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Multiplex protein detection with DNA readout via mass spectrometry

James Flanigan^{1,7}, Masood Kamali-Moghaddam^{2,7}, Ian Burbulis³, Carla Annink², Martin Steffen⁴, Paul Oeth⁵, Roger Brent⁶, Dirk van den Boom⁵, Ulf Landegren², and Charles Cantor^{1,5}

¹ Center for Advanced Biotechnology, Department of Biomedical Engineering, Boston University, Boston, MA 02215, USA

² Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, SE-75185 Uppsala, Sweden

³ The VTT/MSI Molecular Sciences Institute, Berkeley, CA 94704, USA

⁴ Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA 02118, USA

⁵ Sequenom, Inc., San Diego, CA 92121, USA

⁶ Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

Abstract

Multiplex protein quantification has been constrained by issues of assay specificity, sensitivity and throughput. This research presents a novel approach that overcomes these limitations using antibody–oligonucleotide conjugates for immuno-polymerase chain reaction (immuno-PCR) or proximity ligation, coupled with competitive PCR and MALDI-TOF mass spectrometry. Employing these combinations of technologies, we demonstrate multiplex detection and quantification of up to eight proteins, spanning wide dynamic ranges from femtomolar concentrations, using only microliter sample volumes.

Introduction

While crucial for numerous scientific and medical applications, scalable approaches to sensitive and specific protein quantification have been problematic. Primary hurdles include the following: (i) protein complexity and homology can limit assay specificity; (ii) proteins of interest often occur at levels that are orders of magnitude lower than that of their most abundant counterparts and (iii) throughput of protein assays has been limited by lack of multiplex assay formats [1–5]. To address these obstacles, we present a novel multiplex approach for protein measurement, which overcomes many of these limitations by using a combination of established technologies: immuno-polymerase chain reaction (immuno-

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Corresponding author: Flanigan, J. (jflanigan@gmail.com).

⁷These authors contributed equally to this work.

Author contributions

J.F., M.K.-M., U.L. and C.C. designed the study. J.F., M.K.-M. and C.A. carried out the experiments. J.F., M.K.-M., M.S., U.L. and C.C. wrote the manuscript.

Competing financial interests

U. Landegren is founder and stockholder of Olink Bioscience, C. Cantor is CSO and stockholder of Sequenom, Inc.

PCR) and proximity ligation assay (PLA) address both specificity and signal-to-noise issues; competitive PCR enables both relative and absolute quantification of proteins among samples; and mass spectrometry using the MassARRAY platform (Sequenom, San Diego, CA) allows for high throughput, precision and multiplexing.

Immuno-PCR and PLA

both rely on antibody–oligonucleotide chimeric probes: the antibody portions of the probes are used to bind a target protein with high specificity, while conjugated DNA is used as a proxy signal. The DNA strands attached to the antibodies serve as a template for a PCR-generated representation of the bound protein, either directly [6], or after ligation of oligonucleotides on two antibodies that bind the same target molecule [7]. To demonstrate the flexibility of our approach, we conducted experiments with two leading PCR-based platforms: immuno-PCR and PLA reactions using antibody–oligonucleotide conjugates.

Antibody–oligonucleotide conjugates

are chimeric detection probes consisting of antibody linked to DNA in specific conjugation schemes [8–11]. The general approach of this platform begins with the creation of an immuno-sandwich assay using antibody functionalized magnetic beads to capture the antigen and the antibody–DNA conjugate for detection, followed by PCR to amplify the template of the bound detection probes (Fig. 1a). In previously published single-plex immuno-PCR studies, these probes had a dynamic range of detection over 11 orders of magnitude; they could distinguish concentration differences as small as 10%; and they detected femtomolar levels of target proteins in human serum [8–10].

PLA technology

utilizes the simultaneous binding of two or more independent affinity reagents to their target protein to create a signal. In the simplest implementation, antibodies for separate epitopes of a target protein are conjugated to oligonucleotides – having free 3′ ends for one of the antibodies, and free 5′ ends for the other – to form PLA probes. When the PLA probes recognize different epitopes on the same target protein molecule, the oligonucleotides are brought in close proximity, and joined by enzymatic ligation in the presence of a connector oligonucleotide (Fig. 1b). Once ligated, the newly formed DNA strand can be amplified and quantified by real-time quantitative PCR (qPCR) [12–18]. Published studies of protein detection using PLA demonstrate up to three orders of magnitude of greater sensitivity than that of the ELISAs. In addition, the requirement that two PLA probes bind in proximity to generate an amplifiable signal reduces false-positive signals resulting from nonspecific probe binding.

PLA can be carried out homogeneously or on a solid support. In homogeneous PLA, a very small sample volume is required and there is no need for washes or separations [7,12]. In solid-phase PLA (SP-PLA) the target protein is first captured on a solid support followed by removal of excess probes and undesired sample components before the ligation step. The requirement of an additional recognition event further increases the specificity of the assay, and the solid support provides the possibility to capture target molecules from larger sample volumes while removing any interfering substances [13,14,17]. Both homogeneous and SP-PLA have been used for parallel detection of proteins in body fluids [16,18]. Notably, the multiplex SP-PLA has shown to perform with minimal cross-reactivity of the antibodies with noncognate target proteins as compared to sandwich immunoassay [18].

Competitive PCR

enables absolute quantification of DNA, and hence DNA-tagged proteins, over an extremely wide dynamic range [19–22]. This technique utilizes an internal DNA standard (the competitor), which is co-amplified by PCR with the nearly identical target sequence at a range of ratios. The competitor differs from the sequence that represents the detected protein by only one base, so that the efficiency of amplification of the two species is nearly identical, resulting in accurate measurement of the ratio between the two sequence variants. Because the concentrations of the standards are known *a priori*, the concentration of the bound chimeric protein probe can be calculated in both relative and absolute terms (Fig. 1c). Here we apply standard PCR techniques to amplify the nucleic acid signal from the immuno-PCR and PLA reactions, and then compare these with known competitor concentrations.

Mass spectrometry

For multiplex assay readout, we utilize MALDI-TOF mass spectrometry using the MassARRAY platform (Sequenom) [23–26]. This has been used previously in conjunction with competitive PCR in a technique called real competitive PCR [20]. When used for single-nucleotide polymorphism (SNP) detection, competitive PCR coupled with mass spectrometry has been demonstrated to detect and quantify as few as five molecules in a 5 μ l sample, with the potential for high multiplexing with excellent quantitative precision [20,27].

Materials and methods

Reagents

Proteins and affinity purified poly- and monoclonal antibodies were purchased from R&D System (Minneapolis, MN). The complete list of the proteins and antibodies are shown in Table S1. The designs of immuno-PCR and PLA probes are described in the Supplement, and the oligonucleotide sequences are listed in Table S2. The antibody–oligonucleotide conjugation for immune-PCR was carried out using hydrazone chemistry by Solulink (San Diego, CA). The conjugation of oligonucleotides to streptavidin for use in PLA probes was carried out by Olink Bioscience (Uppsala, Sweden) using succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) chemistry.

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SP-PLA protocol

PLA probes were prepared as previously described [28]. Briefly, identical volumes of 100 nM streptavidin–oligonucleotide conjugates were incubated with 100 nM biotinylated antibodies for 1 hour at room temperature, followed by dilution in PLA buffer (1 mM D-Biotin (Invitrogen), 0.1% purified BSA (New England Biolabs), 0.05% Tween 20 (Sigma–Aldrich), 100 nM goat serum IgG (Sigma–Aldrich), 0.1 μ g/ μ l salmon sperm DNA (Invitrogen), and 5 mM EDTA, 1 $\times$ PBS) 15 min before that all reagents were mixed to form PLA probe mix.

A mixture of six polyclonal antibodies directed against TIMP1, IL6, VEGF, TNF α , ErbB2 and PDGF (R&D systems) were immobilized in polycarbonate tubes (AJ Roboscreen, GmbH, Germany) in carbonate buffer (pH 9.6) for 1.5 hours at 37°C, followed by three washes with 1 $\times$ PBS and 0.01% (v/v) Tween 20. The wells of the tubes were blocked with 200 μ l blocking buffer (1% BSA, 1 mM biotin, 100 μ g/ml salmon sperm DNA, 0.05% Tween 20 in PBS) overnight at 4°C. Next, the tubes were incubated with 10 μ l antigen

mixture containing TIMP 1, IL6, VEGF, TNF α , ErbB2 and PDGF (RnD Systems) spiked in buffer or 10% chicken plasma in a serial dilution from 10 nM down to 0.01 pM. After 1.5 hour incubation at 37°C and subsequent washes, 50 μ l PLA probe mix (500 pM of each probe) was added and the incubation was continued for another 1.5 hours at 37°C followed by washes. Next, 15 μ l ligation mix (100 nM of six specific connector oligonucleotides, 0.08 mM ATP, 2.7 U T4 DNA ligase, 10 mM Tris-acetate, 10 mM Mg-acetate and 50 mM K-acetate) was added and the reactions were incubated at 30°C for 10 min followed by 15 min inactivation at 65°C. Thereafter, the connector oligonucleotides were degraded with uracil-DNA excision mix (Epicentre Biotechnologies UEM04100) for 15 min at 37°C, followed by inactivation of the enzyme for 10 min at 95°C. Next, 85 μ l PCR reaction mix (1 $\times$ PCR buffer (Invitrogen), 1 mM MgCl₂, 0.6 U Taq polymerase (Invitrogen), 0.2 mM dNTP (containing dUTP) and 100 nM of each forward and reverse universal primer) was added and PCR was performed (2 min 95°C, 13 cycles of 15 s 95°C and 1 min 60°C). One aliquot of the PCR product was used for specific detection of proteins using competitive PCR with mass spectrometry readout (see below). Another aliquot was used for control qPCR readout, where 1 μ l of 100-fold diluted PCR product was separately added to six different PCR mixes containing specific pairs of primers for each set of probe in PCR mix (1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.6 U Taq polymerase (Invitrogen), 0.2 mM dNTP and 0.5 $\times$ syber gold (Molecular Probes)). The qPCR was performed on an Mx-3000 real-time PCR instrument (Stratagene) with an initial incubation for 2 min at 95°C, followed by 45 cycles of [15 s at 95°C and 1 min at 56°C]. In the experiments with constant VEGF, 10 pM VEGF was spiked in the dilution serial of the antigen mix.

Immuno-PCR detection protocol

Nanolink streptavidin-coated magnetic beads (Solulink) were functionalized with biotinylated monoclonal antibodies using companion Chromalink Biotin Labeling kit (Solulink) according to manufacturer protocol at a final concentration of 1×10^6 beads/ μ l and stored at 4°C in PBS (pH 7.4), .01% (v/v) Tween 20, 0.1% (w/v) BSA and 0.1% (w/v) sodium azide. Beads were next combined into blocking buffer (12.5 mM Tris pH 7.4, 0.5% casein (w/v), 200 mM NaCl, 0.03% Tween 20 (v/v) and 10 μ g/ml calf thymus DNA (w/v)) at 20 $\times$ dilution to maintain individual bead concentration of 5×10^4 beads/ μ l and incubated for 1 hour at 30°C. After vortexing, 10 μ l of bead suspension was incubated with 90 μ l target proteins serially diluted in blocking buffer for 1 hour at 37°C. The beads were then washed with 3 $\times$ 500 μ l blocking buffer, and separated from solution using a magnetic rack. Next, 50 μ l of antibody–oligonucleotide conjugate detection probes (final concentration of 100 nM of each individual probe) was added to the mixture and incubated for an additional 30 min. Finally, beads were washed three times with 750 μ l washing buffer (10 mM Tris pH 7.4 and 0.01% Triton X100 (v/v)), and were ready for competitive PCR. During all blocking and incubation steps, reaction tubes were placed on a rotating device (Denville Lab Rotator) to keep the beads suspended.

Competitive PCR

After completing the immunoassay (either immuno-PCR or SP-PLA detection), we mixed all competitors at equal concentration, and then created a dilution series ranging from 100 nM to 0.01 pM in 10 $\times$ steps in PCR grade water with 0.01% Tween 20. We next created a *competitor-matrix plate* by adding 15 μ l of the competitor dilution at each concentration in the series to 15 μ l of immunoassay in a 96-well plate. In a second 96-well *pre-PCR plate*, we pipetted 5 μ l of PCR master mix in each well to be used. (PCR master mix has a final concentrations of 1.25 $\times$ Hotstar Taq buffer, 1.62 mM MgCl, 0.3 mM dNTP mix (Roche), 0.15 μ M PCR primers and 0.15 U/ μ l Hotstar Taq (Qiagen) in PCR grade water). Into the plate containing the PCR medley, we pipetted 7.5 μ l of target/competitor from the competitor matrix plate, gently mixing before and after each pipetting action to ensure proper

distribution of all components (12.5 μ l total). Next we drew 10 μ l from each well of the PCR/immunoassay/competitor mixture and transferred 2×5 μ l aliquots to a 384-well microtiter plate. This gives two technical replicates of each target/competitor combination. PCR was conducted on the 384-well plate in a thermocycler at 94°C for 15 min; followed by 45 cycles of [94°C for 20 s; 56°C for 30 s; and 72°C for 1 min]; and 72°C for 3 min.

PCR postprocessing and mass spectra acquisition

After PCR amplification, a series of processing steps was performed to interrogate the assays (for which the detailed protocols can be found in Sequenom application guides). First, unincorporated dNTPs that were not consumed in PCR reaction were degraded with shrimp alkaline phosphatase (SAP). Second, the information contained in the amplification products were interrogated by using oligonucleotide primers that anneal to the amplicons up to the position of the synthetic target/competitor polymorphisms. These extension primers and their products can be readily analyzed by MALDI-TOF mass spectrometry and enable unambiguous verification for the presence of the target protein probe. To compensate for mass/peak intensity differential in the time of flight (TOF), we used a regression formula based on empirical data to adjust each primer's concentration by the following factor: $[100/(-300.77 \times \ln(\text{primer_mass}) + 2730.7)]$. Third, after completing the extension reaction, salt adducts were removed from the extension products. Next, using the Nanodispenser (Sequenom), nanoliter volumes of sample from the microtiter plate were dispensed on a SpectroChip (Sequenom) and combined with the chip matrix. Finally, the chip was placed in the mass spectrometer where spectra were acquired on each spot and recorded to an Oracle database (see sample 8-plex mass spectra of targets and probes in Fig. 1d).

Data analyses

The data from competitive PCR and mass spectrometry were analyzed using the Quantitative Gene Expression software (Sequenom) and Microsoft Excel software [29]. The data from qPCR were analyzed with MxPro software (Stratagene) and Microsoft Excel software. The limit of detection (LOD) was calculated as $2 \times$ standard deviation above the signals obtained for the background noise.

Results and discussions

Employing immuno-PCR or SP-PLA in combination with competitive PCR and MALDI-TOF mass spectrometry, we conducted numerous spike-and-recovery experiments with up to eight target proteins in both buffer and serum. The results demonstrate consistent multiplex protein detection and quantification, spanning wide dynamic ranges (up to six orders of magnitude), with high sensitivity (limits of detection at attograms/microliter in buffer and femtograms/microliter in serum), using sample volumes as little as 10 μ l.

Immuno-PCR assays were conducted with competitive PCR and mass spectrometry under a variety of conditions – varying means of target capture, concentration of antigens and concentration of antibody–oligonucleotide conjugate probes. Results for an 8-plex series of spike-and-recovery of antigen into buffer are shown in Fig. 2a. Varying the antigen concentration in log-10 steps from 10 nM down to 1 fM, our resolvable dynamic range consistently spanned 5 logs (from 10 nM to 100 fM) for all proteins, and up to 6 logs (10 nM to 10 fM) for some species (e.g. VEGF in Fig. 2a).

Given the consistent performance of SP-PLA, we performed antigen spike-and-recovery experiments in a background of 10% chicken serum. As above, the antigen concentration varies in log-10 steps from 10 nM down to 10 fM. We observe a dynamic range of 6 logs, from 10 nM to 10 fM (Fig. 2b). We then confirmed the mass spectrometry results using single-plex real-time PCR (Fig. 2c). As an additional control, we performed a second set of

PLA experiments in which we kept the concentration of one of our antigens (in this case VEGF) constant for the entire series, while concentrations of the other antigens were varied in log-10 steps from 10 nM down to 10 fM. The presence of the other highly abundant proteins did not affect detection of VEGF (Fig. 2d).

The immuno-PCR and PLA platforms we employed have complementary strengths. The immuno-PCR advantages include: a relatively simple assay design, good sensitivity (500 attograms/ μ l) and a wide dynamic range (5+ orders of magnitude). The immuno-PCR approach has slightly higher background (100–1000 amplification templates/ μ l) than PLA, and slightly greater variability between assays, which can be due to inefficiency of manual magnetic separation. The SP-PLA shows demonstrable promise in our combined method. Its advantages include: extremely low background levels (10–100 amplification templates/ μ l), resulting in greater than 10-fold higher sensitivity as compared to immuno-PCR, insensitivity to serum components, and wider dynamic range (six orders of magnitude). Disadvantages of SP-PLA include variability resulting from our use of a pre-PCR amplification step, and a slightly more complex protocol (the requirement for degradation of uridine-containing ligation splints).

Employing a combination of protein detection via antibody–oligonucleotide conjugates, competitive PCR and the MassARRAY mass spectrometry platform, we have achieved consistent multiplex protein detection and quantification, spanning wide dynamic ranges, with high sensitivity (limits of detection at attograms/microliter in buffer and femtograms/microliter in serum), using sample volumes as low as 10 μ l. Compared with standard protein detection assays, our combined technology has an approximately 100-fold increase in sensitivity, requires 10–100-fold lower sample volume and offers multiplex and high-throughput opportunities. These attributes could facilitate commercial applications, which might include biomarker discovery and validation, as well as clinical diagnostics involving tumor markers, viral proteins, microbial pathogens, metabolism-related proteins and autoimmune-related proteins.

Future directions include increased multiplexing and increased automation. We have designed immuno-competitive PCR components for parallel detection of 65 targets. The assays could also be reformatted for increased automation to achieve greater consistency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1. General methodology for DNA-based protein detection, competitive PCR and mass spectrometry readout. **(a)** Immuno-PCR assay. **(b)** Solid-phase PLA assay. **(c)** Competitive PCR. **(d)** Mass spectrum of multiplex target and competitor assay.

FIGURE 2.

Protein detection with DNA readout. **(a)** Immuno-PCR 8-plex with MS readout at various antigen concentrations in buffer solution. **(b)** SP-PLA 6-plex with MS readout, antigens spiked in 10% chicken serum. **(c)** SP-PLA 6-plex assay, separated into single-plex after 'pre-PCR' cycles, and evaluated with real-time qPCR. **(d)** SP-PLA 6-plex with VEGF held with constant concentration, MS readout.