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Supporting Information

Transcription immunoassay: Light-up RNA aptamer-based immunoassay using in vitro transcription

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Experimental Procedures

Reagents and materials

T7 RNA polymerase was purchased from Takara (Shiga, Japan). (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-2-methyl-1-(2,2,2-trifluoroethyl)-1H-imidazol-5(4H)-one (DFHBI-1T) was purchased from Tocris Bioscience (Bristol, UK). Recombinant human AFP, anti-human AFP antibody, and biotinylated anti-human AFP antibody were obtained from Meridian Life Science (Memphis, TN, USA). AFP-free human serum was purchased from Cone Bioproducts (Seguin, TX, USA). 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from BD Biosciences (San Jose, CA, USA), and Amplex ultrared reagent was purchased from Invitrogen (Carlsbad, CA, USA). Bovine serum albumin (BSA), b-BSA (biotinylated BSA), streptavidin (STA), and Tween 20 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 3-aminopropyl triethoxysilane (APTES) and glutaraldehyde solution (GA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was obtained from Biosesang (Daejeon, Korea). One Taq polymerase was purchased from New England Biolabs (Ipswich, MA, USA).

Preparation of template DNA for transcription

Spinach-2 (see Table S1 for sequence information), cloned in the pUC57 plasmid, was PCR-amplified with primers against the T7 promoter and T7 terminator. PCR was conducted using forward primer (5′-CCAAGCTTGCATGCAGGCCTCTGCAGTCGA-3′) and reverse primer (5′-CTAGTTATTGCTCAGCGG-3′). PCR was performed according to the manufacturer's protocol, as follows: 5 min of denaturation at 94°C; 30 cycles of 94°C for 30 sec, 53°C for 30 sec, and 68°C for 25 sec; followed by a final extension step at 68°C for 5 min. PCR products including biotin on the 5′ end near the T7 promoter (b-Spi2), biotin on the 5′ end near the T7 terminator (Spi2-b), or biotin on both ends (b-Spi2-b) were amplified. All PCR products were purified using a commercial PCR clean up kit (Promega, Madison, WI, USA) prior to use. The concentration of each purified PCR product was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific)

Characterization of STA/b-BSA Dendrimers

The morphologies of STA/b-BSA dendrimers were characterized by scanning electron microscopy (SEM). In order to clean the silicon wafer surface, the wafer was immersed in pure ethanol and sonicated for 10 min and thoroughly cleaned by rinsing with ethanol and then dried with gentle N_2 flow. The silicon wafer surface was modified by 1% APTES in pure ethanol for 2 h. Residues of APTES were washed out with ethanol flow and then dried with N_2 gas. To modify the amine surface to the aldehyde surface, 2% GA in PBS was reacted for 2 h. The capture antibody (2 μg/mL, 100 μL) was immobilized on the surface through the amino-coupling and subsequently blocked with 2% BSA in PBS. The chip was rinsed with flowing PBST (PBS at pH 7.4 containing 0.1% Tween 20) for three times. AFP (43 fM,

100 μL) was added to the surface and reacted for 1 h. Then, biotinylated anti-AFP antibody (1 μg/mL, 100 μL) was added to the surfaces. For dendrimer structure, 100 μL of b-BSA (100 μg/mL in PBS) and 100 μL of STA (100 μg/mL in PBS) were sequentially added after STA was bound to the biotinylated anti-AFP antibody. Multiple cycles were performed by following the same steps. Finally these silicon wafers were washed with deionized water and dried with N_2 flow. The morphologies of STA/b-BSA dendrimers on the silicon wafers were observed and recorded using an SU8230 scanning electron microscope (Hitachi, Japan).(Figure S5)

In vitro transcription

The PCR products were used for *in vitro* transcription using T7 RNA polymerase. Each reaction mixture was incubated at 37°C for 2 h. Afterward, the 10-μL transcription mixture was mixed with 90 μL of folding buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 125 mM KCl) with DFHBI-1T (final concentration: 1 μM) and incubated at room temperature for 5 min. Fluorescence was measured using a VICTOR microplate reader (Model 2030-0050; PerkinElmer, Waltham, MA, USA).

Transcription immunoassay

First, 96-well plates were coated with 100 μL of anti-AFP antibody (2 μg/mL) at 37°C for 2 h. After washing the plate three times with 200 μL PBST (PBS at pH 7.4 containing 0.1% Tween 20), the wells were blocked with blocking buffer (300 μL of 2% BSA in PBST) at 37°C for 1 h and then washed again. After washing, various concentrations of AFP in PBS were added to the wells and incubated at room temperature for 1 h. After incubation, the wells were washed three times with PBST followed by the addition of 100 μL per well of 1 μg/mL biotinylated anti-AFP antibody diluted with PBS. After incubation for 1 h, the excess detection antibody was removed by washing the wells five times with washing buffer. For transcription immunoassay with a single aptamer, 18 μM of biotinylated PCR product was added to each well and attached to a biotinylated anti-AFP antibody using STA as a linker. For dendrimer structure-based transcription immunoassay, 100 μL of b-BSA (100 μg/mL in PBS) and 100 μL of STA BSA (100 μg/mL in PBS) were sequentially added after STA was bound to the biotinylated anti-AFP antibody. Multiple cycles were performed by following the same steps described above. Biotinylated PCR products were then added after the b-BSA/STA layer was completed. Unbound PCR products were removed by washing the plate five times with PBST. After rinsing with PBST, each well of the plate was supplied with 40 μL of the reaction mixture for transcription. Fluorescence was measured after 2 h of incubation at 37°C.

For conventional enzyme-linked immunosorbent assay (ELISA), 1 μg/mL STA-conjugated horseradish peroxidase (STA-HRP) instead of STA was bound to the biotinylated AFP antibody. After washing, the wells were supplied with 100 μL of Amplex red substrate solution and then incubated at room temperature for 15 min. Fluorescence detection was performed using a VICTOR microplate reader (Model 2030-0050; PerkinElmer) at excitation/emission wavelengths of 485/535 nm. For

colorimetric detection, 100 μL of TMB was added in place of the Amplex red substrate solution and then incubated at room temperature for 15 min. Absorbance at 450 nm was measured using a VICTOR microplate reader (Model 2030-0050; PerkinElmer).

Supplemental Table and Figures

Name	Sequence
Spinach-2	TAATACGACTCACTATAGGGGATGTAACTGAATGAAATGGTGAAGGACG GGTCCAGTAGGCTGCTTCGGCAGCCTACTTGTTGAGTAGAGTGTGAGCT CCGTAACTAGTTACATCCCGCTGAGCAATAACTAG

Table S1. Nucleotide sequence of Spinach-2

Concentration (fM)	Human serum sample	
	Recovery (%)	RSD (%)
0.89	94.6	2.22
7.14	101.9	7.41
14.29	108.6	3.25

Table S2. Recovery rate for concentrations of AFP added to 50% human serum

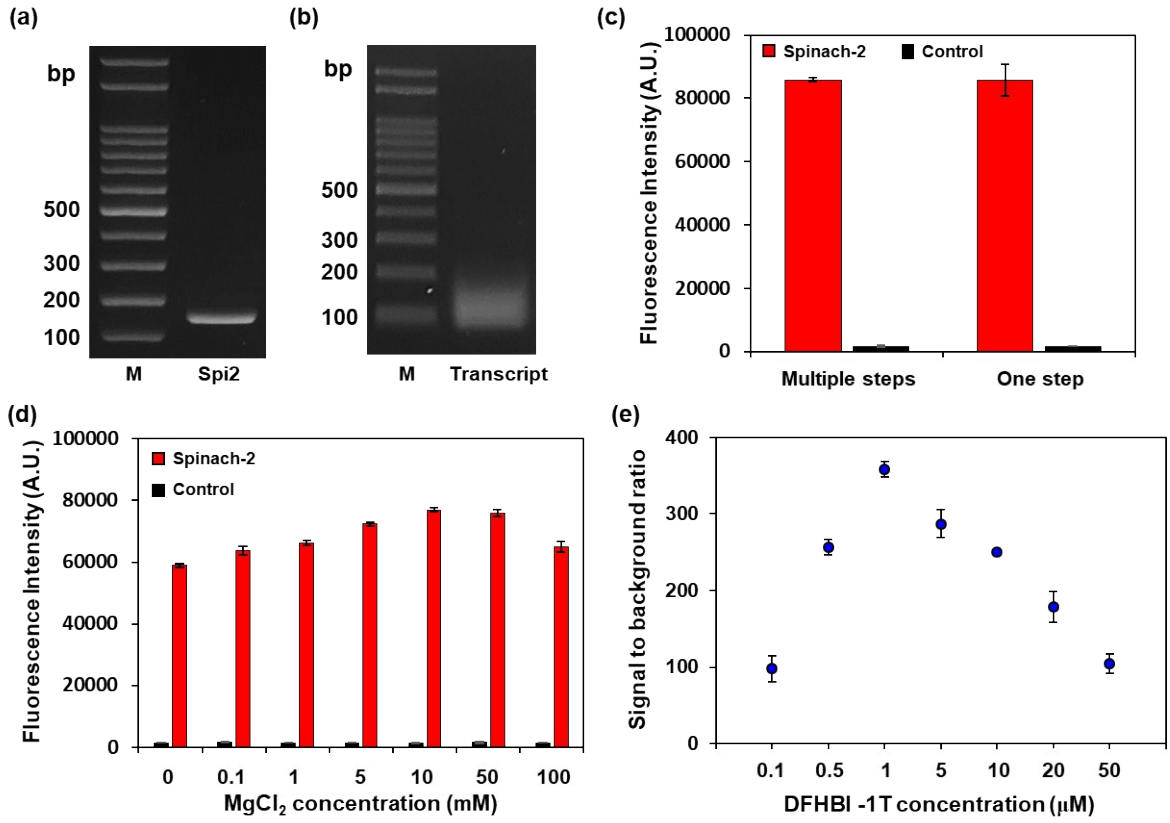

Fig. S1 Optimization of Spinach-2 transcript folding conditions. (a) The template DNA containing the sequence of the T7 promoter, Spinach-2, and T7 terminator was prepared by PCR. The amplified PCR product was confirmed by 1.2% agarose gel electrophoresis. (b) Spinach-2 RNA aptamer was transcribed from template DNA. The Spinach-2 transcript was confirmed by 1.2% formaldehyde gel electrophoresis. M: DNA marker, Spi2: Spinach-2-encoding PCR product, Transcript: transcribed Spinach-2 RNA. (c) The effect of folding protocol on the fluorescence of the Spinach-2 transcript. For multiple steps, transcripts were folded by incubation at 90°C for 2 min, followed by cooling to 65°C and then further cooling to room temperature. For one step, transcripts were incubated at room temperature for 10 min. Optimization of concentrations of $MgCl_2$ (d) and DFHBI-1T (e) in the folding efficiency of Spinach-2 transcript. For *in vitro* transcription, 20 μM of PCR product was incubated with transcription mixture at 37°C for 2 h. The control (background) is the fluorescence signal obtained in the absence of PCR products. Error bars indicate the standard deviations of three independent measurements.

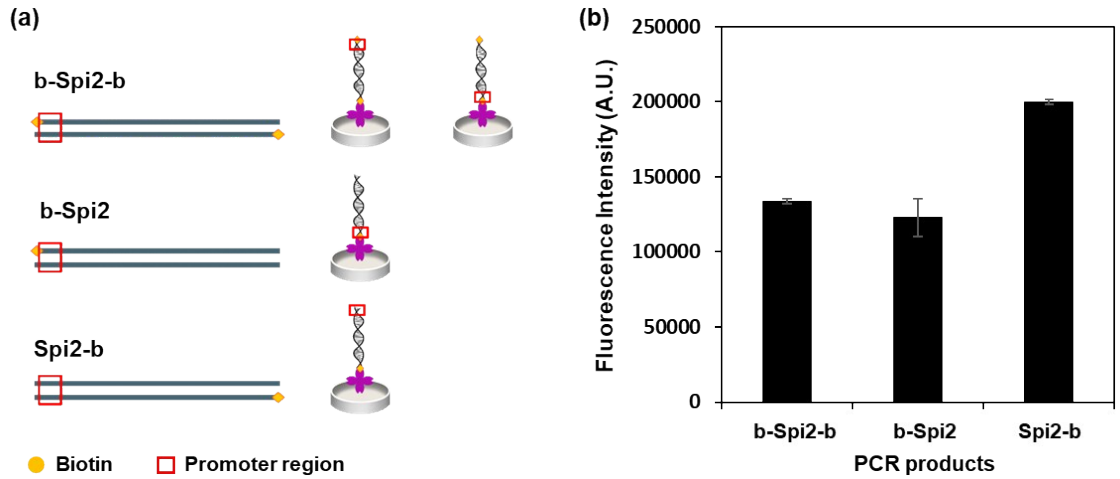

Fig. S2 Transcription efficiency dependent on promoter position of immobilized PCR products. (a) A schematic of the biotinylated PCR products immobilized on the STA surface. Three kinds of biotinylated PCR products were prepared. b-Spi2-b, biotin on both ends of the PCR product; b-Spi2, biotin on the 5′ end near the T7 promoter region of the PCR product; Spi2-b, biotin on the 5′ end near the T7 terminator region of the PCR product. Biotinylated PCR product (1 μM) was immobilized on the STA-coated surface, and transcription efficiency was determined by the fluorescence signal from the transcribed Spinach-2 aptamer. (b) The fluorescence intensity of the Spinach-2 transcripts from three immobilized PCR products. Error bars indicate the standard deviations of three independent measurements.

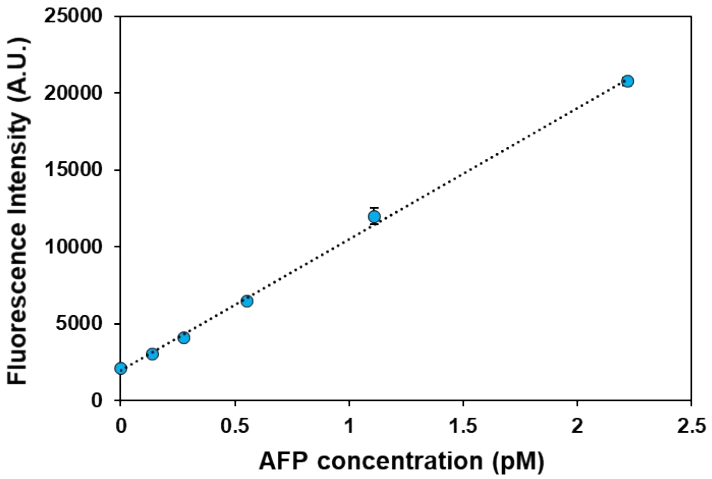

Fig. S3 Transcription immunoassay for the detection of AFP. Standard curve of the transcription immunoassay performed with various concentrations of AFP (0–2.2 pM) under optimized conditions. The regression equation of the immunoassay was $y = 8560.3x + 1936.3$ ($R^2 = 0.9981$) in the linear range. Each data point is the average of N=3 individual measurements, and error bars indicate standard deviations.

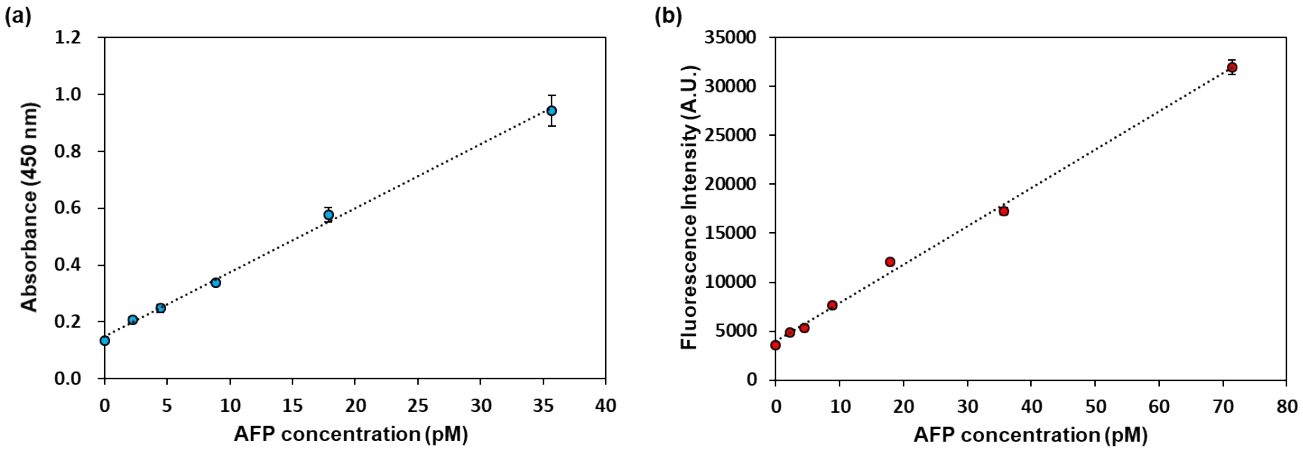

Fig. S4 Conventional ELISA for the detection of AFP. Calibration curves of the (a) colorimetric and (b) fluorescence ELISA performed with various concentrations of AFP. The regression equations were $y = 1243x + 2128$ ($R^2 = 0.9975$) and $y = 1388.2x + 2426.7$ ($R^2 = 0.9967$) of colorimetric and fluorescence ELISA in the linear range, respectively. Each data point is the average of N=3 individual measurements, and error bars indicate standard deviation.

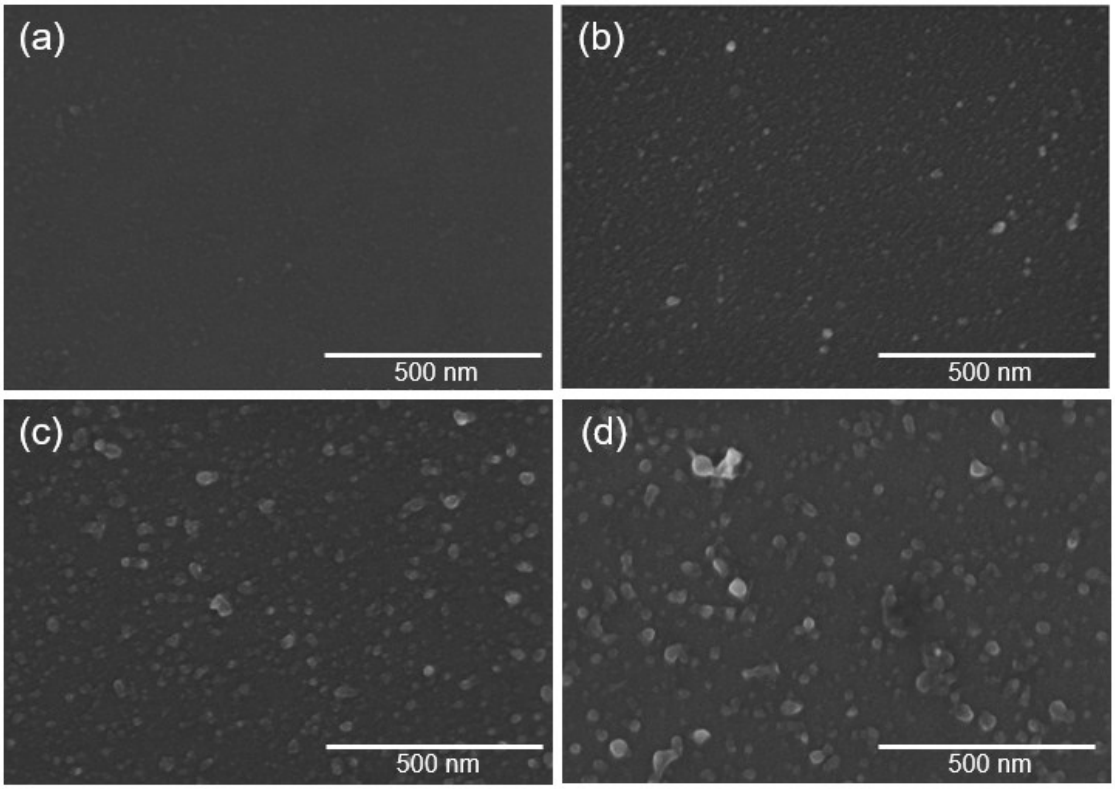

Fig. S5 SEM images of (a) control; capture antibody surface, (b) 1 layer, (c) 4 layer, (d) 12 layer of STA/b-BSA dendrimers on the immunocomplex. One layer is defined as a layer of STA added to a layer of b-BSA. STA and b-BSA are added sequentially to form a dendrimer structure on the immunocomplex as the number of layers increases.

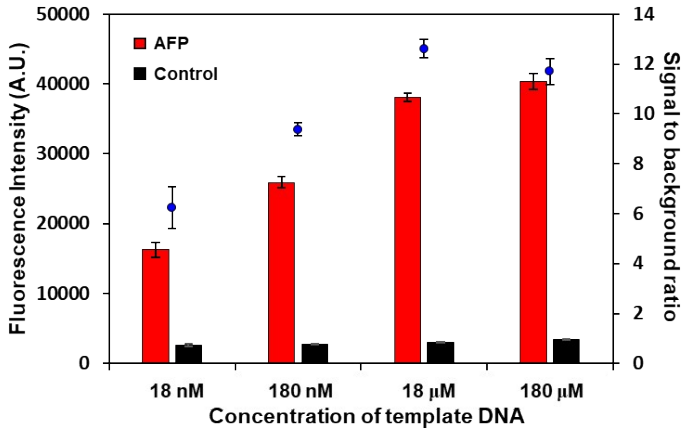

Fig. S6 Optimization of the concentration of template DNA in dendrimer structure-based transcription immunoassay. AFP (43 fM) was analyzed for condition optimization. The control (background) is the fluorescence signal obtained in the absence of AFP. Error bars indicate the standard deviations of three independent measurements.

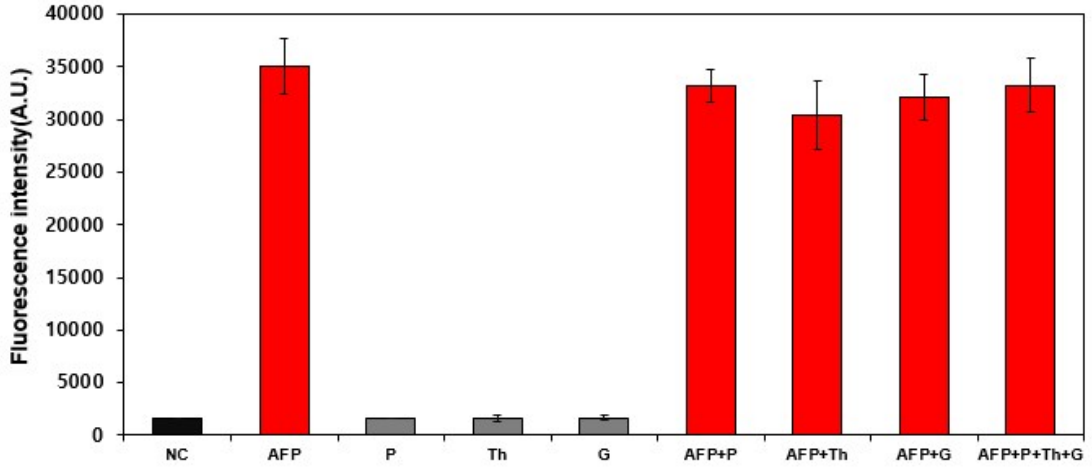

Fig. S7 Specificity analysis of the dendrimer structure-based transcription immunoassay. The concentrations of the AFP was 14 fM while other interfering substances (P; Prostate-specific antigen, Th; Thrombin, G; Glucose) were 1000 folds. Each data point is the average of N=3 individual measurements, and error bars indicate standard deviation.