 1 nM, 500 nM, 300 nM, and 2.5 mg/mL, respectively.

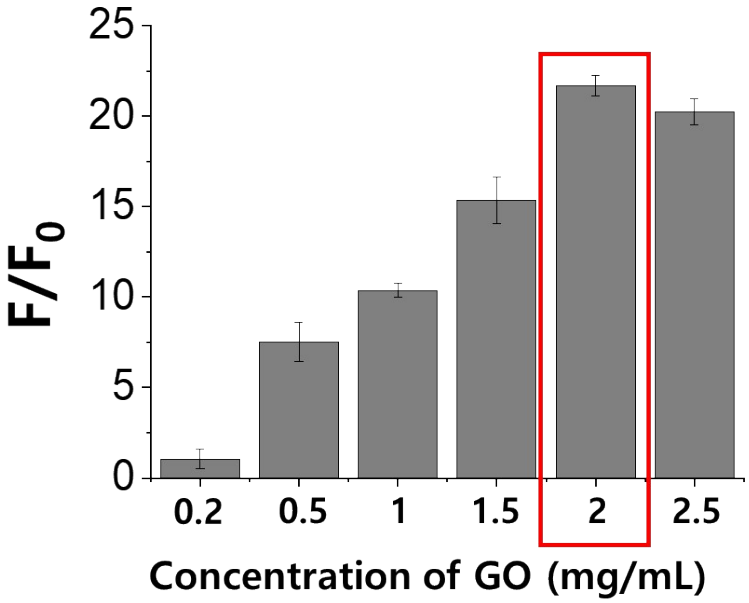

Figure S5. Optimization of GO concentration. Fluorescence signal-to-background ratios (F/F_0) in the presence of GO at different concentrations. The final concentrations of miRNA let-7a, each SHP, each TTP, KF, and NE are 1 nM, 500 nM, 300 nM, 1 U/μL, and 0.375 U/μL, respectively.

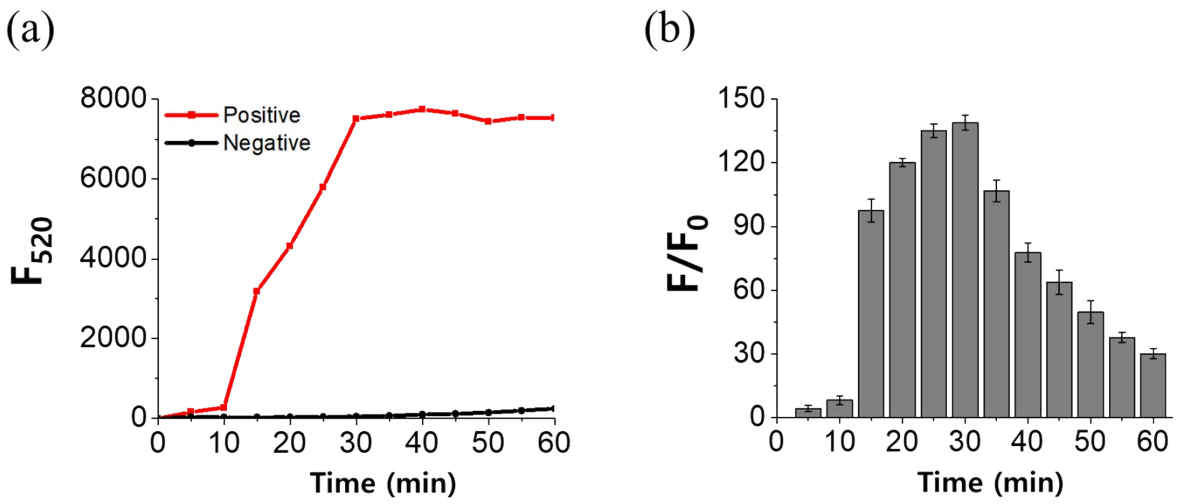

Figure S6. Time-dependent fluorescence signals resulting from self-priming hairpin-triggered cascade reaction. (a) Fluorescence intensities in the absence (Black) and presence (Red) of target miRNA and (b) signal-to-background ratios (F/F_0) at different reaction times. The final concentrations of miRNA let-7a, each SHP, each TTP, KF, NE, and GO are 1 nM, 500 nM, 300 nM, 1 U/μL, 0.375 U/μL, and 2 mg/mL, respectively.

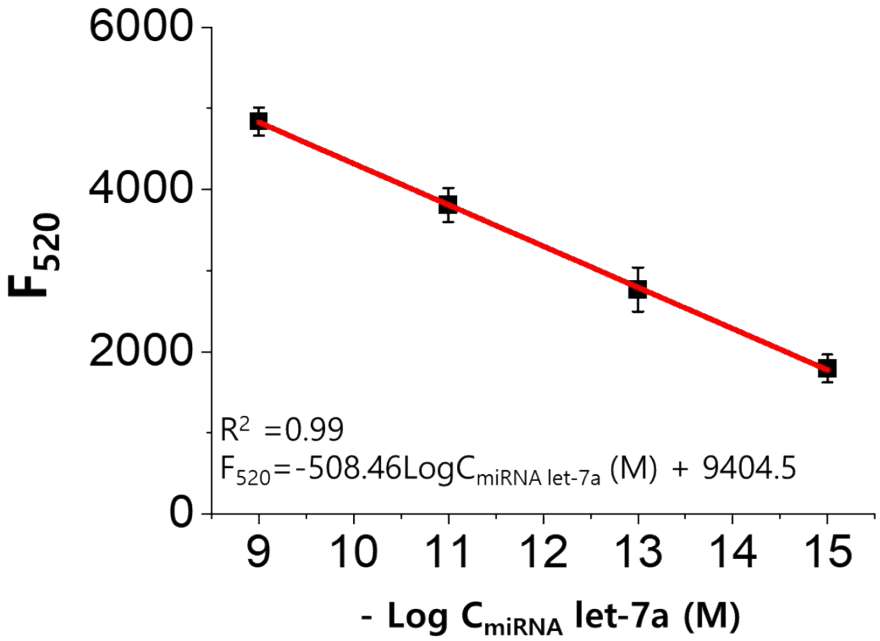

Figure S7. The linear relationship between the fluorescence intensity at 520 nm (F_{520}) and the concentration of target miRNA let-7a spiked in diluted human serum (10%). The final concentrations of each SHP, each TTP, KF, NE, and GO are 500 nM, 300 nM, 1 U/μL, 0.375 U/μL, and 2 mg/mL, respectively.

References

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