Nanoparticle-Based Proximity Ligation Assay for Ultra-Sensitive, Quantitative Detection of Protein Biomarkers

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Supporting Information

Experimental Section

Materials. Bovine serum albumin (BSA, A7906-50G), streptavidin–horseradish peroxidase (HRP) conjugate (S5512-.5mg), hCG (CG10-1VL, using the conversion factor 9.28 IU/ug from the 3rd International Standard), TWEEN® 20 (Molecular Biology Grade, P9416-100ML), and Nunc® MicroWell™ 96 well polystyrene plates (P7366-1CS) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Pierce premium-grade sulfo-NHS-SS-Biotin (PG82077), Zeba™ spin desalting columns (89882), 1-Step™ Ultra TMB-ELISA Substrate Solution (34028) were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL, USA). PCR optical tubes and caps (8 × strips), Brilliant III Ultra-Fast SYBR® Green QPCR master mix, and Agilent Mx3005P QPCR System were purchased from Agilent Technologies, Inc. (Santa Clara, CA, USA). Phosphate-buffered saline (PBS) tablets (T9181), pH 7.4, were purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). Mouse monoclonal anti-β hCG antibody (ABBCG-0402), goat anti-α hCG polyclonal antibody (ABACG-0500), and goat anti-mouse polyclonal antibody (ABGAM-0500) were purchased from Arista Biologicals, Inc. (Allentown, PA, USA). T4 DNA Ligase (M0202L) and 10 × buffer for T4 DNA ligase with 10 mM ATP (B0202S) were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). (D,L)-1,4-Dithiothreitol (DTT), 99.5+%, Molecular Biology Grade CAS# [27565-41-9], was purchased from Soltec Ventures, Inc. (Beverly, MA, USA). EM.STP15-15 nm gold streptavidin particles were purchased from BBI Detection, Inc. (Madison, WI, USA). Bio-Adembeads Streptavidin plus 0321 was purchased from Ademtech SA (Pessac, France). ANANAS Poly-Avidin nanoparticles were purchased from ANANAS Nanotech (Padova, Italy). Infinite® M200 PRO multimode reader and the HydroFlex microplate

washer were purchased from Tecan, Co. (Männedorf, Switzerland). Anonymized serum samples were obtained from the Gulf Coast Regional Blood Center (Houston, TX, USA). All DNA oligos were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa, USA).

PLA detectability of different nanoparticles. Different types of avidin/streptavidin-coated nanoparticles were diluted to varied concentrations in 2% BSA, PBS pH 7.4. To each PCR tube, 2 μL nanoparticle dilution, 1 μL of a mixture containing oligo-A (60 pM) and Oligo-B (60 pM), and 1 μL 60 pM Oligo-C were added. The solutions were mixed and incubated for 30 min at 25°C. Thereafter, 100 μL ligation mix (10 μL 10× buffer for T4 DNA ligase with 10 mM ATP, 24 units T4 DNA ligase, and 90 μL pure water) was added to each of the PCR tubes, mixed, and incubated for 15 min at 25°C. Immediately after ligation, 10 μL reaction solution was mixed with 10 μL 2× QPCR Master Mix (containing 1 μM of primers) in another optical PCR tube, and amplified by PCR (95°C for 10 min, then 50 cycles of 95°C for 15 s and 60°C for 30 s) in an Agilent Mx3005P QPCR System. The -delta C_t values were calculated by subtracting the C_t value of samples from the C_t value of the blank (no particles) control.

Biotinylation of antibodies. Biotinylation of antibodies with Pierce premium-grade sulfo-NHS-SS-Biotin was performed according to the manufacturer's protocol. Briefly, the protein was mixed with sulfo-NHS-SS-Biotin (mole ratio, 1:20), and the reaction was allowed to occur at room temperature for 30 min. The uncoupled sulfo-NHS-SS-Biotin was removed with a Zeba™ desalting column (40 kDa molecular weight) according to the manufacturer's protocol. After biotinylation, the mole ratio of biotin to antibodies was estimated using a 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assay as

approximately 4 to 4.5. Briefly, biotinylated protein samples were mixed with HABA/avidin reagent for at least 2 min at 25°C. The changes in absorbance at 500 nm were recorded and used to calculate the amount of biotin in the samples of biotinylated proteins. The biotinylated antibodies were stored with 1% BSA in PBS pH 7.4 at 4°C.

ELISA. Selected wells of a Nunc® MicroWell™ 96 well polystyrene plate were coated with 100 µl of capture antibody (10 µg/mL mouse monoclonal anti-β hCG antibody for hCG detection), and the plate was incubated at 4°C in PBS pH 7.4 overnight. Thereafter, the antibody solutions were removed from these wells, and 300 µL PBS with 3 % BSA was added. After incubation at 25ºC for 2 h, the wells were washed three times with PBS containing 0.1 % TWEEN® 20. Samples (100 µL) were immediately added to each of the wells after washing, and the plate was incubated for 1.5 h at 25°C. The wells were again washed three times with PBS containing 0.1% TWEEN® 20. Buffer A (PBS pH 7.4 with 1% BSA) was used to make dilutions in the following steps. Detection antibodies (100 µL, 10 ng/mL biotinylated goat anti-α hCG polyclonal antibody (ABACG-0500) for hCG detection) were added to each well, and the plate was incubated for 30 min at 25°C. The wells were washed three times with PBS containing 0.1% TWEEN® 20, and HRP reporters (100 µL, 200 ng/mL streptavidin-HRP for hCG detection) were added to each well. After incubating overnight at 4°C, wells were washed three times with PBS containing 0.1 % TWEEN® 20, and 100 µL 1-Step™ Ultra TMB-ELISA Substrate Solution was added. Following incubation for 20 min at 25°C, 50 µL 2 M sulfuric acid was added to each well, and the absorbance at 450 nm was measured with an Infinite® M200 PRO multimode reader.

iPCR. The first part of the iPCR protocol was the same as described for ELISA. After incubation of the detection antibodies, the wells were washed three times with PBS containing 0.1 % TWEEN® 20. Dilutions were made in Buffer A (PBS pH 7.4 with 1% BSA) unless specifically noted. Thereafter, 100 µL avidin-coated nanoparticles (1.25 $\times 10^7$/mL) were added to each of the wells, and the plate was incubated at 4°C overnight. The wells were then washed three times with PBS containing 0.1 % TWEEN® 20, and 100 µL Target Oligo was added to each of the wells. After incubating for 30 min at 25°C, the wells were washed three times with PBS containing 0.1 % TWEEN® 20, and 100 µL 50 mM DTT in pure water was added to each well. The plate was incubated for 2 h at 25°C. Finally, 10 µL of solution from each well was mixed with 10 µL 2× QPCR Master Mix (containing 1 µM primers) in a new optical PCR tube, and the DNA was amplified by PCR (1 cycle at 95°C for 10 min, then 50 cycles of 95°C for 15 s and 60°C for 30 s) with an Agilent Mx3005P QPCR System. The -delta C_t values were calculated by subtracting the C_t value of samples from the C_t value of the blank (no target analyte) control.

NP-PLA. The first part of the NP-PLA protocol was the same as described for the ELISA experiments. After incubation of the detection antibodies, the wells were washed three times with PBS containing 0.1 % TWEEN® 20. The following dilutions were made in Buffer A (PBS pH 7.4 with 1% BSA) unless otherwise noted. Then, 100 µL avidin-coated nanoparticles (1.25 $\times 10^7$/mL) were added to each of the wells, and the plate was incubated at 4°C overnight. The wells were washed three times with PBS containing 0.1 % TWEEN® 20, and 50 µL of the mixture containing oligo-A (60 pM) and Oligo-B (60 pM) and 50 µL 60 pM Oligo-C were added to each well. After incubating for 30 min at

25°C, the wells were washed three times with PBS containing 0.1 % TWEEN® 20, and 30 µL 50 mM DTT in water were added. Following incubation at 25°C for 2 h, 70 µL ligation mix (10 µL 10× buffer for T4 DNA ligase with 10 mM ATP, 24 units T4 DNA ligase, and 60 µL water) were added to each well. The solutions were mixed, and the plate was incubated for 15 min at 25°C. Immediately after ligation, 10 µL of each reaction solution was mixed with 10 µL 2× QPCR Master Mix (containing 1 µM primers) in another optical PCR tube, and the DNA was amplified by PCR (1 cycle at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 30 s) with an Agilent Mx3005P QPCR System. The -delta C_t values were calculated by subtracting the C_t value of samples from the C_t value of the blank (no target analyte) control.

Melting curve-based competitive PCR. For competitive PCR, 30 copies of the competitor oligo were added as an internal standard to the ligation products of each PCR reaction. Control reactions with 30 copies of the target oligo and/or 30 copies of the competitor oligo were used to determine the positions and the baselines of the target and competitor peaks in the melting curve. The PCR conditions were as follows: 1 cycle of 95 °C for 60 s followed by 1 cycle 55°C for 30 s and ended by a gradual increase in the temperature to 95°C (at the instrument default rate of 0.2°C/sec) and collecting fluorescence data continuously during this increase to 95°C. The melting curves were analyzed with OriginPro 9.0 (OriginLab Corp. Northampton, MA, USA).

Table S1 Oligonucleotides for nanoparticle-based proximity ligation assay (NP-PLA).

Name of oligo	Sequence (5'-3')	5' Modification	3' Modification
Oligo-A	CAGGTAGTAGTACGTCTGTTTC-ACGATGAGACTGGATGAA	Biotin	None
Oligo-B	TCACGGTAGCATAAGGTGCAA-GATAATACTCTCGCAGCAC	Phosphate	Biotin
Oligo-C	CTACCGTGATTCATCCAGAAAA-AAAAAAAAAAAAAAAAA	None	Biotin
Target	CAGGTAGTAGTACGTCTGTTTC-ACGATGAGACTGGATGAATCACGGTAG-CATAAGGTGCAAGATAATACTCTCGCAG CAC	Biotin	None
Competitor	CAGGTAGTAGTACGTCTGTTTA-TATGATTATATTATATTAATGAATGAAT-AATCAATTAAAGATAATACTCTCGCAGC AC	None	None
Primer-F	CAGGTAGTAGTACGTCTGTT	None	None
Primer-R	GTGCTGCGAGAGTATTATCT		

Table S2 Optimization of assay conditions for detection of hCG with NP-PLA.

Detection Antibody (ng/mL)	Streptavidin-coated Magnetic Nanoparticles (x10^7/mL)	C_t hCG (pg/mL) 0	0.2	2	20
2.5	**1.25**	33.88	33.23	32.99	31.29
	12.5	30.85	30.92	31.84	28.93
	125	27.97	27.91	28.52	27.52
5	**1.25**	33.37	32.16	32.07	31.19
	12.5	29.99	29.07	29.12	28.08
	125	27.73	26.67	26.61	26.81
10	**1.25**	34.33	32.44	31.82	30.39
	12.5	29.15	28.85	29.3	28.27
	125	26.99	26.81	26.89	26.97
25	**1.25**	31.05	31.84	31.45	28.83
	12.5	29.35	29.18	28.79	27.07
	125	26.89	27.22	27.02	25.62

Table S1 Use of melting peak-based competitive PCR (mp-cPCR) for quantification of the nanoparticle-based proximity ligation assay (NP-PLA) to detect human chorionic gonadotropin (hCG).

hCG (pg/mL)	Ratio of Melting Peaks (T/C)							
	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Mean	Standard Deviation
0	1.692	1.118	1.457	1.542	1.455	1.443	1.451	0.188
0.1	1.768	1.459	1.520	1.544	1.693	1.766	1.625	0.134
0.25	2.068	2.338	2.021	1.688	1.607	1.838	1.927	0.270
0.5	2.252	1.895	2.083	1.672	1.739	2.085	1.954	0.224
1	2.089	1.844	2.089	1.619	2.416	1.764	1.970	0.286
2.5	2.308	2.345	2.304	2.541	2.274	2.159	2.322	0.125
5	2.741	2.812	2.671	2.458	3.210	3.066	2.826	0.273

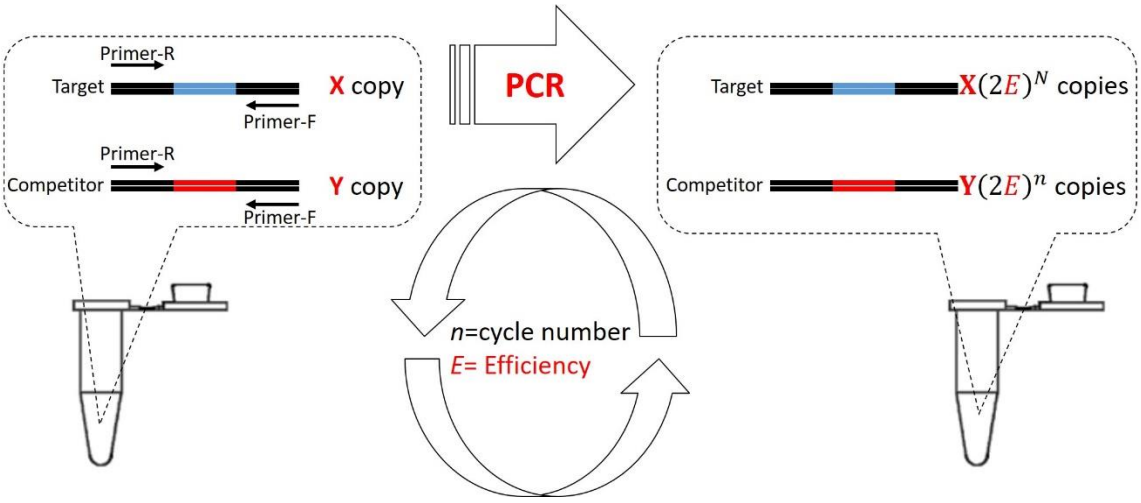

Figure S1 Working principle of competitive polymerase chain reaction (cPCR).

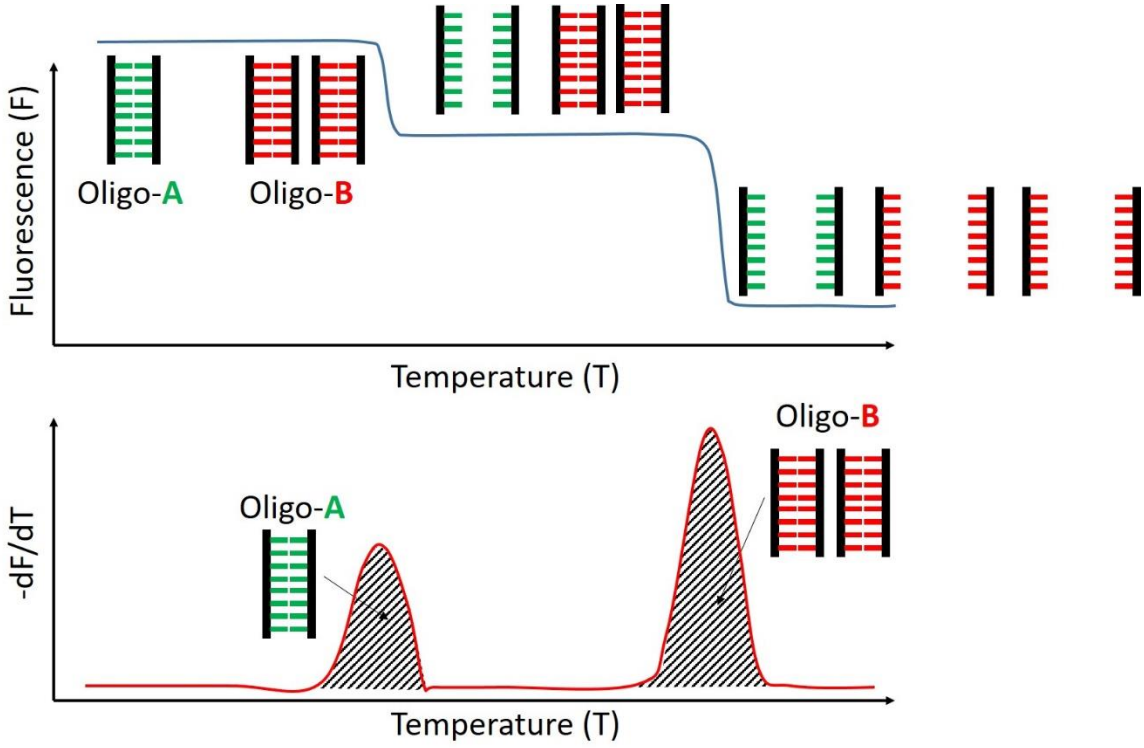

Figure S2 Working principle of melting peak-based competitive polymerase chain reaction (mp-cPCR). Target-only or Competitor-only controls are used to determine the ranges and baselines for integration of peak areas.

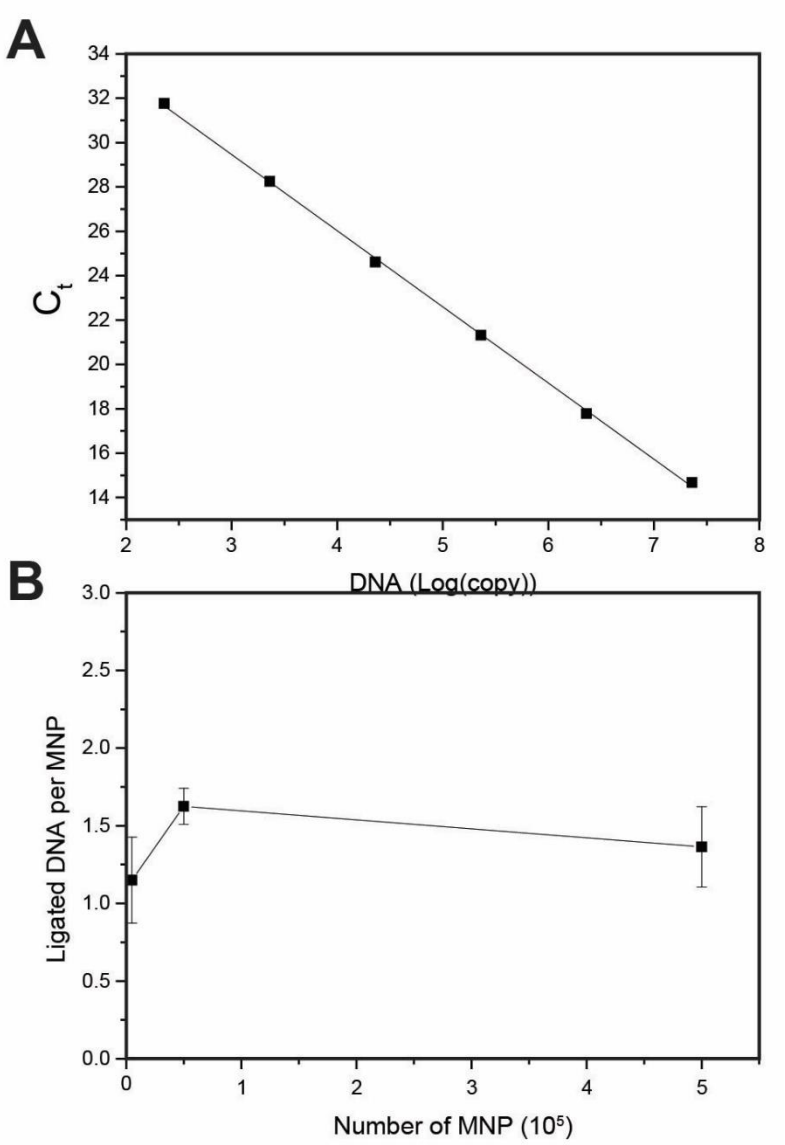

Figure S3 Quantification of the ligation yield on the surface of nanoparticles.

(A) Standard curve of the synthetic full-length PCR template spiked in 10 µL ligation buffer and mixed with 10 µL PCR master mix. PCR was run in the same settings as described in Methods. (B) Ligated PCR template per MNP detected by PCR at different particle counts. Mean ± standard deviation; n = 3.

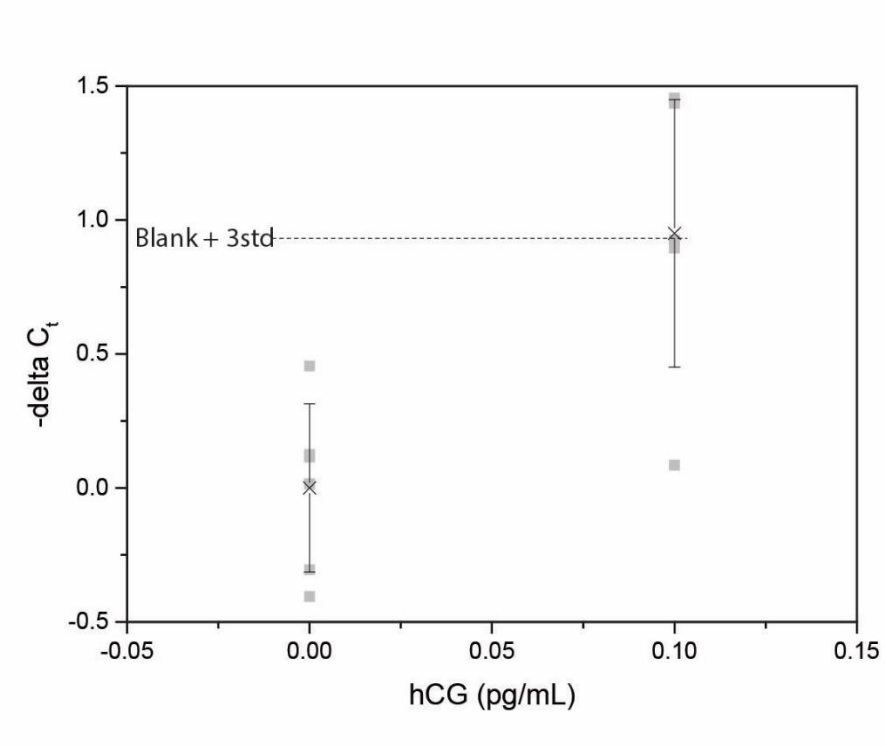

Figure S4 Limit of detection for human chorionic gonadotropin (hCG) using nanoparticle-based proximity ligation assay (NP-PLA). The dashed line indicates the level of the blank signal plus 3 standard deviations of the blank signal. The gray squares represent the individual signals of sextuplicates. The signals at 100 fg/mL are significantly different from the blank signals by two-tailed test with p = 0.0027.

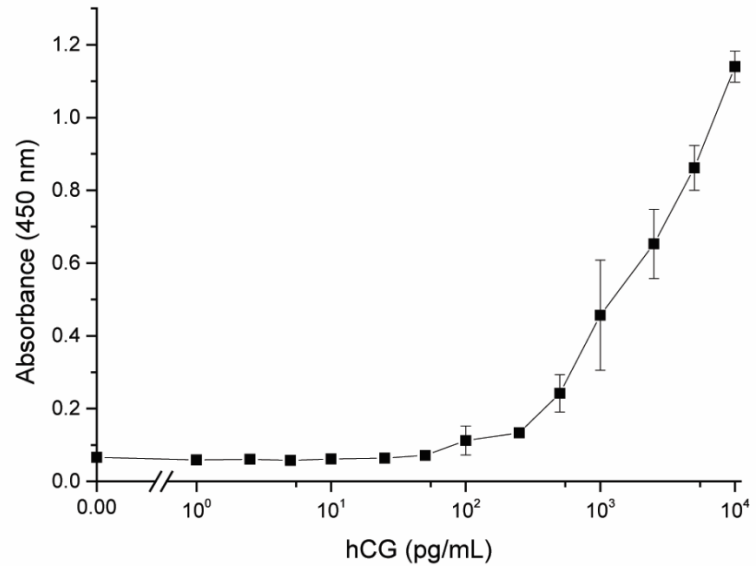

Figure S5 Detection of human chorionic gonadotropin (hCG) using enzyme-linked immunosorbent assay (ELISA). Mean ± standard deviation; n = 3.