Electronic Supplementary Information

A highly sensitive, multiplex immunoassay using gold nanoparticle-enhanced signal amplification

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EXPERIMENTAL SECTION

Materials

Streptavidin-HRP was purchased from Abcam (Cambridge, UK). EZ-Link NHS-PEG12-Biotin was obtained from Thermo scientific (Rockford, IL, USA). AFP and Anti-AFP antibodies were purchased from Fitzgerald (Acton, MA, USA) and FABP and anti-FABP antibodies were from Meridian Life Science (Memphis, TN, USA). Bradford assay solution was purchased from Bio-Rad (Hercules, CA, USA), TMB substrate solution and Stop solution were from GenDepot (Barker, TX, USA), Protector RNase Inhibitor (PRI) was from Roche (Mannheim, Germany), gold nanoparticles were from British Biocell International (Cardiff, UK), and RNase H was from Takara (Otsu, Japan). DNA oligonucleotides (thiol-$(polyethylene\ glycol)_6$-ACTCTATGGG for AFP and thiol-$(polyethylene\ glycol)_6$-AGCGTTGTAG for FABP) and RNA probes (TAMRA-CCCAUAGAGU-BHQ2 for AFP and Cy5-CUACAACGCU-BHQ2 for FABP) were synthesized by Bioneer (Daejeon, Korea). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of biotinylated detection antibody for ELISA and OLISA

70 µL of 1 mg/mL detection antibody (dAb) in PBS was incubated for 1 h at room temperature with 0.7 µL of 20 mM EZ-Link NHS-PEG12-Biotin. The biotinylated antibodies were purified by the use of Zeba Spin Desalting Columns, 40K MWCO (Thermo scientific) and quantified by Bradford assay.

ELISA

Each well of a transparent Maxisorp 96-well microplate (Thermo scientific) was coated with 100 µL of cAbs (3 µg/mL) by incubation overnight at 4 °C, followed by washes with PBS (3×300 µL). The cAb coated-wells were blocked with 200 µL of Blocking Buffer (3% BSA in PBS), incubated for 1 h at room temperature, and rinsed with PBST (0.05% Tween-20 in PBS, 3×300 µL). Protein solutions of varying concentrations (0, 0.625, 1.25, 2.5, 5, and 10 ng/mL) in 100 µL of Assay Buffer (1% BSA in PBST) were added to the wells, followed by incubation for 1 h at room temperature. The plate was then washed with PBST (3×300 µL) before addition of the biotinylated dAbs (100 µL, 3 µg/mL in Assay Buffer) and incubation for 1 h at room temperature. After rinsing with PBST (3×300 µL), streptavidin-HRP (2 µg/mL) in Assay Buffer (100 µL) was added and incubated for 1 h at room temperature. The plate was further washed with PBST (3×300 µL), and each well was incubated in TMB substrate solution (100 µL) for 10 min at room temperature. After addition of TMB stop solution (100 µL) to the each well, optical density at 450 nm was immediately measured by means of a 96-well microplate reader (SpectraMax Plus™, Molecular Devices, Sunnyvale, CA, USA).

Preparation of dAb-GNP-DNA complexs

1 mL of 10 nm gold colloid was incubated for 30 min at room temperature with 14 µg of monoclonal dAb under gently shaking. After adding Tween 20 and phosphate buffer, pH 8.0 at a final concentration of 0.05% and 10 mM, respectively, the particles were then modified with 100 µL of 100 µM 5'-thiolated DNA oligonucleotide under gently shaking at 4°C. After 1 h incubation, the solution was further salted by adding 30 µL of 5 M NaCl, followed by gently shaking overnight at 4°C. Functionalized GNPs were stabilized for 1 h by adding 100 µL of 10% BSA. Next, the solution was centrifuged for 50 min at 4°C and 18,000 g, and supernatant was removed. This centrifugation procedure was repeated two times and the final pellet was resuspended in 1 mL of PBST containing 0.1% BSA. The concentration of GNP was determined by measuring absorbance. The wavelength of absorbance maximum and extinction coefficient were 522 nm and 1.024×10^8 $M^{-1}cm^{-1}$, respectively. Zeta potential of the prepared dAb-GNP-DNA complexes was measured at 25 °C using a particle

analyzer (Zetasizer, Malvern Instruments, UK). The zeta potential values of dAb_{AFP}-GNP-DNA and dAb_{FABP}-GNP-DNA were -24.1 ± 1.0 and -26.6 ± 1.5 mV, respectively.

Determination of the number of DNA oligonucleotides per GNP

To determine the number of the DNA oligonucleotides on a dAb-GNP-DNA complex, RNase H reaction-based method was used. 100 μL of RNase H reaction solutions (100 nM F-RNA-Q probe, 0.4 U of PRI, 6 U of RNase H, 40 mM Tris-HCl, 4 mM $MgCl_2$, 10 mM DTT, 0.003% BSA, pH 7.7) containing serially diluted DNA or GNP were incubated for 15 min at 37°C and then fluorescence intensities were measured by Appliskan (Thermoscientific, Walthan, MA, USA) with excitation/emission filter set of 544/589 and 590/675 nm for TAMRA and Cy5, respectively. The concentrations of the released DNA oligonucleotides were determined by interpolation from a standard linear calibration curve. The average number of oligonucleotides per GNP was calculated by dividing the concentration of oligonucleotides by the concentration of GNP.

OLISA and GNP-OLISA

Maxisorp Black 96-well microplates (Thermo scientific) were coated with cAbs (2 μg/mL) in PBS (100 μL/well) by incubation overnight at 4 °C. Each well was then washed with PBS (3×300 μL), blocked with Blocking Buffer (200 μL), and incubated for 1 h at room temperature. After rinses with PBST (3×300 μL), biomarker solutions of varying concentrations in 100 μL of Assay Buffer were added to the wells, followed by incubation for 1 h at room temperature. The plate was washed with PBST (3×300 μL) before addition of biotinylated dAb (100 μL, 3 μg/mL in Assay Buffer) and incubated for 1 h at room temperature. After rinsing with PBST (3×300 μL), streptavidin (2 μg/mL) in Assay Buffer (100 μL) was added and incubated for 30 min at room temperature. After rinsing with PBST (3×300 μL), biotinylated DNA probe in Assay Buffer (100 μL) was added and incubated 30 min at room temperature. After rinsing with PBST (3×300 μL), the RNase H reaction solutions (100 μL) were added and incubated for 30 min at 37°C. GNP-OLISA procedure is similar to OLISA except for the steps of biotinylated dAb, streptavidin, and biotinylated DNA probe. Instead of these steps, dAb-GNP-DNA complex diluted with Assay Buffer (final 0.025 OD) were added and incubated for 1 h at room temperature. The fluorescence intensities were measured by Appliskan (Thermoscientific, Walthan, MA, USA) with excitation/emission filter sets of 544/589 and 590/675 nm. All assays were performed in triplicates.

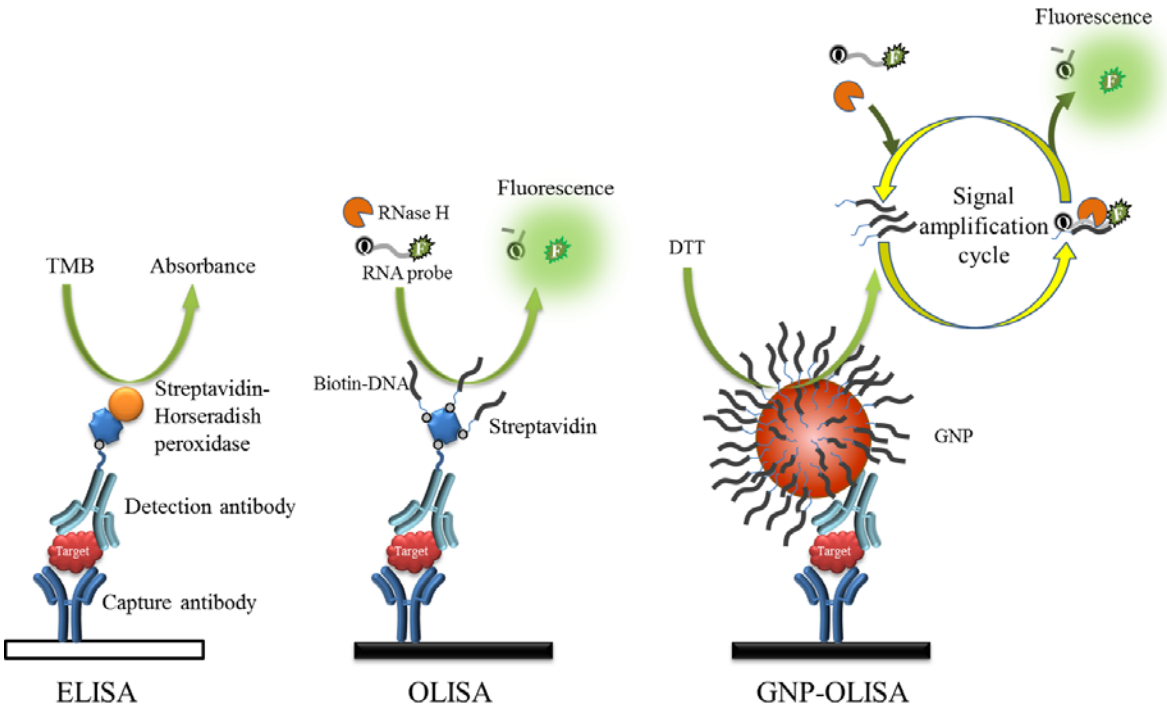

Figure S1. Schematic diagrams of ELISA, OLISA, and GNP-OLISA.