Supporting Information

Fluorescein-switching-based lateral flow assay for the detection of microRNAs

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1. Materials and Methods

All reagents and solvents were obtained from commercial suppliers (TCI, Samchun, Daejung, Sigma-Aldrich, and Alfa) and used without additional purification. Natural and modified oligonucleotides were sourced from Bioneer (Daejeon, South Korea). The mass of synthesized oligodeoxynucleotides was measured using MALDI-TOF MS (Axima LNR MALDI-TOF, Shimadzu) at Bioneer. The Lateral Flow Assay strips were acquired from Milenia Biotec GmbH (HybriDetect - Universal Lateral Flow Assay Kit). PNA monomers were purchased from HLB Panagene Co Ltd. Electrospray ionization mass spectrometry (ESI-MS) was performed in the positive ion mode on a Synapt G2-Si HDMS Q-TOF mass spectrometer (Waters, UK, Manchester). The final concentration of the PNAs was 5 μM in HPLC grade water. Quantification of test line of LFA strips was conducted by ImageJ software.

1.1 Preparation of oligodeoxynucleotides

To reduce oligomer-conjugated FAM, approximately 1 mg of $NaBH_4$ was added to a mixture of 10 μL of 500 μM FAM-modified oligodeoxynucleotides and 40 μL of 0.2 M sodium phosphate buffer (pH 7.03) in a microtube, followed by vortexing. The mixture was then centrifuged at 1400 rpm and 4 ℃ for about one hour. The completion of the reduction was indicated by the disappearance of the yellow color of FAM. To minimize bubbling caused by $NaBH_4$, 50 μL of triethylammonium acetate (TEAA) buffer (pH 7.2) was added when the yellow color vanished. The mixture was then centrifuged for 20 minutes before being injected into a high-performance liquid chromatograph (HPLC) for purification. The reduced oligodeoxynucleotides were purified using an Agilent ZORBAX 300SB-C18 column and HPLC (Agilent 1260) with the following conditions: A = 0.1M TEAA buffer (pH 7.2), B = 0.01% TFA in acetonitrile; gradient: 100:0 at 0 min, 30:70 at 28 min, 0:100 at 29 min, 100:0 at 30 min. The flow rate was 2.5 mL/min, with detection wavelengths of 260 nm and 490 nm. The injection volume was 100 μL.

Riboflavin tetraacetate-conjugated oligodeoxynucleotides were prepared according to our previously reported method.[1] In this experiment, the four target oligonucleotides were prepared at a concentration of 100 μM before use. Oligodeoxynucleotides were quantified by measuring the UV absorbance at 260 nm, with extinction coefficients for A, T, G, and C at 260 nm being 13700, 6600, 11700, and 8800 M^{-1}, respectively.

List of oligodeoxynucleotides used in this study

Name	Sequence (5´ to 3´)	Expected mass (m/z)	Observed mass (m/z)
H1	**FAM-H**-TCG CTC CCC	3148.6016	3148.5628
H2	**FAM-H**-TCG CTC CC	2859.5552	2857.8203
H3	**FAM-H**-TCG CTC C	2570.5088	2570.8667
H4	**FAM-H**-TCG CTC	2281.4625	2282.1093
R1	G CCC CGC CCC-**Rf**	3867.8629	3867.9000
H5	C CAT CTT TAC C-**FAM-H**	3851.7420	3852.6899
H6	CAT CTT TAC C-**FAM-H**	3562.6956	3562.9038
H7	AT CTT TAC C-**FAM-H**	3273.6493	3273.2907
R2	**Rf**-AG ACA GTG TTA	4307.9487	4307.6352
B1	**FAM-H**-TCG CTC C-**Biotin**	3005.6681	3004.9498
B2	**Biotin**-CAT CTT TAC C-**FAM-H**	3967.8444	3967.6400
P1	(N-ter) **FAM**-TCG CTC C (C-ter)	2346.8648	2346.77
P2	(N-ter) **FAM**-TCG CTC (C-ter)	2095.7629	2095.72
H2´	**FAM**-TCG CTC CC	Oligonucleotides were purchased from Bioneer and used without further purification.	
B1´	**FAM**-TCG CTC C-**Biotin**		
B2´	**Biotin**-CAT CTT TAC C-**FAM**		
M1	UAA CAC UAU CUG GUA AAG AUG G		
M3	UUA CAC UAU CUG GUG AAG AUG G		
miR-126	UCG UAC CGU GAG UAA UAA UGC		
miR-34	UGG CAG UGU CUU AGC UGG UUG U		

1.2 Solid phase PNA synthesis

Prepare the following five reagents in advance:

I. 20% piperidine in DMF (2 mL piperidine + 8 mL DMF)

II. HATU activation solution 0.5 M (0.5 mL NMP + 0.095 g HATU)

III. Base solution (0.18 mL 2,6-lutidine + 0.18 mL DIPEA + 0.5 mL NMP)

IV. Cap mix solution (0.51 mL acetic anhydride + 0.72 mL 2,6-lutidine + 10 mL DMF)

V. Monomer solution (0.2 M * Number of monomers * 2 (Couple each monomer twice) * 33 µL (Volume to be added) * M_w) in NMP

A (Fmoc-A(Bhoc)aeg-OH, HLB Panagene)

T (Fmoc-T-aeg-OH, HLB Panagene)

G (Fmoc-G(Bhoc)aeg-OH, HLB Panagene)

C (Fmoc-G(Bhoc)aeg-OH, HLB Panagene)

C-terminal: CCTCGCT (N-terminal) 7mer / C-terminal: CTCGCT (N-terminal) 6mer

Synthesis was conducted from the C-terminal to the N-terminal. Add 2.5 mg (1 eq) of the resin (0.43 mmol/g, Novabiochem, NovaPEG Rink Amide resin) to the column, fill it with DCM, and activate the resin (Preparation of the resin). For monomer preactivation, add 33 μL of monomer solution, 12 μL of HATU activation solution, and 6 μL of base solution to a microtube, vortex, centrifuge, and wait for 5 minutes. Wash the resin with DCM before coupling. Add the preactivated monomer to the column containing the resin and repeat the coupling process twice (Coupling, 20 min). Wash the remaining solution with DCM and DMF, then add the cap mix solution to the column, filling it to approximately two-thirds of its volume (Capping, 10 min, repeat twice). Wash the cap mix solution with DCM and DMF, then add a 20% piperidine solution to the column, filling it to approximately two-thirds of its volume (Fmoc deprotection, 10 min, repeat twice). After washing the remaining solution with DCM and DMF, repeat the process with the next monomer. After completing the synthesis from the C to the T monomer, treat Fmoc-6-Ahx-OH as a monomer and proceed with the coupling reaction.

After Fmoc deprotection, transfer FITC (4 eq), DMF (30 μL), and DIPEA (10 eq) into a microtube, mix thoroughly, and add the resulting solution to the resin. Incubate the reaction overnight at room temperature on a shaker (12 h). Following the overnight reaction, wash with DMF and DCM (orange color), and then fill approximately two-thirds of the column with TFA (3 h). Transfer the solution from the column into a microtube, then wash the column with a small volume of TFA, collecting the reaction solution.

To the collected reaction solution, add ether and centrifuge for 5 minutes. Discard the supernatant, leaving the precipitate. Dry the residue and perform HPLC purification (HPLC, Agilent 1260). Conditions: A = 0.1% TFA in DW, B = 0.1% TFA in acetonitrile; gradient: 100:0 at 0 min, 60:40 at 10 min, 0:100 at 22 min, 100:0 at 30 min. The flow rate was 2.5 mL/min. The detection wavelengths were 260 nm and 490 nm. The injection volume was 100 μL.

1.3 Optimization of templated reaction for best discrimination (for Figure 2)

To optimize the length of the FAM probe, reaction solutions containing 200 nM **H1**, **H2**, **H3**, **H4**, 20 nM **R1**, and 0-40 nM miR-6090 DNA were prepared in 1× PBS buffer (pH 7.2). The total volume is 200 μL.

In the FAM optimization experiment targeting RNA (miR-6090, miR-141), 200 nM of reduced FAM probe, 40 nM of riboflavin-conjugated oligodeoxynucleotides, and 20 mM $MgCl_2$ were prepared in 1× PBS buffer (pH 7.2). The total volume is 200 μL.

The reaction conditions for the experiment utilizing a FAM probe with PNA are as follows: 200 nM of reduced PNA probe (6, 7 mer), 20 nM **R1**, and 0-40 nM miR-6090 DNA/RNA were prepared in 1× PBS buffer (pH 7.2). The total volume is 200 μL. The reagents were added in the order of PBS buffer (pH 7.2), target DNA or RNA, reduced FAM, $MgCl_2$, and distilled water, and then allowed to react.

The templated reaction was performed in a 96-well microtiter plate (Thermo Fisher, Nunc™, round bottom). The fluorescence enhancement was directly measured using microplate readers (SpectraMax M2) in the fluorescence endpoint mode at 25 ℃. During the experiment, the samples were illuminated using a blue LED (1 W). The samples were prepared in triplicates. Parameters: λ_{exc} = 490 nm, cut-off = 515 nm, λ_{emi} = 526 nm, PMT gain = medium, six flashes/read, and shake-off.

1.4 Experiments for reduction kinetics (for Figure 3)

NADH and $NaBH_4$ were used as reducing agents, with the FAM oligonucleotide utilized as **H2′**. To observe the reduction of FAM fluorescence by reducing agents, reaction solutions containing 200 nM **H2′** and 60 mM NADH were prepared in 1× PBS buffer (pH 7.2). The total volume is 200 μL. In the experiment using $NaBH_4$, measurements were conducted at two concentrations. Reaction solutions containing 200 nM **H2′** and $NaBH_4$ (60 mM/500 mM) were prepared in 1× PBS buffer (pH 7.2). The total volume is 200 μL. The samples were prepared in triplicates. Parameters: λ_{exc} = 490 nm, cut-off = 515 nm, λ_{emi} = 526 nm, PMT gain = medium, six flashes/read, and shake-off. The aforementioned experiments were tested under two conditions: with and without blue light exposure.

For Figure S3, add 20 pmol of **H2′** (10 μM, 2 μL, final concentration of 200 nM) and $NaBH_4$ or NADH (2 M, 2 μL, final concentration of 1M) to a 96-well microtiter plate, and incubate for one hour. In this process, the reaction containing NADH was exposed to blue light irradiation. Add 40 nM **R1** (1.9 μM, 2.1 μL), 20 mM $MgCl_2$ (200 mM, 10 μL), and 1× PBS buffer (pH 7.2, 25 μL) to the mixture. The total volume is 100 μL. Finally, add the miR-6090 target (1 μM, 2 μL, final concentration of 20 nM) and proceed the templated reaction (4 h, blue light).

1.5 Optimization of quantities of B2 for lateral flow assay (for Figure 4b)

Add (40, 10, 1, 0.5, 0.25, 0.1, 0.05) pmol of **B2** and $NaBH_4$ (final concentration of 500 mM) to a

microtube, and incubate for one hour. The mixtures were diluted to be 1× PBS buffer (pH 7.2), with a total volume of 50 µL. Immerse the LFA strip in the mixture solution and check the results after approximately 5 minutes. As a comparative control, Solutions containing (40, 10, 1, 0.5, 0.25, 0.1, 0.05) pmol of **B2′** were prepared in 1× PBS buffer (pH 7.2), with a total volume of 50 µL.

1.6 Lateral flow assay test for nucleic acid targets (for Figure 4d, 4e, and S4)

1. miR-6090 DNA/RNA Target

Add 20 pmol of **B1** (20 µM, 1 µL, final concentration of 200 nM) and $NaBH_4$ (2 M, 1 µL, final concentration of 1 M) to a 96-well microtiter plate, and incubate for one hour. Add 20 nM (1.9 µM, 1.1 µL) / 40 nM (1.9 µM, 2.1 µL) **R1**, 20 mM $MgCl_2$ (200 mM, 10 µL), and pH 7.2 1× PBS buffer (25 µL) to the mixture. The total volume is 100 µL. Thoroughly mix and divide the solution into two 50 µL portions. Add miR-6090 DNA/RNA Target (final concentration of 50 nM) to one portion and adjust the volume of the other with distilled water. Expose the samples to blue light for 30 minutes. After 30 minutes, transfer 1 µL from each 50 µL aliquot to a new tube. Add 99 µL of pH 7.2 1× PBS buffer, mix, and then transfer 25 µL of this solution into a microtube for LFA testing. Immerse the LFA strip in the mixture solution and check the results after approximately 5 minutes.

2. miR-141 DNA/RNA Target

Add 20 pmol of **B2** (16 µM, 1.3 µL, final concentration of 200 nM) and $NaBH_4$ (2 M, 1.3 µL, final concentration of 1 M) to a 96-well microtiter plate, and incubate for one hour. Add 15 nM (1.5 µM, 1.3 µL) / 50 nM (1.2 µM, 4.2 µL) **R2**, 20 mM $MgCl_2$ (200 mM, 10 µL), and pH 7.2 1× PBS buffer (25 µL) to the mixture. The total volume is 100 µL. Thoroughly mix and divide the solution into two 50 µL portions. Add miR-141 DNA/RNA Target (final concentration of 50 nM) to one portion and adjust the volume of the other with DW. Expose the samples to blue light for 30/40 minutes. After 30/40 minutes, transfer 1 µL from each 50 µL aliquot to a new tube. Add 99 µL of pH 7.2 1× PBS buffer, mix, and then transfer 25 µL of this solution into a microtube for LFA testing. Immerse the LFA strip in the mixture solution and check the results after approximately 5 minutes.

Using the aforementioned conditions, other experiments were conducted to monitor the detection threshold and sequence specificity of the LFA systems.

1.7 Lateral flow assay test for nucleic acid targets in cell lysates (for Figure S6)

1. miR-6090 target

Add 20 pmol of **B1** (20 μM, 1 μL, final concentration of 200 nM) and $NaBH_4$ (2 M, 1 μL) to a 96-well microtiter plate, and incubate for one hour. Add 40 nM (1.9 μM, 2.1 μL) **R1**, 20mM $MgCl_2$ (200mM, 10 μL), pH 7.2 1 × PBS buffer (25 μL) to the mixture. Total volume is 100 μL.

Thoroughly mix and divide 100 μL of the solution into two 50 μL portions. Add miR-6090 RNA target diluted with BV-2 cell lysates (final concentration of 50, 30, 10, 5, 2, 1, 0 nM) to 50 μL portion. The samples were prepared in triplicates.

Expose the samples to blue light for 1 hour.

After 1 hour, transfer 1 μL from each 50 μL aliquot to a new tube. Add 399 μL of pH 7.2 1 × PBS buffer, mix, and then transfer 25 μL of this solution into a microtube for LFA testing.

Immerse the LFA script in the mixture solution and check the results after approximately 5 minutes.

2. miR-141 target

Add 20 pmol of **B2** (19 μM, 1.1 μL, final concentration of 200 nM) and $NaBH_4$ (2 M, 1.1 μL) to a 96-well microtiter plate, and incubate for one hour. Add 50 nM (2.3 μM, 2.2 μL) **R2**, 20mM $MgCl_2$ (200mM, 10 μL), pH 7.2 1 × PBS buffer (25 μL) to the mixture. Total volume is 100 μL.

Thoroughly mix and divide 100 μL of the solution into two 50 μL portions. Add miR-141 RNA target diluted with BV-2 cell lysates (final concentration of 50, 30, 10, 5, 2, 1, 0 nM) to 50 μL portion. The samples were prepared in triplicates.

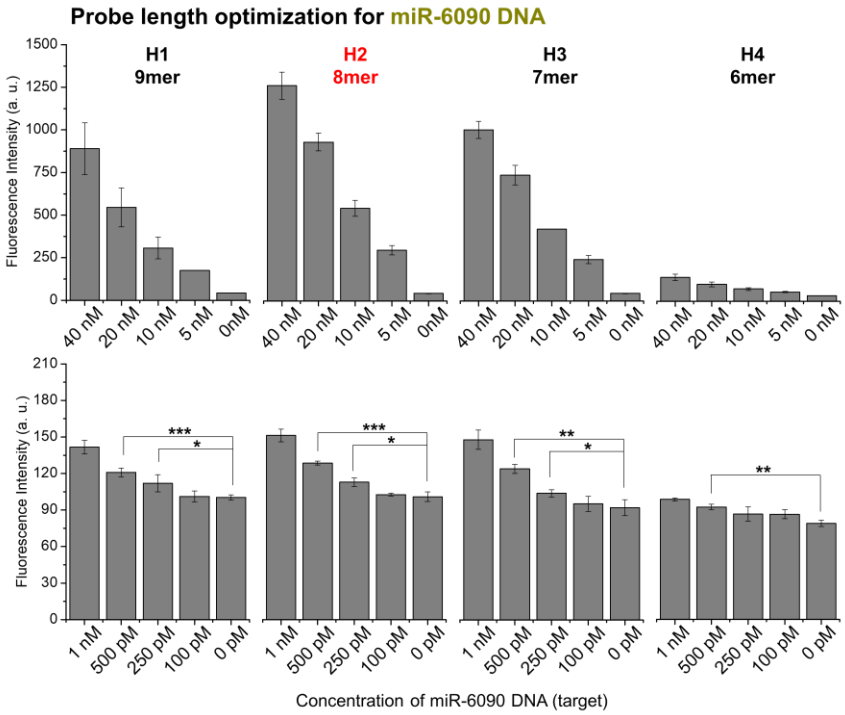
Expose the samples to blue light for 1 hour.

After 1 hour, transfer 1 μL from each 50 μL aliquot to a new tube. Add 399 μL of pH 7.2 1 × PBS buffer, mix, and then transfer 25 μL of this solution into a microtube for LFA testing.

Immerse the LFA script in the mixture solution and check the results after approximately 5 minutes.

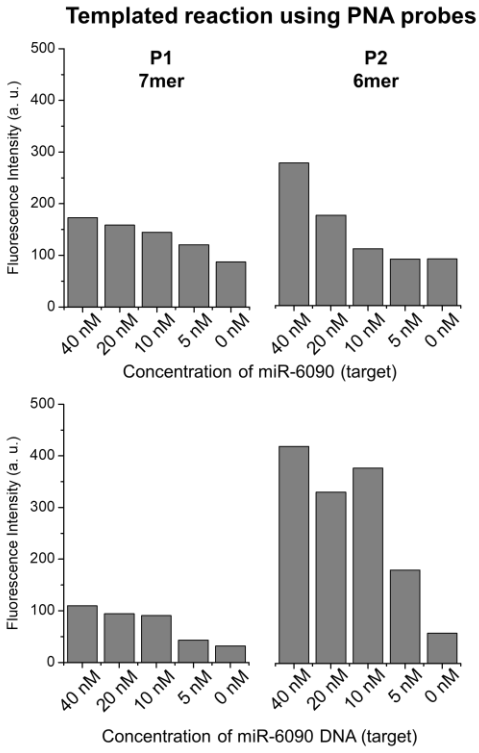
2. Supplementary figures

Figure S1: Results of probe length optimization for miR-6090 DNA. The 8mer probe, **H2**, showed the best performance. Reaction conditions: 200 nM of fluorescein probe, 20 nM of **R1**, pH 7.2 1×PBS, 200 μL, 25 ℃, λ_{exc}: 490 nm, λ_{emi}: 526 nm, cut-off: 515 nm. Statistical significance was calculated using a two sample t-test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).



Name	Sequence
miR-6090 DNA	5′-GGG GAG CGA GGG GCG GGG C-3′
R1	3′-**Rf**-CCC CGC CCC G-5′
H1	3′-CCC CTC GCT-**FAM-H**-5′
H2	3′-CC CTC GCT-**FAM-H**-5′
H3	3′-C CTC GCT-**FAM-H**-5′
H4	3′-CTC GCT-**FAM-H**-5′

Figure S2: Nucleic acid-templated reaction using PNA probes. Even in the presence of 40 nM of targets, fluorescence enhancement was not significant (less than 400) compared with that of DNA probes. Reaction conditions: 200 nM of **P1** or **P2**, 20 nM of **R1**, pH 7.2 1×PBS, 200 μL, 25 ℃, λ_{exc}: 490 nm, λ_{emi}: 526 nm, cut-off: 515 nm.



Name	Sequence
miR-6090 DNA	5′-GGG GAG CGA GGG GCG GGG C-3′
miR-6090	5′-GGG GAG CGA GGG GCG GGG C-3′
R1	3′-**Rf**-CCC CGC CCC G-5′
P1	Ct-C CTC GCT-**FAM-H**-Nt
P2	Ct-CTC GCT-**FAM-H**-Nt

Figure S3: Results of templated reaction using $NaBH_4$ or NADH as the reducing agent. The templated reaction using the fluorescein probe reduced from **H2′** by $NaBH_4$ exhibited a consistent increase in fluorescence, whereas the templated reaction using the fluorescein probe reduced from **H2′** by NADH resulted in a decrease in fluorescence even in the presence of the miR-6090 target, which implied that NADH significantly suppresses the target signal during the templated reaction. Based on this result, $NaBH_4$ was selected as the reducing agent.

Reaction conditions for templated reactions: 200 nM of fluorescein probe, 1M $NaBH_4$ or NADH, 40 nM of **R1**, 20 mM $MgCl_2$, pH 7.2 1× PBS, 100 μL, 25 °C, 20 nM miR-6090, 4 h of blue light irradiation, λ_{exc}: 490 nm, λ_{emi}: 526 nm, cut-off: 515 nm.

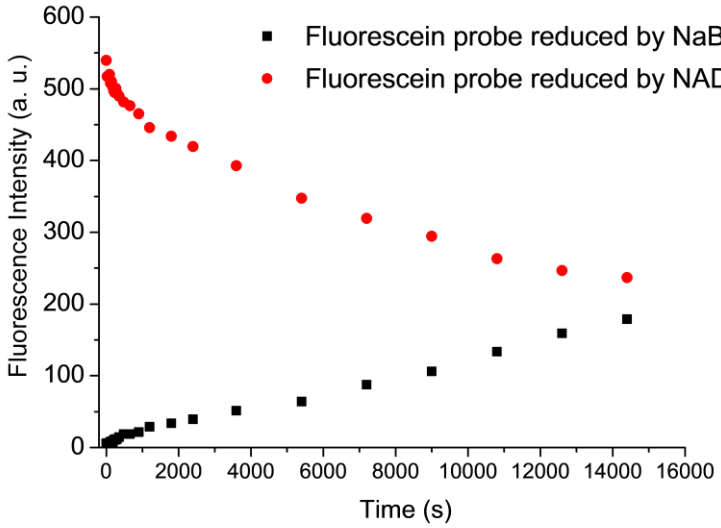


Figure S4: Sensitivity of the designed LFA for DNA detection. 0.05 pmol of fluorescein probe was loaded on the LFA strip. Conditions for templated reaction: 200 nM of FAM probes, 20 nM of **R1** for miR-6090 DNA or 15 nM of **R2** for miR-141 DNA, 20 mM $NaBH_4$, pH 7.2 1×PBS, 20 mM $MgCl_2$, 50 μL, 25 °C, 30 min of blue light irradiation. The LFA results were checked 5 minutes after loading.

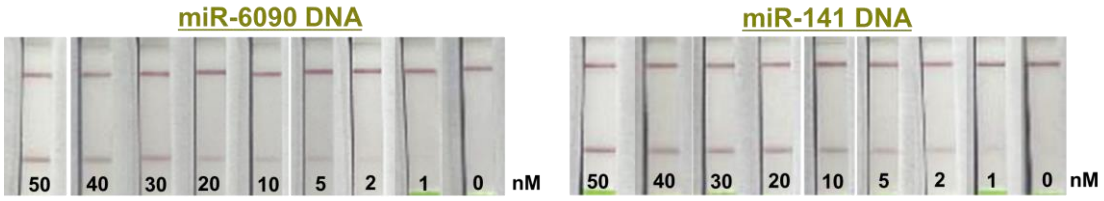


Figure S5: Test line intensity results showing a linear correlation between line intensity and miRNA concentration within the range of 0–5 nM were obtained using miRNAs in deionized (DI) water or in cell lysate (BV-2 cells).

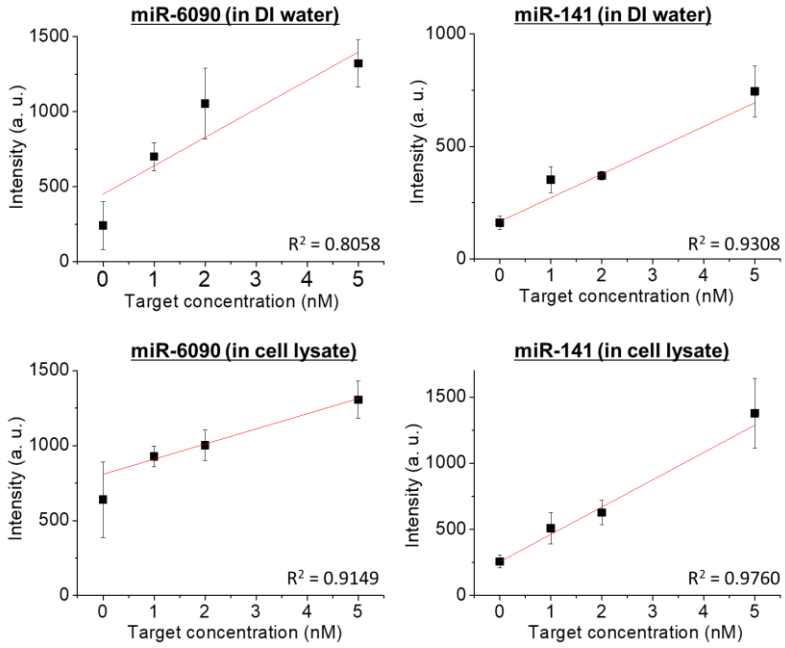


Figure S6: Results of the fluorescein-switching-based LFA using miRNA targets contained in cell lysates (BV-2 cells) are presented. Detailed procedures are provided in the Materials and Methods section. A linear correlation between line intensity and miRNA concentration within the range of 0–5 nM is shown in Figure S5. Detection thresholds of 5 nM (250 fmol) and 1 nM (50 fmol) were observed for miR-6090 and miR-141, respectively.

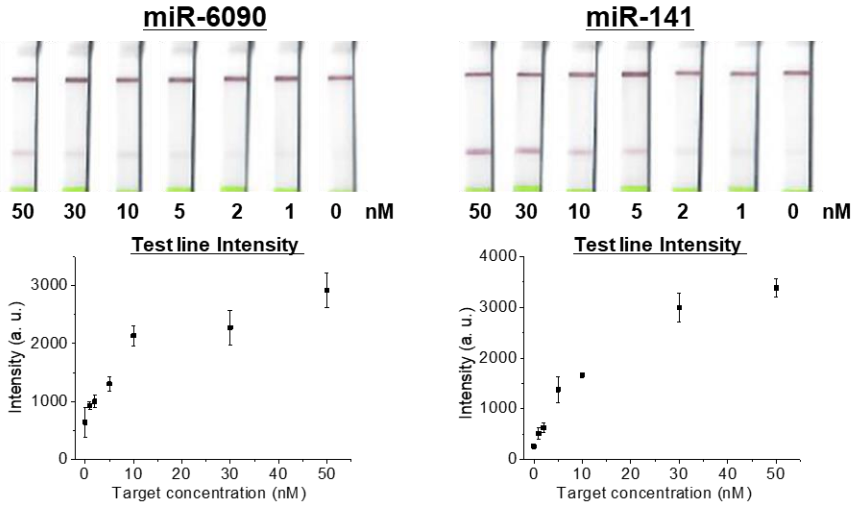
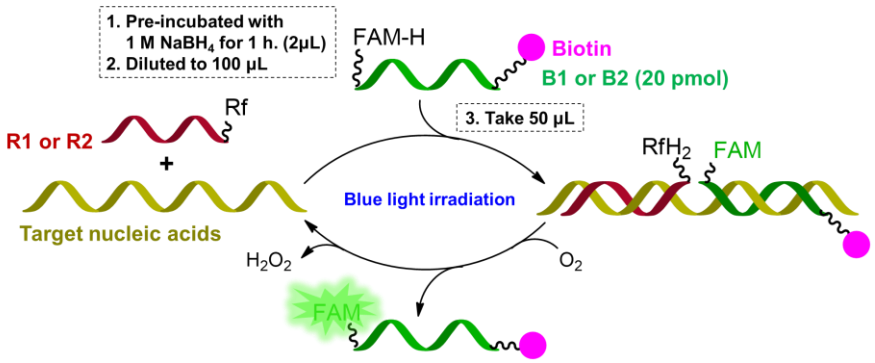


Figure S7: Fluorescence measurement of templated reaction samples for the LFA tests. (a) miR-6090 and (b) miR-141 were used for the target sequences. By fluorescence, 1 nM of the target sequences were clearly detected. Templated reaction conditions for miR-6090: 200 nM of **B1**, 20 mM $NaBH_4$, 40 nM of **R1**, 20 mM $MgCl_2$, pH 7.2 1× PBS, 50 μL, 25 °C, miR-6090 (50,40,30,20,10,5,2,1,0) nM, 3 h of blue light irradiation. Templated reaction conditions for miR-141: 200 nM of **B2**, 20 mM $NaBH_4$, 50 nM of **R2**, 20 mM $MgCl_2$, pH 7.2 1× PBS, 50 μL, 25 °C, miR-141 (50,40,30,20,10,5,2,1,0) nM, 3 h of blue light irradiation, λ_{exc}: 490 nm, λ_{emi}: 526 nm, cut-off: 515 nm.



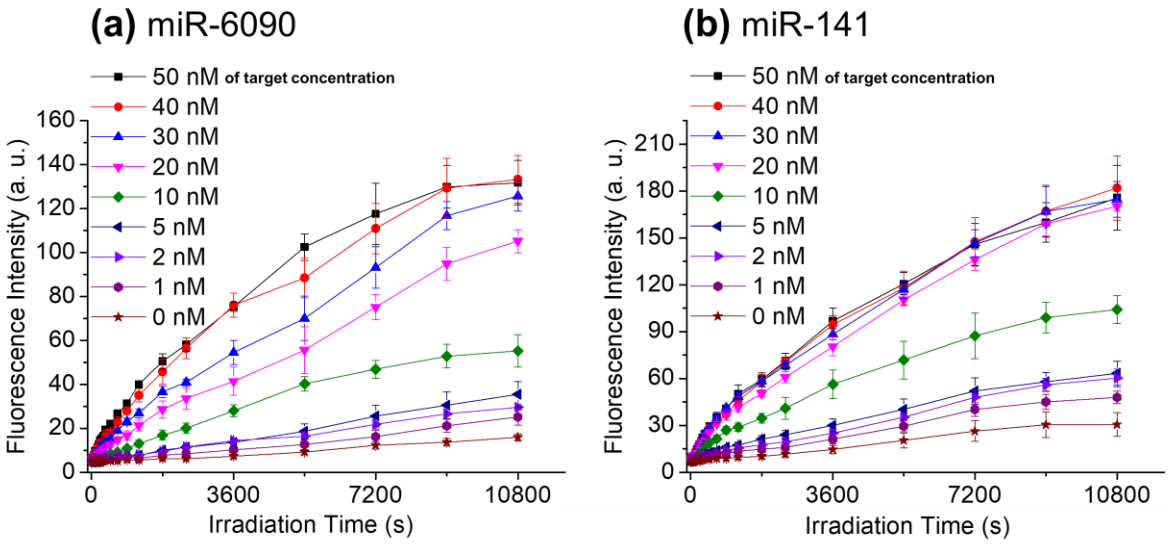
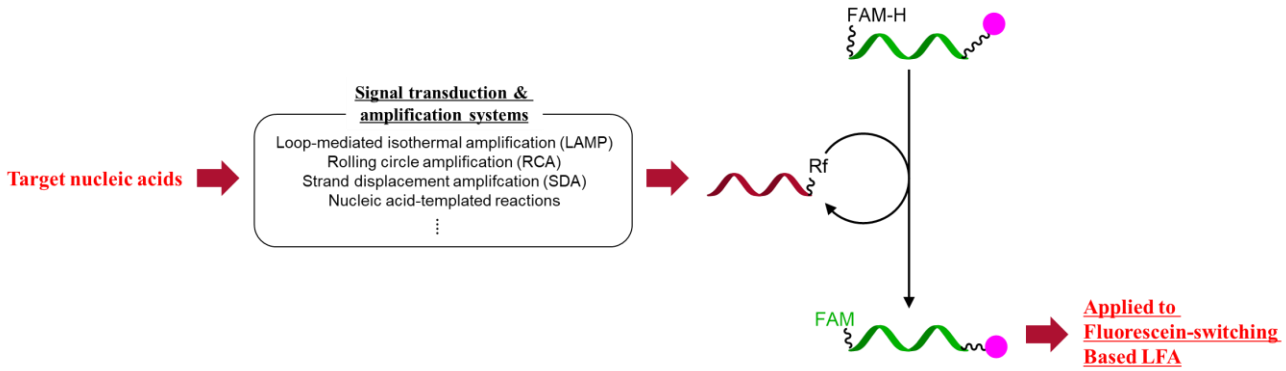


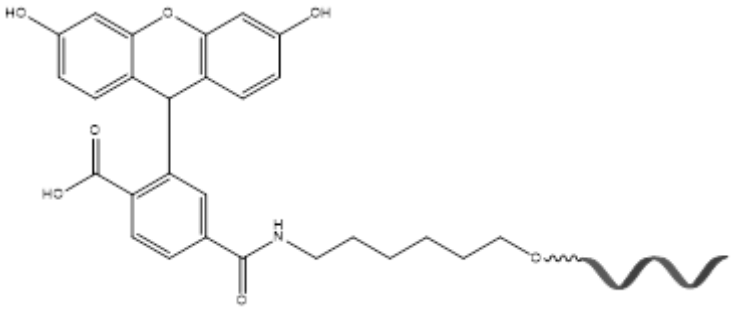
Figure S8: An example of a nucleic acid-based diagnostic system that integrates signal transduction and amplification systems with the developed fluorescein-switching-based LFA. This can be achieved by integrating with a system that amplifies target nucleic acid signals to produce riboflavin oligonucleotides.

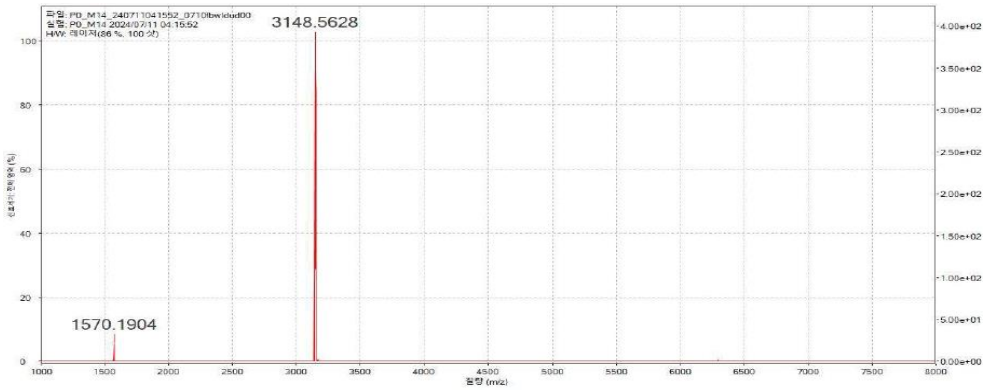


3. Mass results of the synthesized DNAs and PNAs

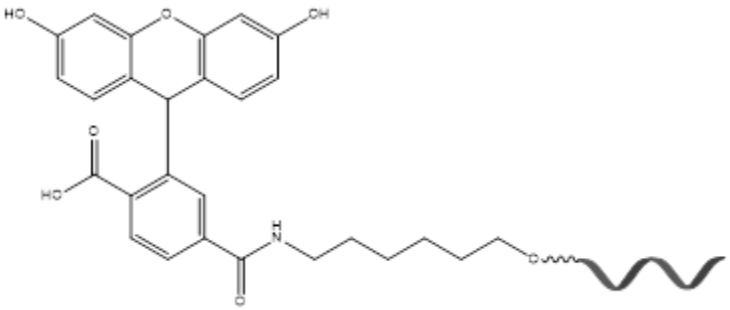
3.1 MALDI-TOF spectra of the synthesized DNAs

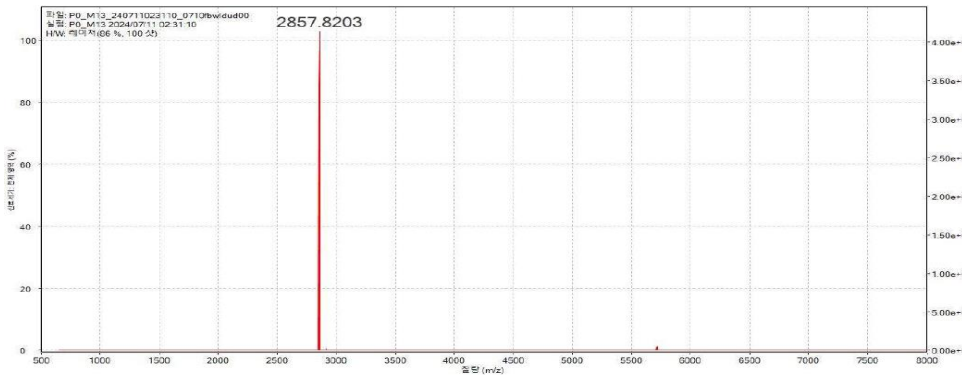
H1 (FAM-H-TCG CTC CCC); exact mass for $C_{111}H_{137}N_{28}O_{63}P_9$: 3148.6016; MALDI-TOF m/z: 3148.5628



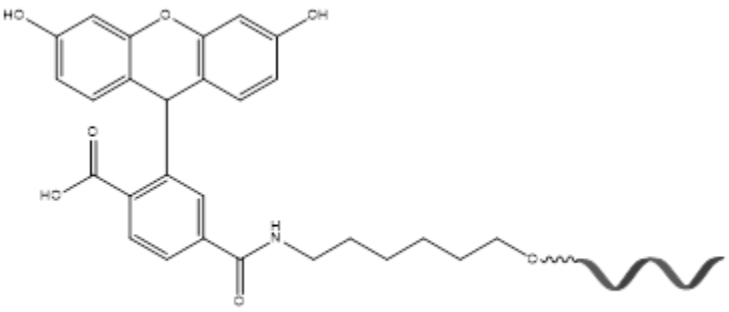


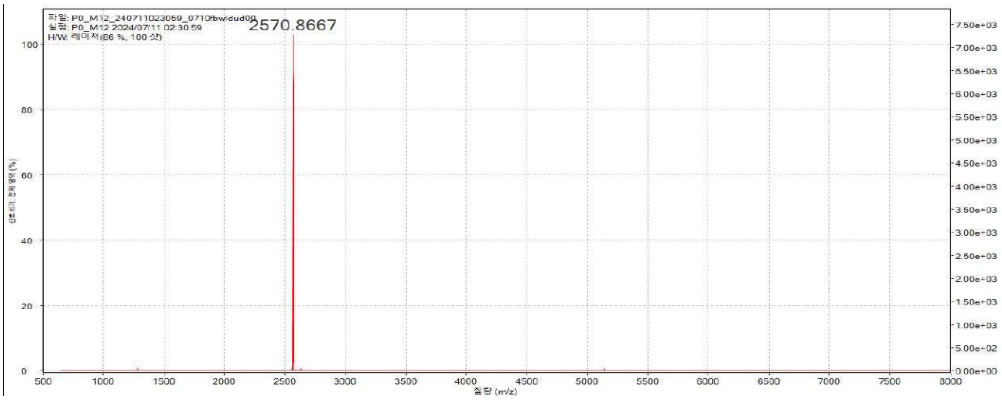
H2 (FAM-H-TCG CTC CC); exact mass for $C_{102}H_{125}N_{25}O_{57}P_8$: 2859.5552; MALDI-TOF m/z: 2857.8203



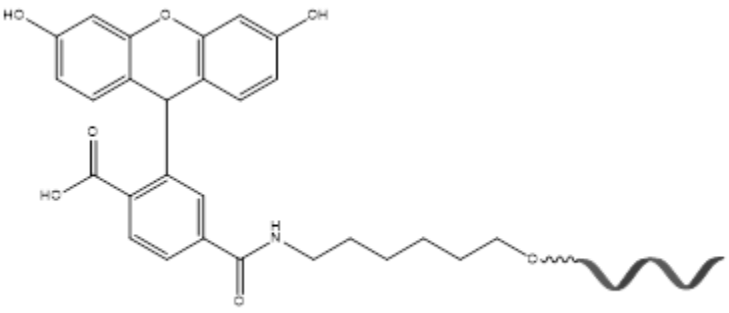


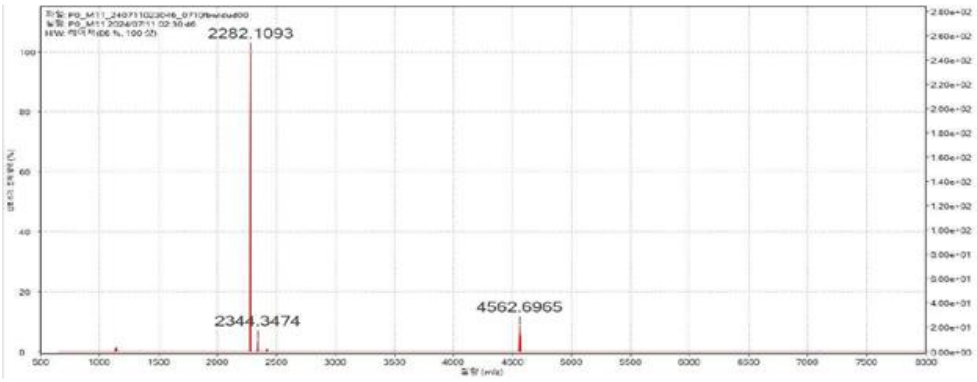
H3 (FAM-H-TCG CTC C); exact mass for $C_{93}H_{113}N_{22}O_{51}P_7$: 2570.5088; MALDI-TOF m/z: 2570.8667



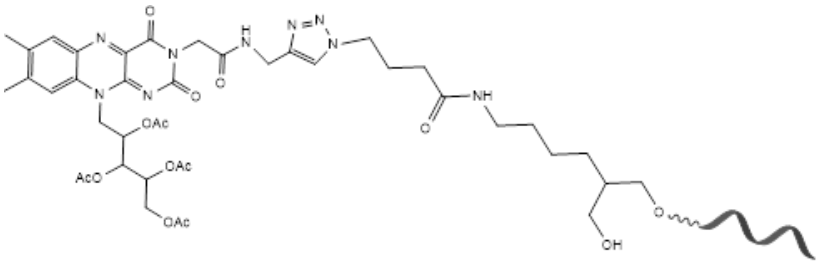


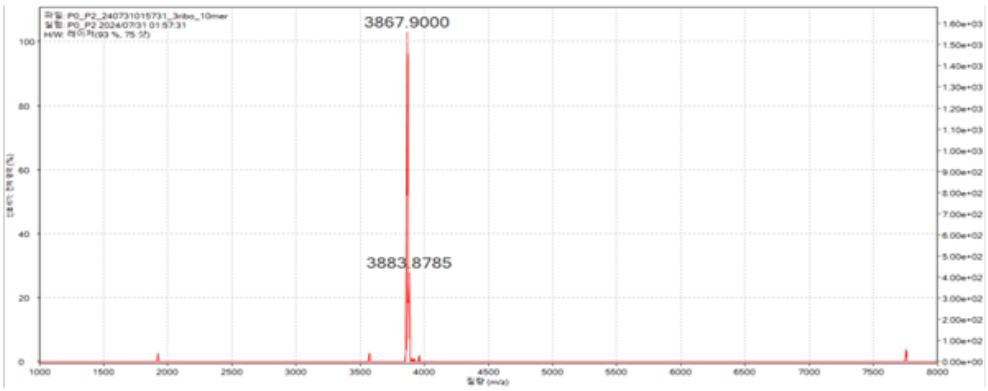
H4 (FAM-H-TCG CTC); exact mass for $C_{84}H_{101}N_{19}O_{45}P_6$: 2281.4625; MALDI-TOF m/z: 2282.1093



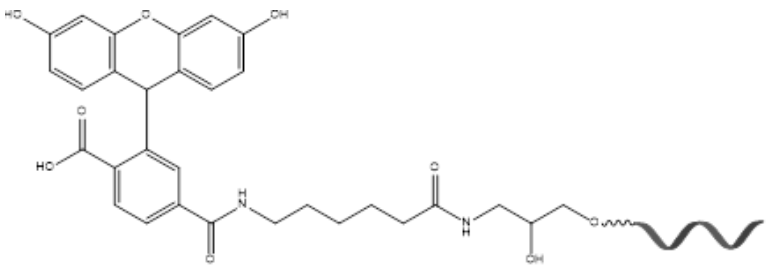


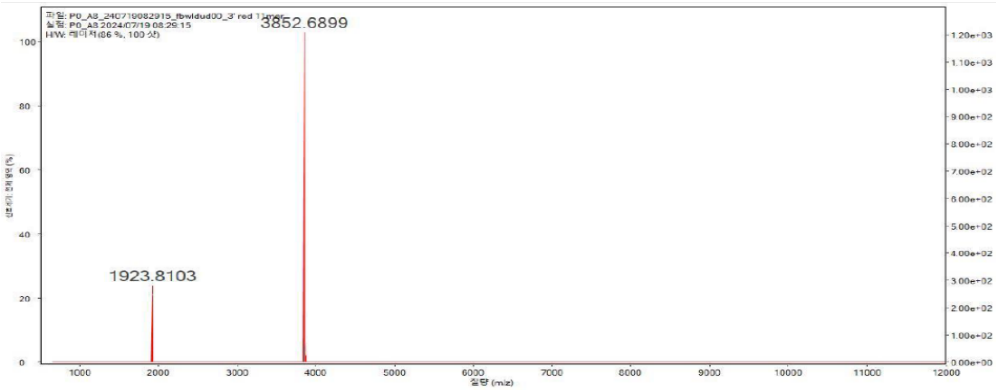
R1 (G CCC CGC CCC-**Rf**); exact mass for $C_{133}H_{175}N_{43}O_{74}P_{10}$: 3867.8629; MALDI-TOF m/z: 3867.9000



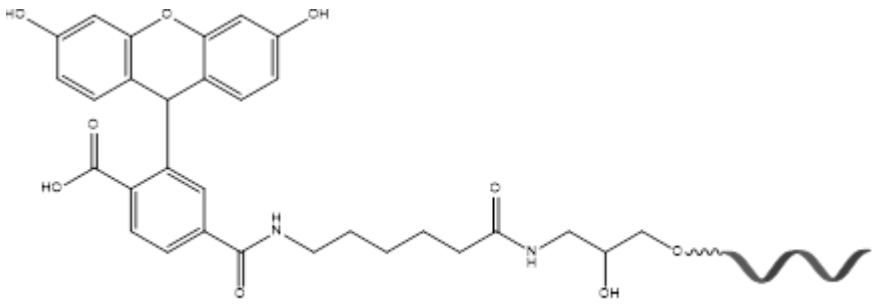


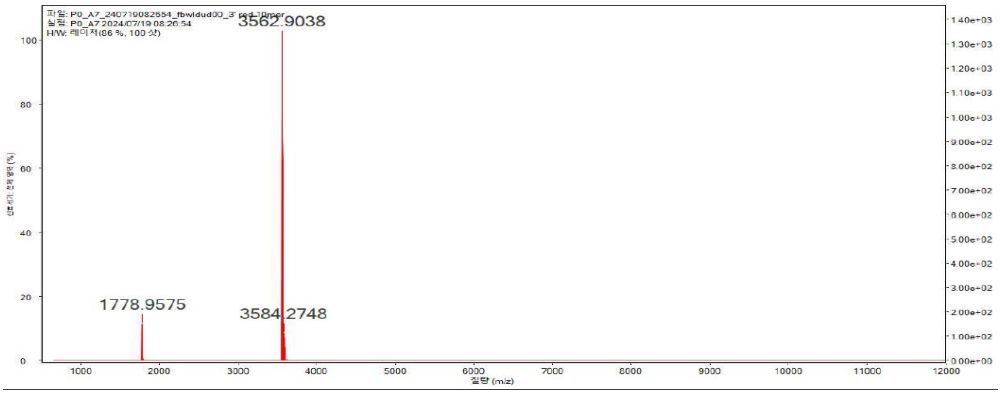
H5 (C CAT CTT TAC C-**FAM-H**); exact mass for $C_{135}H_{168}N_{35}O_{77}P_{11}$: 3851.7420; MALDI-TOF m/z: 3852.6899



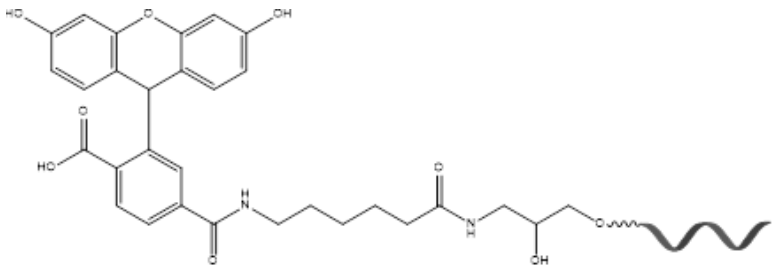


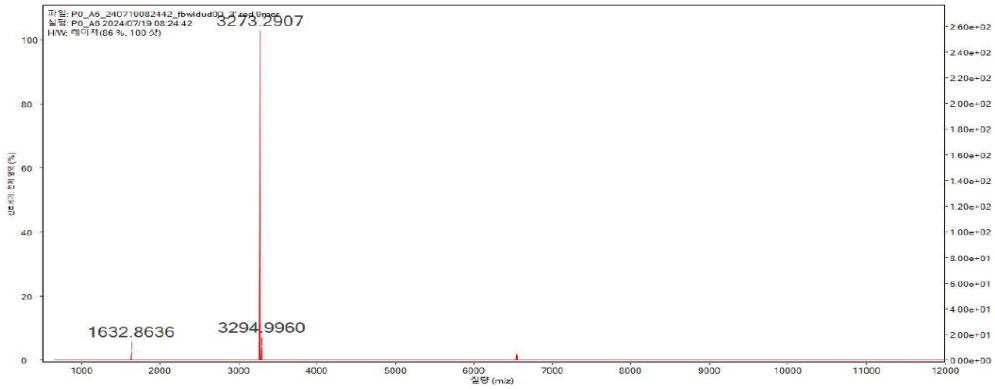
H6 (CAT CTT TAC C-**FAM-H**); exact mass for $C_{126}H_{156}N_{32}O_{71}P_{10}$: 3562.6956; MALDI-TOF m/z: 3562.9038



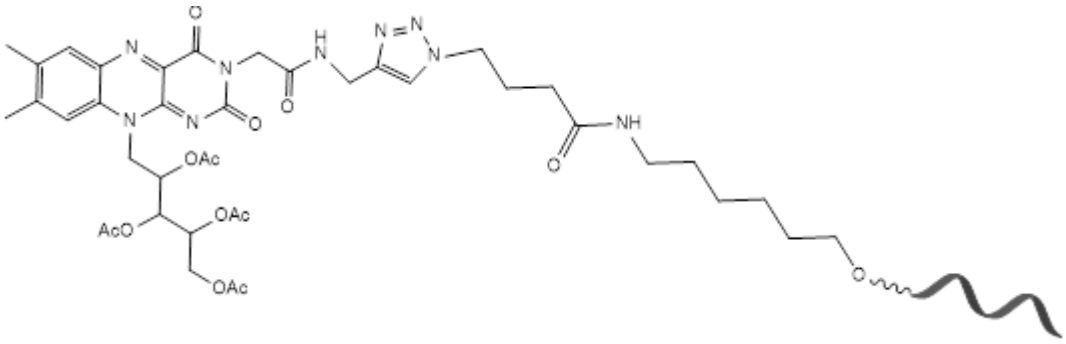


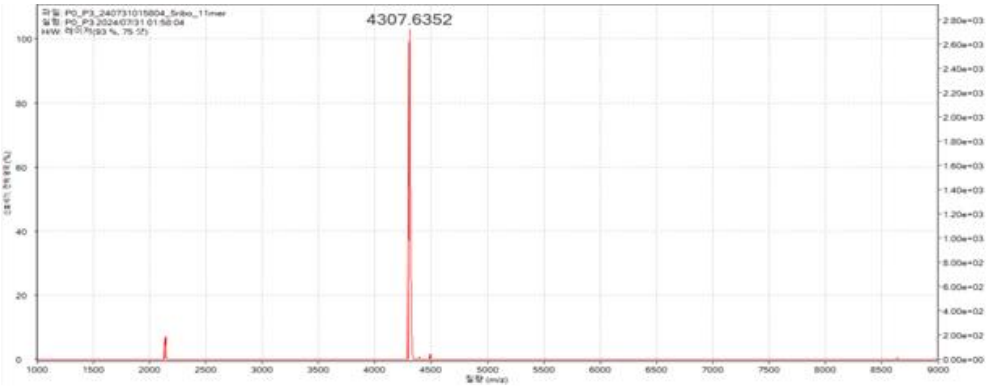
H7 (AT CTT TAC C-**FAM-H**); exact mass for $C_{117}H_{144}N_{29}O_{65}P_9$: 3273.6493; MALDI-TOF m/z: 3273.2907



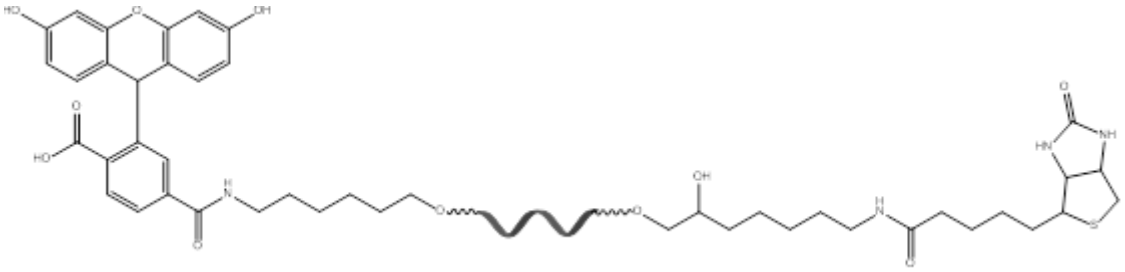


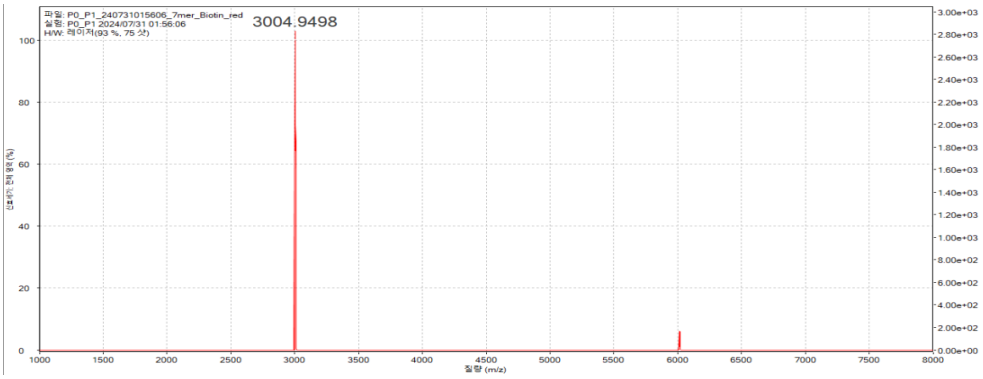
R2 (Rf- AG ACA GTG TTA); exact mass for $C_{149}H_{188}N_{53}O_{78}P_{11}$: 4307.9487; MALDI-TOF m/z: 4307.6352



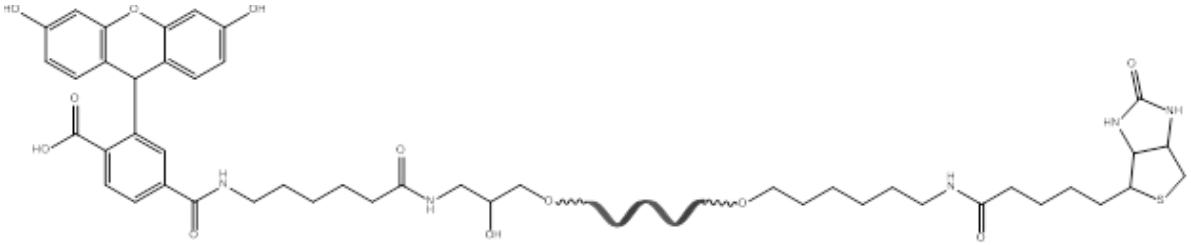


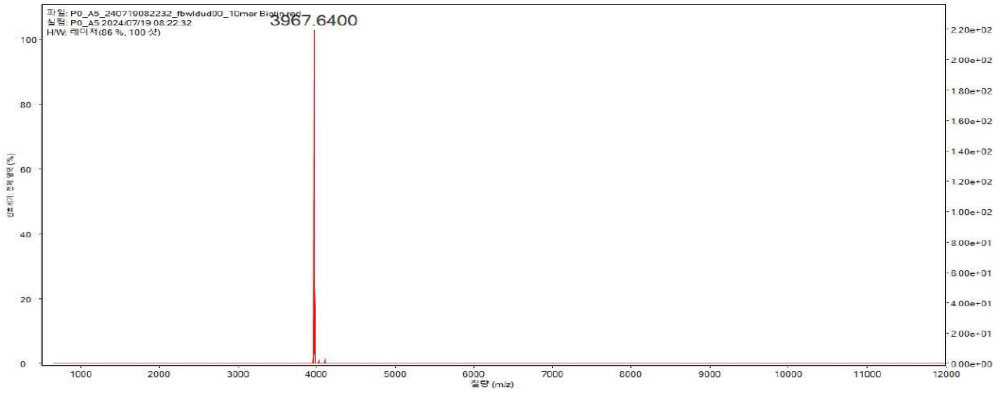
B1 (FAM-H-TCG CTC C-**Biotin**); exact mass for $C_{110}H_{143}N_{25}O_{57}S_1P_8$: 3005.6681; MALDI-TOF m/z: 3004.9498



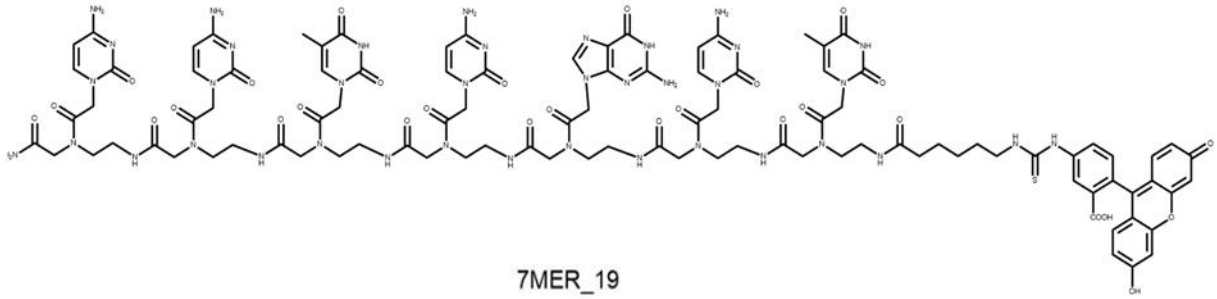


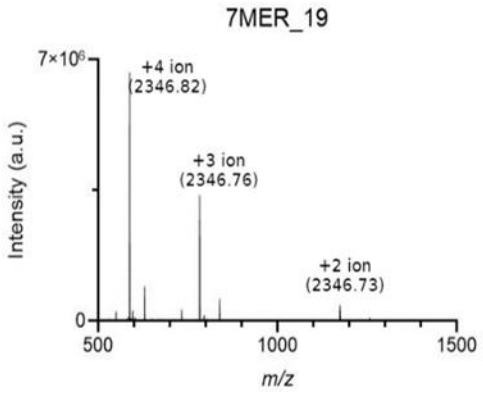
B2 (Biotin-CAT CTT TAC C-**FAM-H**); exact mass for $C_{142}H_{184}N_{35}O_{76}S_1P_{11}$: 3967.8444; MALDI-TOF m/z: 3967.6400



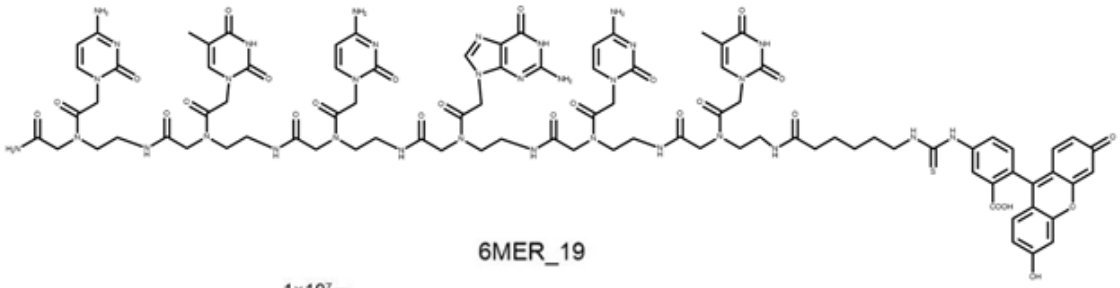


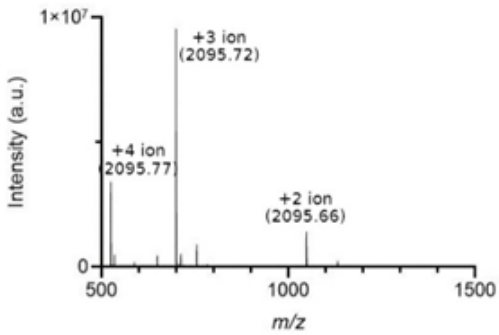
P1 ((N-ter) **FAM**-TCG CTC C (C-ter)); exact mass for $C_{100}H_{118}N_{38}O_{29}S$: 2346.8648, ESI-MS found: 2346.77.





P2 ((N-ter) **FAM**-TCG CTC (C-ter)); exact mass for $C_{90}H_{105}N_{33}O_{26}S$: 2095.7629; ESI-MS found: 2095.72





4. References

(1) Kim, H.; Choi, H.; Min, K. S.; Han, W. J.; Park, J. W.; Kim, K. T. Riboflavin-catalyzed templated reaction to translate nucleic acid cues into signals of rhodamine derivatives. *Chem. Commun.* **2022**, *58*, 13743-13746.