Supporting Information for

Palindromic hyperbranched rolling circle amplification enabling ultrasensitive microRNA detection

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Contents

Materials and methods

1. Materials

All DNA and RNA oligonucleotides used in this study were synthesized and purified with PAGE by Bioneer® (Daejeon, Korea). The sequences of the oligonucleotides are listed in Table S1. T4 DNA ligase, 10X T4 DNA ligase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, pH 7.5), Phi29 DNA polymerase (Phi29 DNAP), and 10X Phi29 DNAP reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 40 mM DTT, pH 7.5) were purchased from New England Biolabs Inc. (Beverly, MA, USA). SYBR Green I (10,000X) was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade and used without further purification.

2. Preparation of DP

First, the hairpin probe (HP) solution containing 25 μM of hairpin probe 1 (HP1) and hairpin probe 2 (HP2) in 1X T4 DNA ligase reaction buffer was heated at 95˚C for 5 min followed by cooling to 25˚C (0.1˚C/s), allowing the annealing of HP1 and HP2. The ligation reaction was conducted at 25˚C for 10 min by mixing the HP solution and DNA ligase in a final volume of 50 μL containing 1 μM HP1 and HP2, 40 U/μL T4 DNA ligase, and 1X T4

DNA ligase reaction buffer. The produced DP was stored at 4 °C prior to use in the PH-RCA reaction.

3. Experimental procedures for the PH-RCA reaction

The PH-RCA reaction was conducted in a final volume of 40 μL containing 100 nM DP, 500 nM PP, 250 μM dNTPs, 1X SYBR Green I, 5 U/μL Phi29 DNAP, 1X Phi29 DNAP reaction buffer, and target miRNA at varying concentrations. The reaction mixture was incubated at 30 °C for 30 min and the fluorescence signal from SYBR Green I staining was measured using a Tecan Infinite M200 pro microplate reader (Mnnedorf, Switzerland).

4. Gel electrophoresis

For polyacrylamide gel electrophoresis (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 ethidium bromide (EtBr) staining, gels were scanned using UV transilluminator (Bio-rad, CA, USA).

For agarose gel electrophoresis, a 5 μL aliquot of the reaction solution was resolved on 2% EtBr agarose gel in 1X TBE buffer at 100 V for 40 min. Gels were scanned using UV transilluminator (Bio-rad, CA, USA).

5. Melting curve analysis

The PH-RCA reaction solution containing 1X SYBR Green I (40 μL) was heated at 30 ^{o}C and the fluorescence signal was measured on a CFX ConnectTM Real-Time System (Bio-Rad, CA, USA) as the temperature was increased from 30 ^{o}C to 90 ^{o}C with an increment of 0.5 ^{o}C. The first derivative plot [-d(RFU)/dT] was used to determine the melting temperature.

6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) protocol for miRNA detection from the cell lines

qRT-PCR was conducted using THUNDERBIRD^{TM} Probe One-step qRT-PCR Kit according to the manufacturer's protocol. The reaction was conducted in 20 μL of sample solution containing 5.2 μL of DW, 10 μL of reaction buffer (2X), 1 μL of stem-loop primer (10 μM), 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), 0.8 μL of TaqMan probe (10 μM), 0.4 μL of extracted total RNA analyte (1 μg/μL), 0.5 μL of DNA polymerase (0.2 U/μL), and 0.5 μL of RT enzyme (0.5 U/μL). The solution was incubated at 50 ^{o}C for 10 min, heated up to 95 ^{o}C for 1 min, and subjected to the temperature cycle step (40 cycles) consisting of 95 ^{o}C for 15 sec and 60 ^{o}C for 45 sec. The fluorescent signal from TaqMan probe was measured at the end of every temperature cycle by using CFX Connect^{TM} Real-Time PCR Detection System (Bio-Rad, CA, USA).

Table S1. Oligonucleotide sequences used in this work.

Name	Oligonucleotide sequence (5' → 3')
miR-141	UAA CAC UGU CUG GUA AAG AUG G
miR-21	UAG CUU AUC AGA CUG AUG UUG A
miR-200c	UAA UAC UGC CGG GUA AUG AUG GA
miR let-7a	UGA GGU AGU AGG UUG UAU AGU U
miR let-7b	UGA GGU AGU AGG UUG UGU GGU U
miR let-7c	UGA GGU AGU AGG UUG UAU GGU U
miR let-7d	AGA GGU AGU AGG UUG CAU AGU U
Blocked target miR-141	UAA CAC UGU CUG GUA AAG AUG G $[PO_4]^a$
Single-base mismatched miR-141	UAA CA<u>G</u>[b] UGU CUG GUA AAG AUG G
HP1 for DP	$[PO_4]^a$ CGC GCG TAA TTT TCC ATC TTT ACC AGA CAG TGT TAA AAA TTA CG
HP2 for DP	$[PO_4]^a$ CGC GCG CGT AAT TTT CCA TCT TTA CCA GAC AGT GTT AAA AAT TAC GCG
HP1 for CDP	$[PO_4]^a$ GTC CCG GCC TTT TCC ATC TTT ACC AGA CAG TGT TAA AAA GGC CG
HP2 for CDP	$[PO_4]^a$ GGA GCC CAG ATT TTT CCA TCT TTA CCA GAC AGT GTT AAA AAT CTG GG
CP 14 mer	AAA AAA AAA AAA AA
PP 14 mer	TAC GCG CGC GCG TA
PP 12 mer	ACG CGC GCG CGT
PP 10 mer	CGC GCG CGC G
PP 8 mer	GCG CGC GC
PP 6 mer	CGC GCG

	NPP 8 mer	CGC GAC CC

[a] [PO_4] indicates the modification of phosphate group at 5' or 3'-end.

[b] Underlined letter in single-base mismatched miR-141 indicates the mismatched base with target miR-141.

Table S2. Detection performance of this method when compared with alternative RCA-based methods.

Method	LOD	Linear range	Reference
SDA-RCA	0.18 pM	0.1 pM – 10 nM	S1
Ligation-RCA	0.8 fM	1 fM – 10 pM	S2
Target-primed BRCA	10 fM	0.025 pM – 1 pM	S3
P-ERCA	0.24 zM	0.3 zM – 18 zM	S4
CEAM-RCA	12 fM	25 fM – 1 pM	S5
PH-RCA	18.5 aM	100 aM – 1 nM	This work

Figure S1. Dumbbell probe comparison test of PH-RCA. (a) Fluorescence emission spectra from SYBR Green I staining of the PH-RCA reaction products with DP containing the palindromic domain and control dumbbell probe (CDP) containing the non-palindromic domain. (b) The fluorescence intensity at 523 nm obtained from DP containing the palindromic domain and CDP containing the non-palindromic domain. The final concentrations of target miR-141, DP, CDP, PP, NPP, and Phi29 DNAP are 1 nM, 10 nM, 10 nM, 1 μM, 1 μM, and 5 U/μL, respectively.

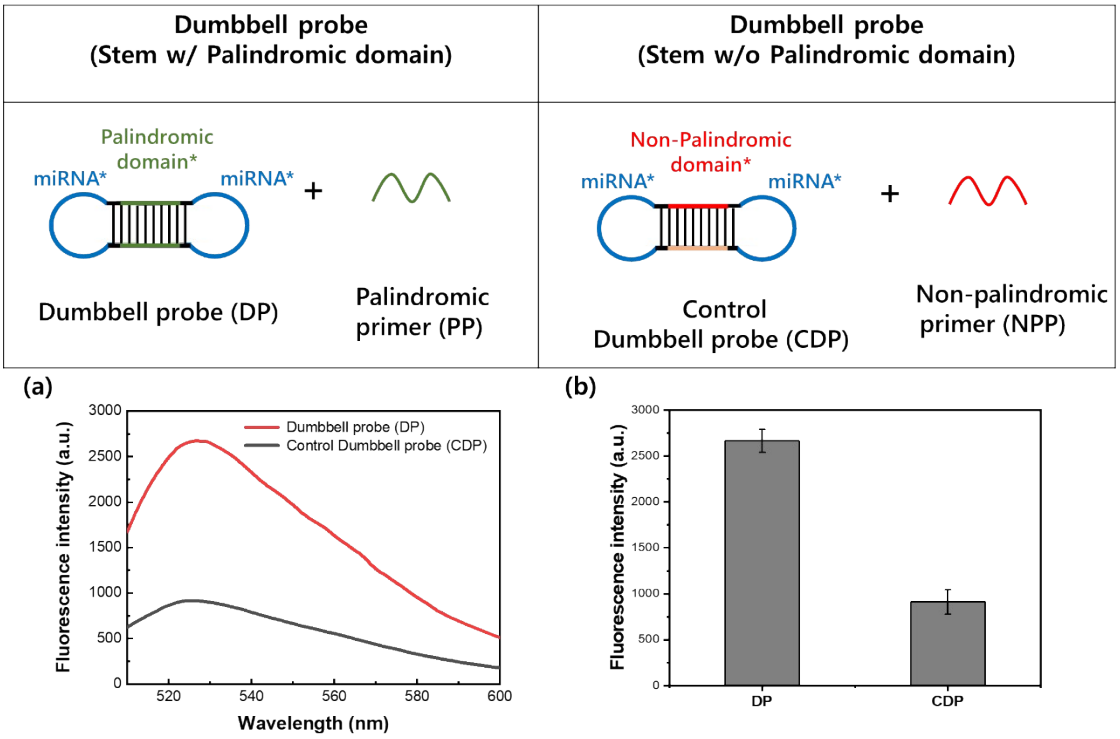


Figure S2. Polyacrylamide gel electrophoresis image of PH-RCA products obtained after the PH-RCA reaction (L: Ultra low range ladder, 1: Target miR-141, 2: HP1, 3: HP2, 4: PP, 5: DP, 6: DP + Target miR-141, 7: DP + PP, 8: DP + Target miR-141 + PP, 9: DP + Target miR-141 + Phi 29 DNAP, 10: DP + Target miR-141 + PP + Phi 29 DNAP, 11: DP + PP + Phi 29 DNAP). The final concentrations of target miR-141, DP, PP, and Phi29 DNAP are 100 nM, 100 nM, 1 μM, and 5 U/μL, respectively.

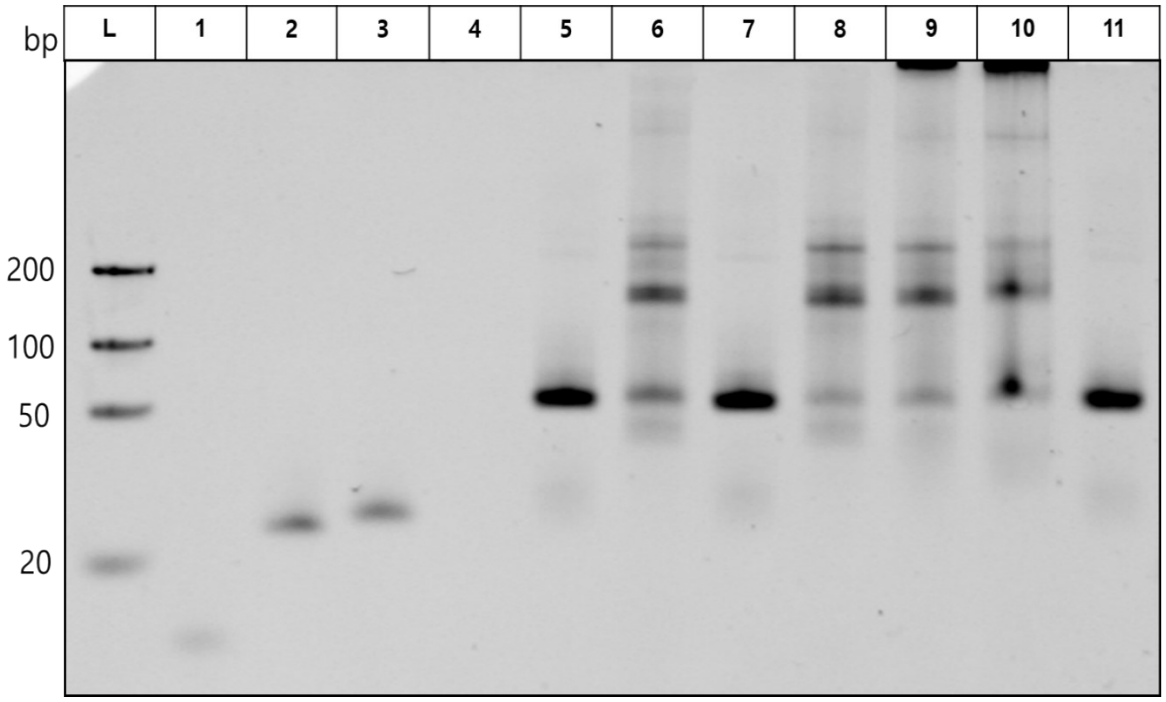


Figure S3. Melting curve analysis for characterization of DP. (a) Fluorescence emission spectra from SYBR Green I for confirming the formation of DP (1: DP, 2: HP1, 3: HP2). (b) Fluorescence emission spectra from SYBR Green I during the PH-RCA reaction (1: DP, 2: DP+ Target miR-141, 3: DP + PP, 4: DP + Target miR-141 + PP, 5: DP + PP + Phi 29 DNAP, 6: DP + Target miR-141 + PP + Phi 29 DNAP). The final concentrations of target miR-141, HP1, HP2, DP, PP, and Phi29 DNAP are 100 nM, 100 nM, 100 nM, 100 nM, 1 μM, and 5 U/μL, respectively.

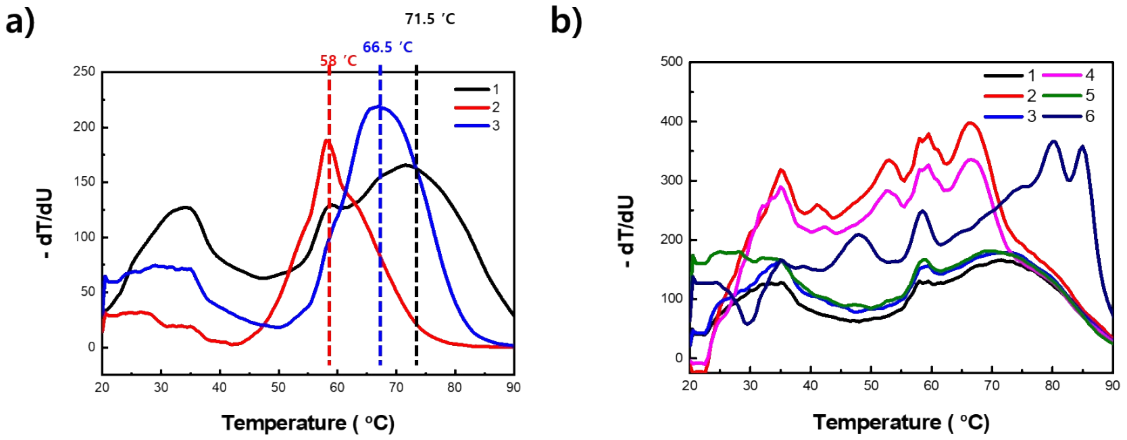


Figure S4. Optimization of the primer length, the palindromic domain length in DP, the concentration of DP, the concentration of PP, and reaction time. The F/F_0 value, where F_0 and F represent the fluorescence intensities at 523 nm from SYBR Green I in the absence and presence of target miR-141, respectively. The final concentrations of target miR-141, DP, PP, and Phi29 DNAP are 1 nM, 10 nM, 1 μM, and 5 U/μL, respectively.

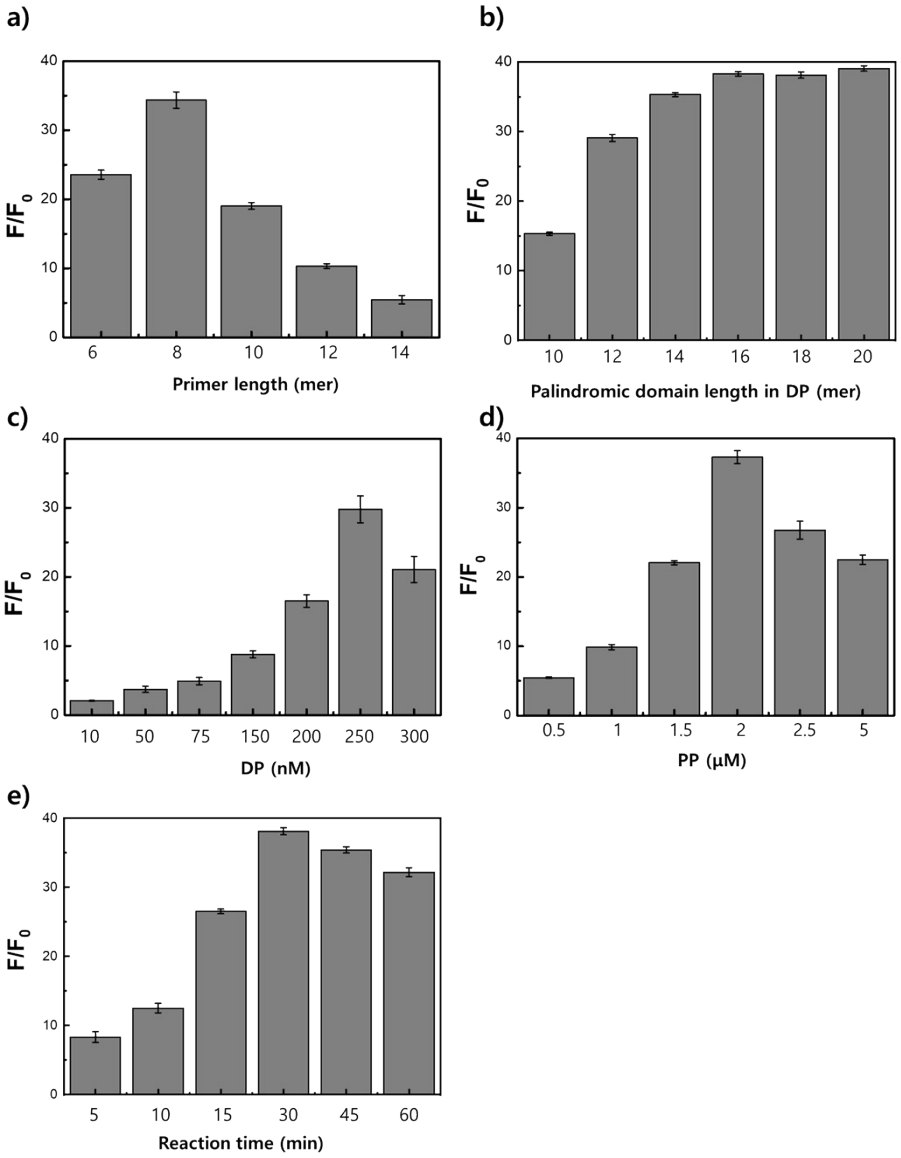


Figure S5. Reaction time to detect the samples at various target miR-141 concentrations with the PH-RCA and qRT-PCR methods. The final concentrations of DP, PP, and Phi29 DNAP are 10 nM, 1 μM, and 5 U/μL, respectively.

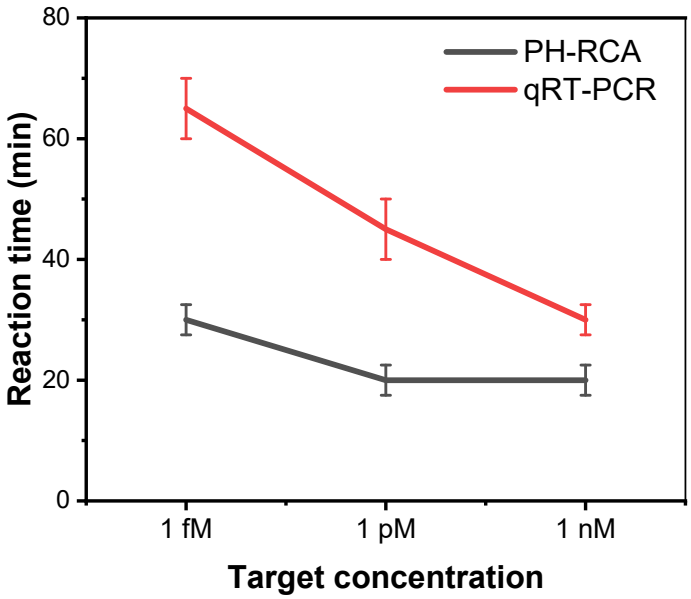
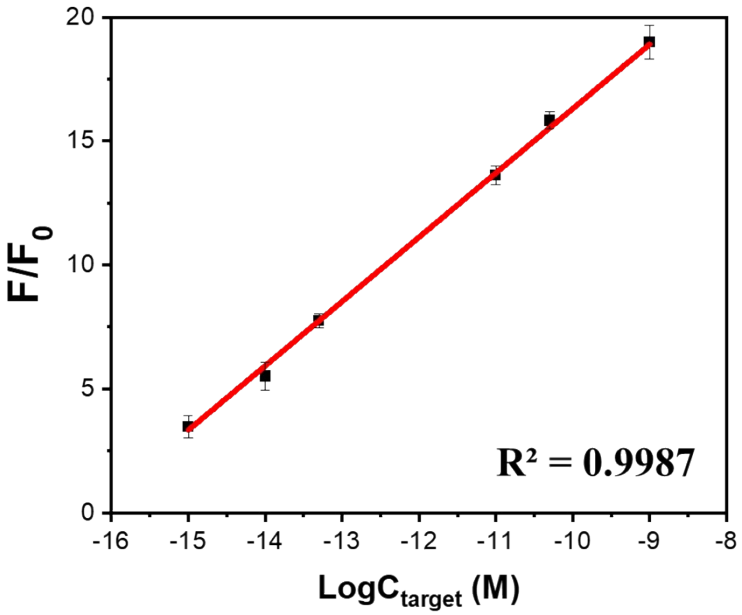


Figure S6. The linear relationship between the F/F_0 value and target miR-141 concentration spiked in human serum, where F_0 and F represent the fluorescence intensities at 523 nm from SYBR Green I in the absence and presence of target miR-141 spiked in human serum, respectively. The final concentrations of target miR-141, DP, PP, and Phi29 DNAP are 1 nM, 10 nM, 1 μM, and 5 U/μL, respectively.



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