Supporting Information for

Novel method for miRNA detection based on target-triggered transcription of light-up RNA aptamer

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Materials and methods

Materials

All DNA oligonucleotides used in this work were synthesized and PAGE-purified by Bioneer (Daejeon, Korea). The sequences of oligonucleotides studied in this work are listed in Table S1. Duplex-specific nuclease (DSN) was purchased from Evrogen (Konkov, Moscow, Russia). Klenow (exo-) DNA polymerase and NEBuffer 2 were purchased from New England Biolabs Inc. (Beverly, MA, USA). EZ™ T7 High Yield In Vitro Transcription kit including transcription buffer, $MgCl_2$, rNTP, T7 RNA Polymerase, DTT, and enhancer solution was purchased from Enzynomics (Daejeon, Korea). Malachite green chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). MCF-7 (human breast cancer cell line), MRC-5 (human lung normal cell line), and HeLa (human cervical cancer cell line) cell lines were obtained from Korean Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM) and Fetalgro Bovine Growth Serum (FBS) were purchased from Welgene Inc. (Gyeongsan-si, Korea) and RMBIO® (Missoula, MT, USA), respectively. Easy-Blue™ Total RNA Extraction Kit and THUNDERBIRD™ Probe One-step qRT-PCR Kit were purchased from Intron Biotechnology Inc. (Daejeon, Korea) and Toyobo (Osaka, Japan), respectively. All other chemicals were of analytical grade and used without further purification.

TTRApt reaction protocol for miRNA detection

The reaction solutions for miRNA detection were separately prepared as part A, part B,

and part C solutions. Part A solution consisted of 0.9 μL of distilled water (DW), 0.4 μL of target recognition probe (2 μM), 0.1 μL of DSN (0.4 U/μL), and 0.2 μL of 10X NEBuffer 2. Part B solution consisted of 0.4 μL of DW, 0.4 μL of T7 promoter-containing signaling probe (2 μM), 0.2 μL of 10X NEBuffer 2, 0.6 μL of dNTP (2.5 mM), and 0.4 μL of Klenow (exo-) DNA polymerase (2 U/μL). Part C solution consisted of 1.5 μL of DW, 4 μL of 5X Transcription buffer, 2 μL of 10X $MgCl_2$, 2 μL of DTT (100 mM), 1 μL of 20X Enhancer solution, 4 μL of rNTP (100 mM), 0.5 μL of T7 RNA Polymerase (200 U/μL), and 1 μL of malachite green chloride (5 mM). 0.4 μL of an analyte miRNA solution at varying concentrations was added to part A solution, which was then incubated at 55 °C for 10 min, followed by the inactivation of DSN at 95 °C for 5 min. Part B solution was next added to the analyte-containing Part A solution and incubated at 37 °C for 10 min. Finally, Part C solution was added to the mixed solution and incubated at 37 °C for 10 min. After the incubation, the fluorescence emission spectra were scanned from 635 nm to 735 nm at an excitation wavelength of 605 nm using Tecan Infinite M200 pro microplate reader (Mannedorf, Switzerland).

For the experiments to detect target miRNAs from the cell lines, MCF-7, MRC-5, and HeLa cell lines were cultured in DMEM supplemented with 10 % FBS under humidified atmosphere containing 5 % CO_2 at 37 ^oC and collected during the exponential growth phase. From the cultured 1×10^6 cells prepared based on cell counting using LUNA-II™ (Logos Biosystems Inc., Gyeonggi-do, Korea), the total RNAs were extracted by following the

protocol of Easy-Blue™ Total RNA Extraction Kit. The concentration of the extracted total RNAs was determined by using a NanoDrop® spectrophotometer (Wilmington, DE, USA) and diluted to 1 μg/μL. Next, 0.4 μL of extracted total RNA solution (1 μg/μL) was applied as an analyte miRNA solution.

Polyacrylamide gel electrophoresis

For polyacrylamide gel electrophoresis (PAGE), a 10 μL aliquot of the reaction solution was resolved on 15 % polyacrylamide gel in 1X TBE at 120 V for 100 min. After GelRed staining, gel was scanned using ChemiDoc™ (Bio-rad, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) protocol for miRNA detection from the cell lines

RT-PCR was conducted using THUNDERBIRD™ 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, and 0.5 μL of RT enzyme mix. The solution was incubated at 50 °C for 10 min, heated up to 95 °C for 1 min, and subjected to the temperature cycle step (40 cycles) consisting of 95 °C for 15 sec and 60 °C for 45 sec. The fluorescent signal from TaqMan probe was measured at the end of every temperature cycle by using CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA).

Table S1. Oligonucleotide sequences used in this study.

Name	Sequence (5' → 3')
Target recognition probe (TRP)	ACA TAG ACA CAC CTT GAG CAA **ACT ATA CAA CCT ACT ACC TCA** - PO_4
T7 promoter-containing signaling probe (T7SP)	*GGA TCC ATT CGT TAC CTG GCT CTC GCC AGT CGG GAT CC*T CTC CCT ATA GTG AGT CGT ATT AGT TTG CTC AAG GTG TGT CTA TGT - PO_4
Complementary T7SP	ACA TAG ACA CAC CTT GAG CAA ACT AAT ACG ACT CAC TAT AGG GAG AGG ATC CCG ACT GGC GAG AGC CAG GTA ACG AAT GGA TCC
Untailed TRP	**AAC TAT ACA ACC TAC TAC CTC A** - PO_4
TRP tailed with random sequence	ATC GTA TGA ACG AAT CGA CAA **ACT ATA CAA CCT ACT ACC TCA** - PO_4
Let-7a (Target miRNA)	uga ggu agu agg uug uau agu u
Let-7b	uga ggu agu agg uug ugu ggu u
Let-7c	uga ggu agu agg uug uau ggu u
Let-7d	aga ggu agu agg uug cau agu u
Let-7e	uga ggu agg agg uug uau agu u
Let-7f	uga ggu agu aga uug uau agu u
Let-7g	uga ggu agu agu uug uac agu u
Stem-loop primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC

	GCA CTG GAT ACG ACA ACT A
Forward primer	GCC GCT GAG GTA GTA GGT TGT A
Reverse primer	GTG CAG GGT CCG AGG T
TaqMan probe	(FAM) - TGG ATA CGA CAA CTA TAC – (BHQ1)

* The red-colored characters indicate the trigger sequence. The bold characters indicate the target-specific region. The italic letters represent the complementary sequence of malachite green aptamer (MGA). The underlined bases are the T7 promoter sequence. The orange-colored characters represent the complementary sequence of trigger. The capital and small letters indicate DNA and RNA, respectively.

Table S2. Comparison of this strategy with alternative light-up RNA aptamer-based methods for miRNA detection.

Key method	Assay time	Detection limit	Limitations	Reference
Spinach-based fluorescent light-up biosensors	~ 3 h	3 pM	- Long reaction time - Low sensitivity	[1]
Amplified Tandem Spinach-Based Aptamer Transcription	~ 4 h	67.3 fM	- Long reaction time - Low sensitivity	[2]
Cascade Transcription Amplification of RNA Aptamer	~ 3 h	5.12 aM	- Long reaction time	[3]
The RNA-regulated fluorescence of malachite green	~ 2 h	50 fM	- Long reaction time - Low sensitivity	[4]
Graphene oxide assisted light-up aptamer selection against Thiofavin T	55 min	2.6 nM	- Low sensitivity	[5]
Multiplex detection of microRNA using light-up RNA aptamers	~ 2 h	0.955 fM	- Long reaction time	[6]
TTRApt reaction	**35 min**	**59.4 aM**	**-**	**This strategy**

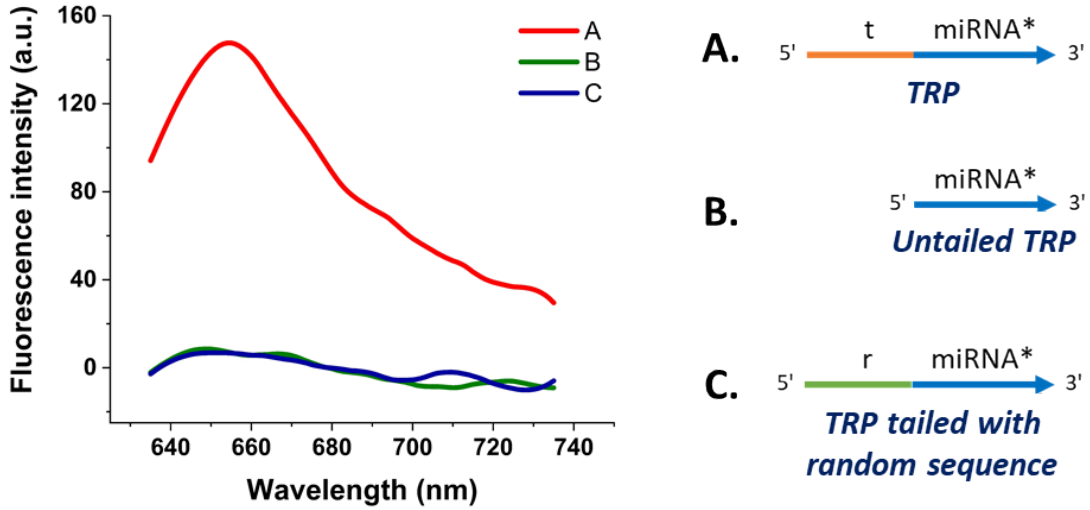

Figure S1. Fluorescence emission spectra from MG after the TTRApt reactions by employing differently designed TRPs. (A) TRP. (B) Untailed TRP. (C) TRP tailed with random sequence. The final concentrations of target miRNA, TRP, and T7SP are 1 nM, 20 nM, and 20 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 3 U, and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C.

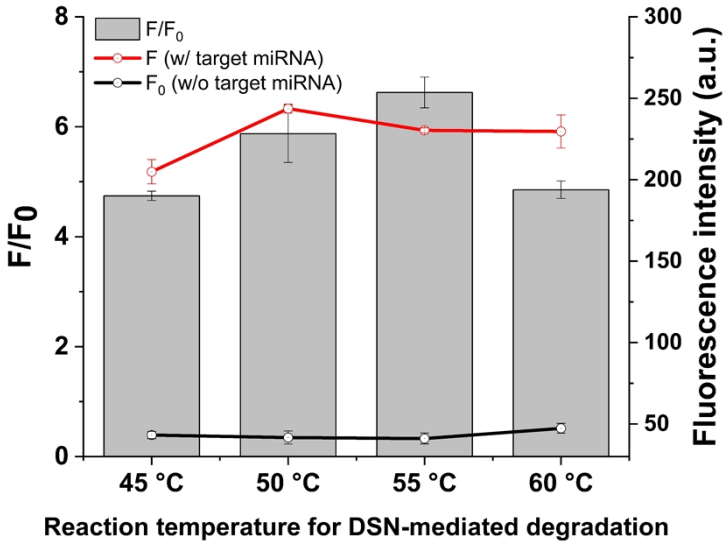

Figure S2. The effect of reaction temperature for DSN-mediated degradation reaction by examining the signal-to-background ratios (F/F_0, where F and F_0 are the fluorescence intensities at 654 nm from the samples with and without target miRNA, respectively) at different conditions. The final concentrations of target miRNA, TRP, and T**7S**P are 1 nM, 20 nM, and 40 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction times for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription are 10 min, 15 min, and 10 min, respectively. The error bars were determined from triplicate measurements.

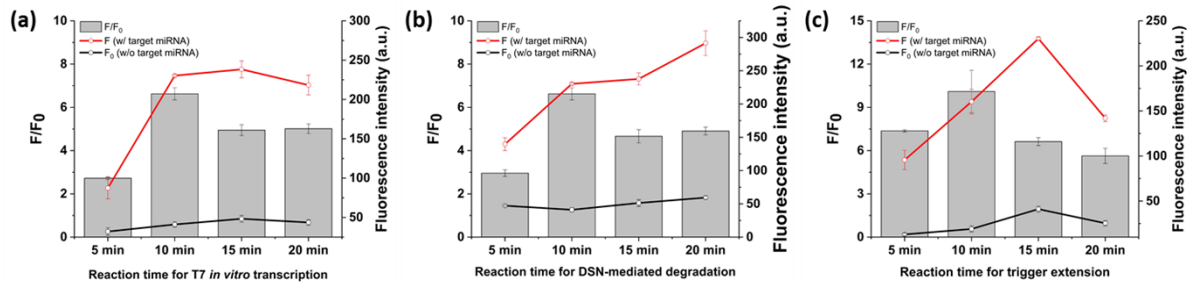

Figure S3. Optimization of reaction times by examining the signal-to-background ratios (F/F_0, where F and F_0 are the fluorescence intensities at 654 nm from the samples with and without target miRNA, respectively) at different conditions. (a) The signal-to-background ratios (F/F_0) for varying reaction times for T7 *in vitro* transcription. The final concentrations of target miRNA, TRP, and T**7S**P are 1 nM, 20 nM, and 40 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction times for DSN-mediated degradation and trigger extension are 10 min and 15 min, respectively. The reaction temperature for DSN-mediated degradation is 55 °C. (b) The signal-to-background ratios (F/F_0) for varying reaction times for DSN-mediated degradation. The final concentrations of target miRNA, TRP, and T**7S**P are 1 nM, 20 nM, and 40 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction times for trigger extension and T7 *in vitro* transcription are 15 min and 10 min, respectively. The reaction temperature for DSN-mediated degradation is 55 °C. (c) The signal-to-background ratios (F/F_0) for varying reaction times for trigger extension. The final concentrations of target miRNA, TRP, and T**7S**P are 1 nM, 20 nM, and 40 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction time for DSN-mediated degradation and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. The error bars were determined from triplicate measurements.

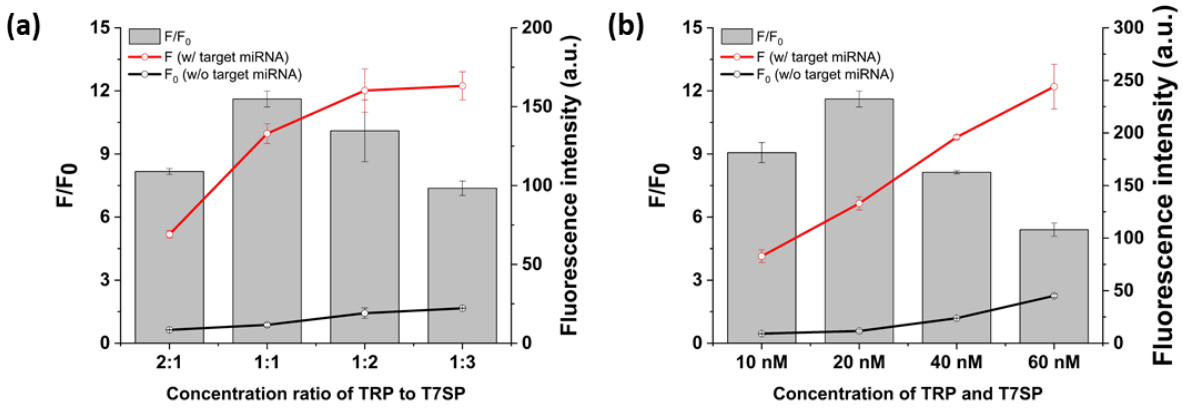

Figure S4. Optimization of probe concentrations by examining the signal-to-background ratios (F/F_0, where F and F_0 are the fluorescence intensities at 654 nm from the samples with and without target miRNA, respectively) at different conditions. (a) The signal-to-background ratios (F/F_0) for varying concentration ratios of TRP to T**7S**P. The final concentrations of target miRNA and TRP are 1 nM and 20 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. (b) The signal-to-background ratios (F/F_0) for varying concentrations of TRP and T**7S**P. The final concentration of target miRNA is 1 nM. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 4 U, and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. The error bars were determined from triplicate measurements.

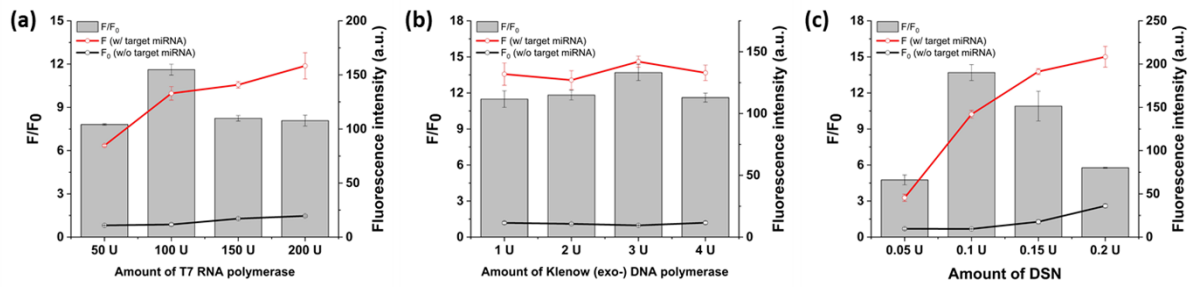

Figure S5. Optimization of amounts of enzymes by examining the signal-to-background ratios (F/F_0, where F and F_0 are the fluorescence intensities at 654 nm from the samples with and without target miRNA, respectively) at different conditions. (a) The signal-to-background ratios (F/F_0) for varying amounts of T7 RNA polymerase. The final concentrations of target miRNA, TRP, and T7SP are 1 nM, 20 nM, and 20 nM, respectively. The amounts of DSN and Klenow (exo-) DNA polymerase are 0.1 U and 4 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. (b) The signal-to-background ratios (F/F_0) for varying amounts of Klenow (exo-) DNA polymerase. The final concentrations of target miRNA, TRP, and T7SP are 1 nM, 20 nM, and 20 nM, respectively. The amounts of DSN and T7 RNA polymerase are 0.1 U and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. (c) The signal-to-background ratios (F/F_0) for varying amounts of DSN. The final concentrations of target miRNA, TRP, and T7SP are 1 nM, 20 nM, and 20 nM, respectively. The amounts of Klenow (exo-) DNA polymerase and T7 RNA polymerase are 3 U and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. The error bars were determined from triplicate measurements.

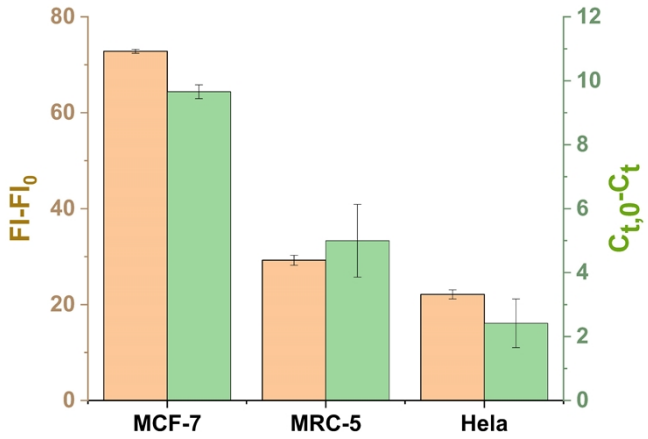

Figure S6. Practical applicability test of the TTRApt reaction for target miRNA detection. Orange bars represent the degrees of fluorescence intensity increase (FI - FI_0, where FI and FI_0 are the fluorescence intensities at 654 nm from the sample with and without the target miRNA extracted from the cell lines, respectively) obtained from the TTRApt reactions. Green bars represent the degrees of threshold cycle number decrease ($C_{t,0} – C_t$, where C_t and $C_{t,0}$ are threshold cycle numbers from real-time fluorescence signal curve from the sample with and without the target miRNA extracted from the cell lines, respectively) obtained from the conventional RT-PCRs. The final concentrations of total RNA, TRP, and T7SP are 20 ng/μL, 20 nM, and 20 nM, respectively. The amounts of DSN, Klenow (exo-) DNA polymerase, and T7 RNA polymerase are 0.1 U, 3 U, and 100 U, respectively. The reaction time for DSN-mediated degradation, trigger extension, and T7 *in vitro* transcription is 10 min. The reaction temperature for DSN-mediated degradation is 55 °C. The error bars were determined from triplicate measurements.

References

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