Analytically Sensitive Protein Detection in Microtiter Plates by Proximity Ligation with Rolling Circle Amplification

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BACKGROUND: Detecting proteins at low concentrations in plasma is crucial for early diagnosis. Current techniques in clinical routine, such as sandwich ELISA, provide sensitive protein detection because of a dependence on target recognition by pairs of antibodies, but detection of still lower protein concentrations is often called for. Proximity ligation assay with rolling circle amplification (PLARCA) is a modified proximity ligation assay (PLA) for analytically specific and sensitive protein detection via binding of target proteins by 3 antibodies, and signal amplification via rolling circle amplification (RCA) in microtiter wells, easily adapted to instrumentation in use in hospitals.

METHODS: Proteins captured by immobilized antibodies were detected using a pair of oligonucleotide-conjugated antibodies. Upon target recognition these PLA probes guided oligonucleotide ligation, followed by amplification via RCA of circular DNA strands that formed in the reaction. The RCA products were detected by horseradish peroxidase-labeled oligonucleotides to generate colorimetric reaction products with readout in an absorbance microplate reader.

RESULTS: We compared detection of interleukin (IL)-4, IL-6, IL-8, p53, and growth differentiation factor 15 (GDF-15) by PLARCA and conventional sandwich ELISA or immuno-RCA. PLARCA detected lower concentrations of proteins and exhibited a broader dynamic range compared to ELISA and iRCA using the same antibodies. IL-4 and IL-6 were detected in clinical samples at femtomolar concentrations, considerably lower than for ELISA.

CONCLUSIONS: PLARCA offers detection of lower protein levels and increased dynamic ranges compared to ELISA. The PLARCA procedure may be adapted to routine instrumentation available in hospitals and research laboratories.

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Antibody-based protein detection assays are valuable tools for clinical diagnostics and research applications. However, the molecular complexity and wide protein concentration ranges of biofluids such as plasma *(1)* complicate detection. The challenge of specific detection increases rapidly with decreasing concentrations of proteins of interest. In particular, early detection of disease via leakage markers specifically emanating from affected tissues places stringent demands on both the analytical specificity and sensitivity. Therefore, bioassays are needed that combine high analytical sensitivity and specificity, as well as high precision and broad dynamic ranges. Although dedicated instrumentation is becoming available for highly sensitive protein measurement, a technology that confers improved performance using existing instrumentations could have broad utility.

Analytically sensitive protein detection assays can be designed so that binding of specifically captured proteins by even single detection reagents suffices to give rise to detectable signals. For example, sandwich immune reactions confined to femtoliter reaction chambers with enzymatic signal amplification have enabled digital readout of single detected molecules *(2–4)*. Similarly, eluted products of sandwich immunoreactions have allowed fluorophore-labeled detection complexes to pass through a laser beam in which single fluorescent antibodies can be recorded and quantified *(5, 6)*. Despite the single molecule detection capability, the aforementioned techniques require many molecules to be present in a sample for adequate detection over background.

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Alternative assays use DNA molecules as the reporter of the proteins detected. Examples of such assays are immuno-rolling circle amplification (iRCA)[6] (7, 8) and immuno-PCR (9, 10), in which affinity reagents are conjugated to DNA molecules that can be amplified for the sensitive detection of labeled antibodies.

These DNA-assisted assays have all shown analytical sensitivity in the femtomolar concentration range. Contribution to background commonly seen in conventional immunoassays, in the form of nonspecific fluorescence or absorbance from media, is avoided in the aforementioned assays because detectable signals can be elicited only by specific detection reagents. Nonetheless, other potential sources of nonspecific signals remain in the form of absorption of detection reagents to solid supports or cross-reactivity for irrelevant molecules in the samples by the antibody pairs (11).

Proximity ligation assays (PLAs) are immunoassays in which pairs of antibody-conjugated DNA oligonucleotides give rise to amplifiable reporter DNA strands only upon coordinated target binding, whereas individual reagents are undetectable. The DNA ligation products that form can be amplified and detected by real-time PCR or by means of rolling circle amplification (RCA) (12). In a similar fashion, DNA polymerases may be used to create reporter DNA strands upon pairwise binding by 2 oligonucleotide-conjugated antibodies in a proximity extension assay (PEA) (13). Homogenous PLA or PEA reactions lend themselves for convenient multiplex detection of proteins in single microliter samples with no need for washes, and multiplex PEA are commercially available. The analytical specificity may be extended, and large sample volumes can be used to enhance analytical sensitivity by first capturing target proteins on a solid support in simplex (12, 14) or multiplex (15, 16) before pairs of PLA probes are introduced, followed by washes to remove excess reagents, ligation, and then detection via real-time PCR.

A variant of PLA, in situ PLA, is used to image proteins, protein–protein interactions, and posttranslational modified proteins in situ by microscopy (17–21). For such localized detection reactions, amplification through RCA of DNA circles that form via ligation in a target-dependent manner is used in place of PCR. Each RCA product is a concatamer of typically hundreds of complements of the DNA circle that templated its synthesis, collapsed into a submicrometer ball of single-stranded DNA. These amplification products can be detected using fluorophore- or enzyme-labeled oligonucleotide probes, hybridizing to their repeated sequence, and are visible as bright fluorescent or colored spots by microscopy. RCA-mediated

PLA has also been used in flow cytometry (22–24) and for enhanced Western blotting (25).

We report here an adaptation of the solid-phase PLA protocol for protein detection using a standard microtiter plate reader, a format established in many clinical and research laboratories.

Materials and Method

PATIENT SAMPLES

The study was conducted in accordance with the Declaration of Helsinki, and it was approved by the institutional ethics committees of the institutions recruiting the patients. The Charité approval number was EA1/069/11, the University of Graz number was 23–015ex 10/11, and the Uppsala University number was 01/367. Patients signed informed consent before material collection.

Tissue lysates were prepared from 22 primary tumors and 22 normal fresh-frozen colon tissue samples [collected within the OncoTrack (26) project] together with zirconium oxide beads and lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 8, 1% Triton X-100, 0.1% sodium deoxycholate) using a Bullit Blender homogenizer (Next Advance). Homogenized tissues were centrifuged at 13000g for 10 min at 4 °C, and the supernatants were stored at −80 °C. The total protein concentration was measured (Pierce BCA Protein Assay Kit), and the samples were diluted to 2 g/L.

Blood samples from 25 patients with prostate cancer, ages 34–70 years, and from 24 age-matched healthy male controls (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue9) were collected in Vacutainer tubes containing K2-EDTA (Cat. #454410, Becton Dickinson). The tubes were centrifuged at 1500g for 10 min at room temperature, and the plasma was transferred to new tubes, which were stored at −80 °C.

ANTIBODIES, ANTIGENS, AND OLIGONUCLEOTIDES

The antigens and their cognate affinity-purified polyclonal antibodies are presented in Table 2 in the online Data Supplement, and the oligonucleotide systems for proximity ligation assay with rolling circle amplification (PLARCA) and iRCA are listed in Table 3 in the online Data Supplement.

PREPARATION OF PLA PROBES

PLA probes were prepared by covalently conjugating oligonucleotides to antibodies. Next, 25 mmol/L dibenzylcyclooctyne N-hydroxysuccinimide ester (Jena Bioscience) was dissolved freshly in 4 mmol/L DMSO (Sigma-Aldrich) in a 25-fold molar excess of dibenzylcyclooctyne N-hydroxysuccinimide ester. Antibodies were diluted in 1× PBS at 2 μg/μL, and a 33.3-fold molar excess of the freshly prepared dibenzylcyclooctyne was added and in-

cubated at room temperature for 30 min in the dark. The activated antibodies were purified using Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL (Thermo Scientific), having been equilibrated with 1× PBS as directed by the manufacturer. Purified antibodies were divided in 2 aliquots of 1 μg/μL and mixed with a 2.5-fold molar excess of either the S3 primer or the S3 2'-O-methyl RNA base-blocked azide-modified oligonucleotides, and incubated overnight at 4 °C.

PHOSPHORYLATION OF OLIGONUCLEOTIDES

The DNA (stock concentration 100 μmol/L) oligonucleotides were mixed to a final concentration of 10 μmol/L in 1× T_4 polynucleotide kinase reaction buffer A (Fermentas) with 0.25 U/μL T_4 polynucleotide kinase (Fermentas), 10 mmol/L ATP, and incubated for 30 min at 37 °C, followed by inactivation at 75 °C for 10 min in a thermocycler. The phosphorylated oligonucleotides were immediately placed on ice before being stored at −20 °C for further use. The oligonucleotides can also be ordered with 5' phosphates as required for their ligation.

PLA WITH ABSORBANCE READOUT

Specific antibodies, 200–800 ng added per well, were coated in a 96-well microtiter plate in coating buffer (100 mmol/L carbonate buffer, pH 9.6) at 4 °C overnight before the wells were blocked with 200 μL of 1% BSA in 1× PBS for 1 h at room temperature. The plates were then rinsed in washing buffer (1× PBS, 0.01% BSA, and 0.05% Tween 20), and the top surface was blotted using tissue paper after each step in the assay. Dilution series of proteins were prepared in PLA buffer or 50% or 10% chicken serum (Invitrogen) as indicated for the individual experiments. All dilution series contained negative controls with no protein added to determine the background. The assays were initiated by incubating 100 μL samples in antibody-coated microtiter wells for 1 h at room temperature on a shaker (80 rpm) followed by 3 washes. The PLA probes were diluted in PLA buffer (0.1% BSA; New England BioLabs), 0.05% Tween (Sigma-Aldrich), 0.1 μg/μL salmon sperm DNA (Invitrogen), 100 nmol/L goat IgG (Sigma-Aldrich), and 5 mmol/L EDTA (1× PBS) to a final concentration of 1 nmol/L, from which 100 μL was added to each well, followed by incubation for 1 h at room temperature on a shaker. The wells were washed again, and 100 μL of a ligation mix (0.5 U/μL T_4 DNA ligase; New England BioLabs) in 1× T_4 ligase buffer without dithiothreitol but with 0.25 g/L BSA, 0.5 mmol/L ATP, and 200 nmol/L of the 2 circle-forming oligonucleotides, was added into each well and incubated for 30 min at 37 °C followed by washes. Next, 100 μL of RCA mix [0.5 U/μL Phi29 polymerase (Fermentas) in 1× Phi29 polymerase buffer (Fermentas) with 0.25 g/L BSA, 0.4 mmol/L dNTPs (Fermentas), and ddH$_2$O] was added,
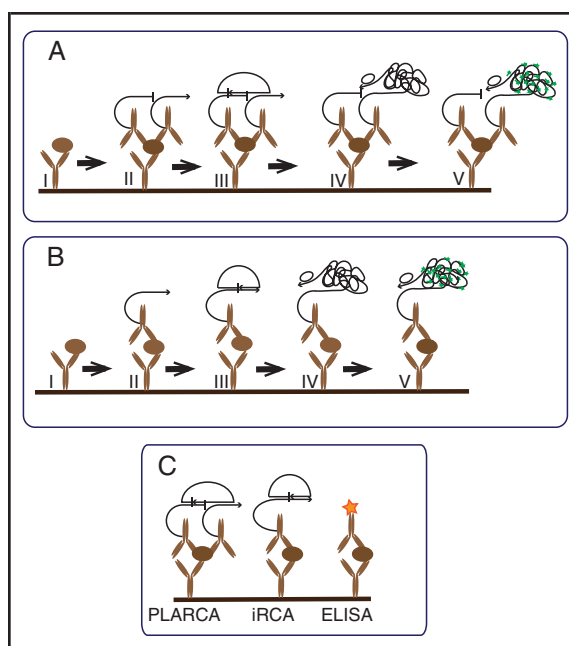

Fig. 1. Schematic descriptions and comparison of PLARCA, iRCA, and standard sandwich ELISA.

Schematic description of the PLARCA assay (A). Antibodies are immobilized via hydrophobic interaction in wells of microtiter plates, followed by washes and blocking of nonspecific binding (I). Bound antibodies are used to capture target proteins from added samples, followed by washes to remove unbound sample components. Next, a pair of PLA probes (antibody-DNA conjugates) is added that can recognize captured target molecules, followed by washes (II). DNA oligonucleotides are then added along with a ligase allowing the oligonucleotides to be ligated into DNA circles, templated by an oligonucleotide on one of the PLA probes (III). A DNA polymerase is added to initiate replication of the DNA circles via RCA, primed from that antibody-conjugated oligonucleotide that did not template ligation (IV). Finally, the RCA products are detected by adding horseradish peroxidase-labeled oligonucleotides to the RCA product, followed by washes with addition of the 3`3`,5`5`-tetramethylbenzidine substrate (V). Schematic description of iRCA (B). Same as under (A) except that a single oligonucleotide-modified antibody is used both to template ligation and to prime RCA. Schematic comparison of PLARCA, iRCA, and a standard sandwich ELISA (C).

followed by incubation for 1–1.5 h at 37 °C and then by washes. A 2× hybridization buffer containing 40% formamide and 4× sodium saline citrate (0.15 mol/L NaCl and 0.015 mol/L sodium citrate = 1× SSC) was prepared and stored in the dark. Then, 100 μL of detection mix (50 pmol/L horseradish peroxidase-labeled detection oligonucleotide, 1× hybridization buffer) was added and incubated for 30 min at 37 °C, followed by washes. Finally, 50 μL of the horseradish peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich), equili-
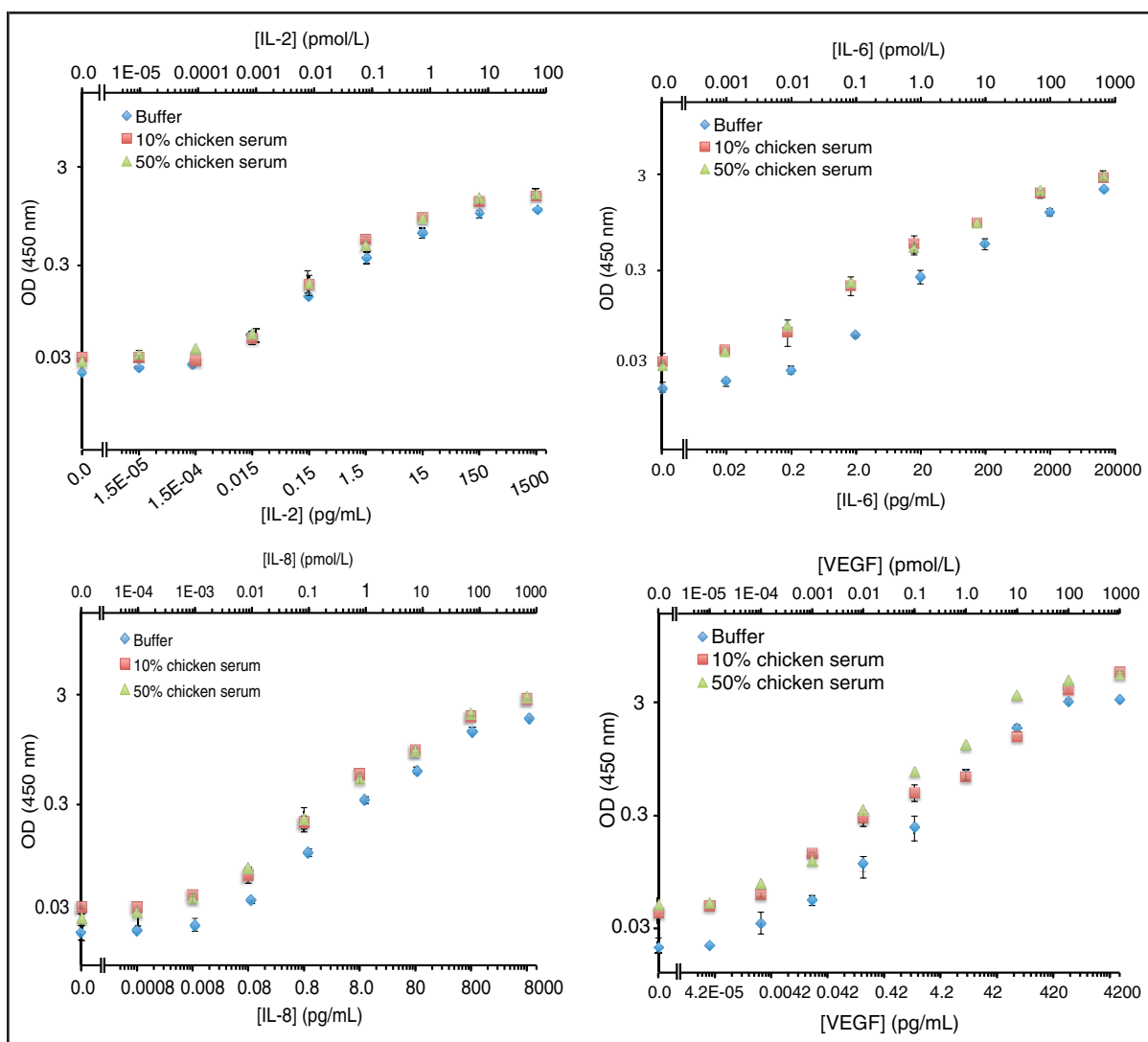
Fig. 2. Validation of PLARCA performance in complex biological samples.

Comparison of results from assays for interleukin (IL)-2, IL-6, IL-8, and vascular endothelial growth factor in buffer (blue) or 10% (red) or 50% (green) chicken serum. The y axes show optical density (OD) measured at 450 nm, whereas the x axes show protein concentrations in molarity (top) and weight per volume (bottom). The means of triplicate measurements are shown with SDs indicated with error bars.

brated at room temperature, was added to each well, incubated for 10 min at room temperature, and then stopped with an equal volume of 2 mol/L H_2SO_4 or H_3PO_4. The colored product was then measured using an absorbance plate reader within 30 min at 450 nm and with absorbance at 650 nm as reference.

iRCA

The assay was performed similarly to the PLARCA described above with the exception that only one PLA conjugate, with the S3 primer, was used to template the formation of a DNA circle via a single ligation, and to prime RCA, as illustrated (Fig. 1B).

SANDWICH ELISA

The R&D Quantikine kits for IL-4, IL-6, and growth differentiation factor (GDF-15) were used according to the manufacturer's instructions (see Table 2 in the online Data Supplement). The proteins were spiked in 10% and 50% calibrator diluent to ensure fair comparison.

DATA ANALYSIS

The absorbance data were recorded using a Safire II microtiter plate reader, and the optical densities (OD 450 nm) were exported and further analyzed with Microsoft Excel and StatPlus LE. A 4-parametric logistic linear regression was used to calculate the limits of detection

Table 1. Assay summary of the analytical performance of PLARCA of IL-2, IL-6, IL-8, and VEGF measured in buffer and 10% and 50% serum.[a]

	IL-2			IL-6			IL-8			VEGF		
	Buffer	10%	50%	Buffer	10%	50%	Buffer	10%	50%	Buffer	10%	50%
LLOQ (pg/mL)	0.333	0.794	0.968	0.910	0.794	0.4753	0.413	0.492	0.471	0.042	0.079	0.095
LOD (pg/mL)	0.031	0.145	0.045	0.074	0.200	0.133	0.131	0.247	0.236	0.047	0.081	0.102
Intraassay CV (%) (n = 6)	8.5	5.0	8.0	4.3	11.5	9.8	8.3	10.0	7.6	5.3	6.3	7.7
Interassay CV (%) (n = 6)	9.9	6.8	14.0	10.8	18.2	14.1	15.1	24.2	21.2	7.9	13.6	9.3

[a] IL, interleukin; VEGF, vascular endothelial growth factor; LLOQ, lower limits of quantification.

(LOD) and the lower limits of quantification (LLOQ). Statistical significances of measurements for the patient and control samples were calculated using the Wilcoxon signed rank test. To calculate the P values, all the data points below the LODs of the assays were put at the LODs. The LOD values were calculated as the protein concentrations on the fitted curves that corresponded to OD values representing the average OD of 3 negative control samples plus 3 standard deviations of those values. Similarly, the LLOQ were calculated as the protein concentrations on the fitted curves corresponding to the average OD background of 3 negative control samples plus 10 standard deviations.

Results

The method described here, which we term PLARCA, relies on target protein capture by immobilized antibodies, followed by detection of the protein via pairs of antibody–DNA conjugates (PLA probes) that give rise to circular DNA strands in the same manner as for in situ PLA with signal amplification via RCA. The amount of RCA products is measured by hybridization with DNA probes conjugated to horseradish peroxidase, acting on the substrate 3,3′,5,5′-tetramethylbenzidine to generate colorimetric reaction products that are read out with an absorbance microplate reader (Fig. 1).

We prepared PLA probes by covalently coupling antibodies to DNA oligonucleotides using click chemistry through residues introduced in the antibodies via a bifunctional dibenzylcyclooctyne N-hydroxysuccinimide ester cross-linker. The quality of each batch of conjugates was validated with agarose gel electrophoresis for each new assay (see Fig. 1 in the online Data Supplement).

Two designs of DNA oligonucleotides coupled to the antibody were tested to identify sequences with optimal signal-to-noise ratio for use in the experiments described here. To investigate the assay sensitivity, pure IL-2, IL-6, IL-8, and vascular endothelial growth factor protein preparations were diluted over a million-fold concentration range in buffer or in 10% and 50% chicken serum before analysis (Fig. 2) to represent a complex biological matrix. With the increase in matrix complexity from buffer to chicken serum, we observed a slightly increased background for some of the analytes (Fig. 2) but with limited effect on the LOD and LLOQ of the assays (Table 1).

The analytical characteristics of the assays are summarized in Table 1, with the LOD defined as the concentration of protein detected at 3 SDs over the background. We compared the performance of PLARCA with conventional sandwich ELISAs for IL-4, IL6, and GDF-15 (Fig. 3A), and with iRCA for IL-8 and p53 (Fig. 3B), all spiked in 50% chicken serum. Monoclonal antibodies, used as capture reagents for PLARCA, were immobilized in microtiter wells overnight in coating buffer. PLA probes were prepared from polyclonal antibody preparations as described in Materials and Methods. Assay diluent, substrate, and stop solution from the ELISA kit were used to compare PLARCA, iRCA, and ELISA. We compared the performance of PLARCA with that of ELISA kits from R&D Systems for detection of IL4, IL-6, and GDF-15 (Fig. 3). Although ELISA took less time than PLARCA, PLARCA in serum demonstrated >86-, 15-, and 2-fold increased analytical sensitivity for detection of IL-4, IL-6, and GDF-15, respectively, compared with ELISA. PLARCA offered 2 orders of magnitude greater dynamic range for IL-4 and IL-6 and 1 order of magnitude greater dynamic range for GDF-15 (Table 2). The mean %CV for ELISA was about 5% for the intraassay variation compared with about 10% for PLARCA.

We also compared PLARCA with iRCA assays for IL-8 and p53. PLARCA showed >3.5- and 3-fold increased analytical sensitivity for detection compared with iRCA for IL-8 and p53, respectively (Fig. 3B and Table 2).
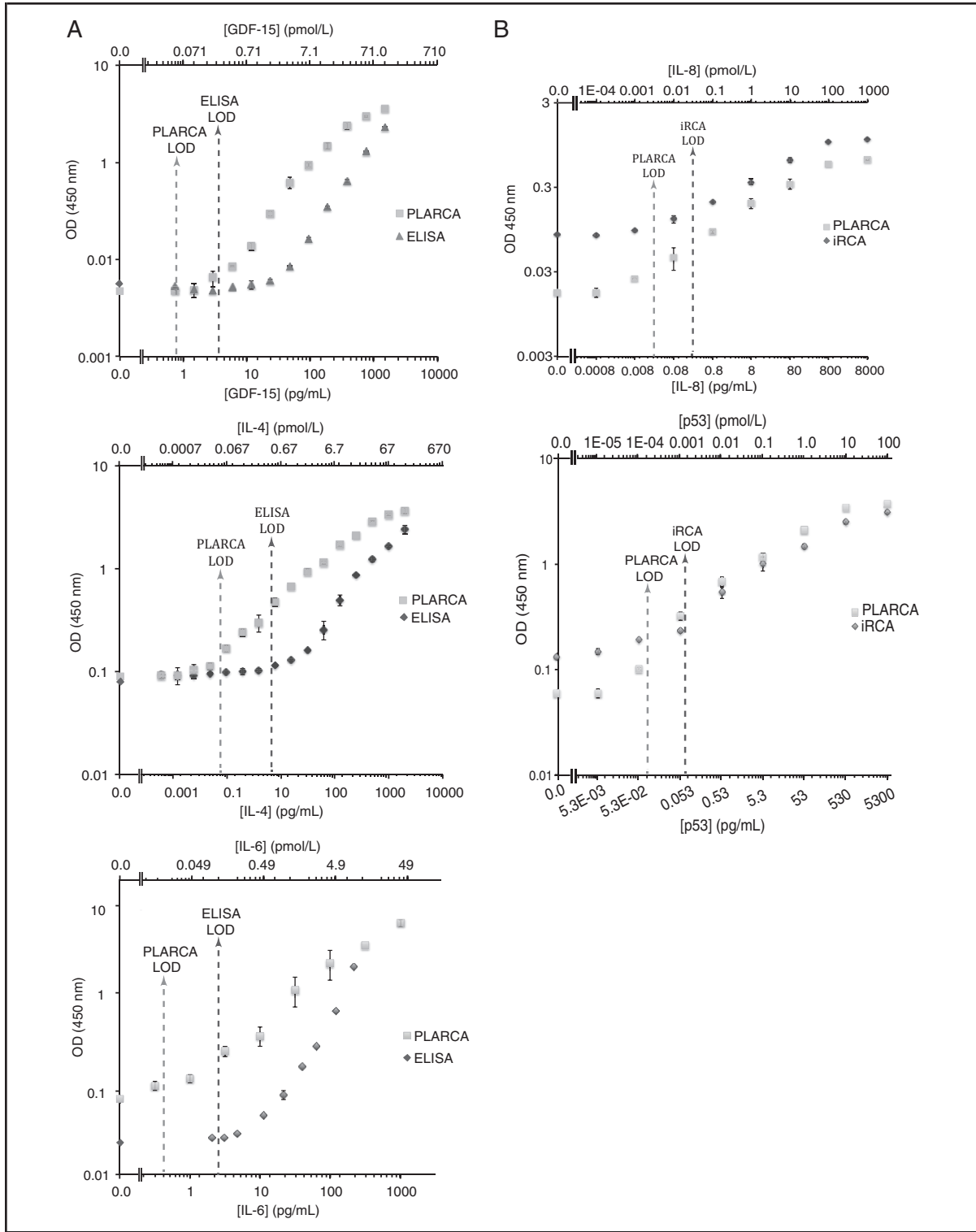
Fig. 3. Performance of PLARCA vs ELISA and iRCA.

Comparison of PLARCA (squares) and ELISA (diamonds) for measuring levels of interleukin (IL)-4, IL-6, and GDF-15 (A). Comparison between PLARCA (squares) and iRCA (diamonds) for measuring concentrations of IL-8 and p53 (B). All analytes for PLARCA, iRCA, and ELISA were spiked in 50% calibrator diluent for serum. The means of triplicate measurements are shown with SDs indicated with error bars.

Table 2. Comparison of LOD, LLOQ, dynamic range, and time for PLARCA in measurements of IL-4, GDF-15, and IL-6 with ELISA and of IL-8 and p53 with iRCA.[a]

	IL-4		GDF-15		IL-6		IL-8		p53	
	PLARCA	ELISA	PLARCA	ELISA	PLARCA	ELISA	PLARCA	iRCA	PLARCA	iRCA
LOD (pg/mL)	0.099	8.47	0.899	1.37	0.089	1.373	0.041	0.161	0.020	0.143
LLOQ (pg/mL)	0.129	10.59	1.43	1.85	0.1928	9.03	0.237	0.902	0.765	1.617
Dynamic range	10^4	10^2	10^3	10^2	10^4	10^2	10^5	10^4	10^5	10^4
Time (h)	6.25	4.5	6.25	4.5	6.25	4.5	6.25	6.26	6.27	6.28

[a] LLOQ, lower limits of quantification; IL, interleukin.
All assays were performed in 50% serum.

We further compared the performance of PLARCA with our previously described microparticle-based solid-phase PLA protocol *(14)* for IL-6 and vascular endothelial growth factor in 10% chicken serum, showing comparable analytical sensitivity for detection and dynamic ranges (see Fig. 2 in the online Data Supplement).

Finally, to evaluate the performance of PLARCA in clinical samples, we measured concentrations of IL-6 in tissue lysates prepared from tumor tissues and surrounding healthy intestinal samples from 22 patients with colon cancer (see Table 1 in the online Data Supplement). PLARCA distinguished IL-6 concentrations between lysates of tumor and nontumor tissues with a *P* value <0.001. Furthermore, detectable IL-6 concentrations were found in most of the tumor and control tissue lysates using PLARCA, whereas the concentrations of IL-6 in most of the samples were below the detection limit for ELISA (Fig. 4A). In addition, we also measured the concentrations of IL-4 and IL-6 in plasma samples from 25 patients with prostate cancer and 24 samples from healthy controls (see Table 1 in the online Data Supplement). ELISA recorded detectable concentrations of IL-4 (Fig. 4B) and IL-6 (Fig. 4C) in 16% and 32% of the patients, respectively, whereas PLARCA successfully detected protein concentrations in >80% and 84% of the samples, respectively.

Discussion

We report here a new format for PLA, suitable for detecting proteins in low femtomolar concentrations in complex media, using antibody-conjugated oligonucleotides *(12)* that jointly but not individually are able to elicit signal amplification via RCA *(17)*. The assays are performed in standard microtiter wells with removal by washes of excess reagents and sample components that could interfere with the assay, followed by recording of the absorbance in a standard microplate reader. Our results show that PLARCA has the potential to detect and measure proteins at very low concentrations and over broad detection ranges, permitting detection of promising protein biomarkers in plasma using routine laboratory equipment. The requirement for detection by sets of 3 affinity reagents in PLARCA can serve to enhance the analytical specificity of detection compared with that of sandwich ELISA and/or iRCA by reducing risks of cross-reactivity, while the solid supports also permit analysis of large sample volumes and washes to remove excess reagents. This is in contrast to homogenous PLA and PEA that are typically limited to sample volumes of 1 μL because of the need to reduce background via dilution of the reactions before the enzymatic step. PLA measurements also reduce risks of background signals because of nonspecifically bound detection reagents, as only pairs of PLA probes can produce detection signals, whereas single nonspecifically bound detection reagents are undetectable. The requirement for specific DNA hybridization and ligation ensures that only cognate pairs of detection reagents give rise to the signal. This has proven highly useful when more PLA probe pairs are added in multiplex reactions *(27, 28)*. Across all investigated proteins, PLARCA consistently achieved low detection limits, broad dynamic ranges, and high precision. Moreover, interfering substances from plasma had only a limited influence on the results. PLARCA had intraassay CV% of about 5% to 11%, considerably less than our previously reported solid-phase PLA with real-time PCR readout for some assays *(14)*.

Compared with conventional ELISA and iRCA, PLARCA offered >100-fold greater analytical sensitivity for detection of some cytokines. In samples from patients with colon cancer, PLARCA better differentiated between lysates prepared from tumor tissues and surrounding healthy tissues compared with ELISA. PLARCA could also detect proteins at concentrations that were below the detection limits for ELISA. Recently an ELISA-PLA protocol was reported for detection of posttranslational modifications of proteins by directly adapt-
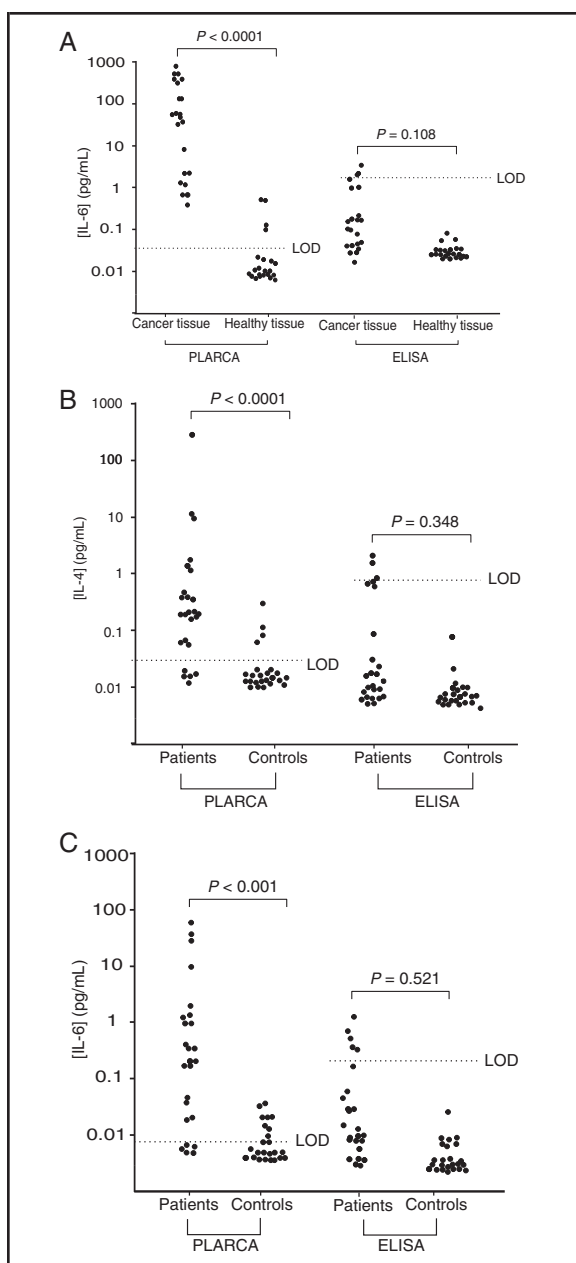
Fig. 4. Measurement of cytokine concentrations in colorectal and prostate cancer.

Measurement of interleukin (IL)-6 PLARCA and ELISA in 22 tissue lysates from colon cancer tissue and 22 normal colonic samples from the same patients (A). Measurement of IL-4 (B) and of IL-6 (C) with PLARCA and ELISA in plasma samples from 25 prostate cancer patients and 24 healthy control subjects. PLARCA and ELISA LODs were calculated from the standards in Table 2 for IL-4 and IL-6. The age ranges for these patients and control subjects are illustrated in Table 2 in the online Data Supplement. Each dot represents the mean of a triplicate. P values were calculated using a 2-sample Wilcoxon rank sum test.

ing the in situ PLA protocol to bind capture and detection antibodies in microtiter plates, for which higher sensitivity than ELISA was demonstrated (29). In contrast to our present study, target molecules were recognized by only 2, rather than 3, antibodies, limiting the analytical specificity of detection.

PLARCA assays can theoretically be easily established for individual proteins, provided high-quality affinity reagents are available. Protein–DNA conjugation is now a routine procedure using conjugation chemistries such as the click chemistry used in this study. Assays with the 3 affinity reagents required for each target protein can be established using combinations of monoclonal and polyclonal antibodies as described herein. Alternatively, we have previously shown that a single polyclonal antibody preparation raised against all or a substantial portion of the target protein can regularly be divided in 3 aliquots and used for a capture antibody preparation and 2 PLARCA probes. As demonstrated for PLARCA here and previously for solid-phase PLA (28), these triple-binder assays work well for proteins with sizes ranging from as little as 8.6 kDa (IL-8) to 43.7 kDa (p53).

In conclusion, we have developed a new assay format for protein detection called PLARCA, which offers an opportunity to detect proteins at low femtomolar concentrations in buffer and in biological samples. PLARCA could be implemented in any laboratory where microplate readers are in use.

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