

Solid-phase proximity ligation assays for individual or parallel protein analyses with readout via real-time PCR or sequencing

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Solid-phase proximity ligation assays share properties with the classical sandwich immunoassays for protein detection. The proteins captured via antibodies on solid supports are, however, detected not by single antibodies with detectable functions, but by pairs of antibodies with attached DNA strands. Upon recognition by these sets of three antibodies, pairs of DNA strands brought in proximity are joined by ligation. The ligated reporter DNA strands are then detected via methods such as real-time PCR or next-generation sequencing (NGS). We describe how to construct assays that can offer improved detection specificity by virtue of recognition by three antibodies, as well as enhanced sensitivity owing to reduced background and amplified detection. Finally, we also illustrate how the assays can be applied for parallel detection of proteins, taking advantage of the oligonucleotide ligation step to avoid background problems that might arise with multiplexing. The protocol for the singleplex solid-phase proximity ligation assay takes ~5 h. The multiplex version of the assay takes 7–8 h depending on whether quantitative PCR (qPCR) or sequencing is used as the readout. The time for the sequencing-based protocol includes the library preparation but not the actual sequencing, as times may vary based on the choice of sequencing platform.

INTRODUCTION

Proteomes—the entireties of proteins expressed by a cell, tissue or an organism—typically constitute highly complex sets of macromolecules with many building blocks (amino acids), members (proteins) and wide differences in abundance among the proteins, as seen, for example, in the plasma proteome¹.

It is also clear that, despite the strong potential scientific and medical value of protein analysis, current analytical methods only access a fraction of the information embodied in proteomes. Examples of shortcomings of current assays are as follows:

- The inability to detect proteins that represent a very small fraction of all proteins in a sample. This is desirable for purposes such as detection of low concentrations of tissue-specific leakage markers in blood that could signal tissue damage at early time points.
- The difficulty of distinguishing closely similar but sometimes functionally distinct forms of proteins, which might arise through alternative splicing, differential processing or post-translational modification.
- Problems in monitoring interactions within protein complexes that may reveal the activity states of cellular signaling cascades.
- Limits in the number of proteins that can be investigated in parallel in a biological specimen, reducing the scope of analyses and complicating studies of the covariation of concentrations of different protein species.

Proximity ligation² and proximity extension assays³ (PLA and PEA, respectively) can address the above limitations by offering a means to construct highly sensitive and specific detection reactions for sets of protein variants or protein complexes. These assays use sets of affinity reagents that are directed against proteins of interest and modified through the addition of short DNA strands, referred to

as PLA probes. Upon joint detection of a target protein or protein complex by two or more such affinity reagents, the attached DNA strands are brought in sufficient proximity to undergo joint enzymatic reactions, i.e., ligation or polymerization reactions. These enzymatic reactions are designed to give rise to DNA products that can be detected via amplification reactions, whereas any unreacted probes fail to give rise to detection signals.

Proximity-based detection reactions differ from immuno-PCR⁴ or immune rolling-circle amplification (RCA) techniques⁵. In contrast to these earlier techniques, proximity reactions depend on the simultaneous recognition of target molecules by two or more oligonucleotide-modified affinity reactions in order to trigger the formation of amplifiable reaction products. Therefore, individual detection reagents fail to be detected, thus avoiding background from single nonspecifically bound reagents.

Proximity reactions have been designed for localized detection in cells or tissues of single proteins, of colocalized pairs of proteins or of post-translationally modified proteins via *in situ* PLA⁶. In these reactions, oligonucleotides attached to pairs of antibodies with bound target epitopes in close proximity are used to template the formation, via ligation, of DNA circles that are then locally amplified via RCA reactions.

Both PLA and PEA reactions can be configured to detect individual or panels of proteins present in solution without the need for a solid phase or for washes^{2,7–10}. In these assays, the incubation of a sample with PLA probes is followed by a dilution step to increase the average distance between any PLA probes that have failed to bind jointly to a target molecule. This serves to reduce background. By contrast, pairs of PLA probes that remain in proximity by having bound a target molecule can now undergo enzymatic reactions that create reporter DNA strands for subsequent

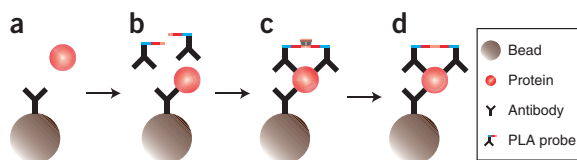


Figure 1 | Principle of spPLA. (a) Functionalized beads are first incubated with the sample. (b) Upon sample incubation and washing, the beads are incubated with PLA probes. (c) Upon proximal binding, PLA probes are allowed to hybridize to a short connector oligonucleotide. (d) The oligonucleotides on the PLA probes are then ligated and detected with real-time PCR or sequencing.

amplification reactions. PEA and *in situ* PLA assays are commercially available from Olink Bioscience.

In the case of PLA, oligonucleotides attached to antibodies that have both bound their target are joined by ligation by adding a connector oligonucleotide and a ligase. In PEA, the antibody-bound oligonucleotides undergo a templated polymerization reaction in which one of the oligonucleotides serves as a primer and the other as a template. In both assays, the target protein-specific reporter strands that form are typically quantified via real-time PCR.

Here we provide protocols for another form of PLA, namely solid-phase PLA (spPLA)¹¹, which offers highly specific and sensitive detection of single or multiple proteins in, e.g., blood plasma, cerebrospinal fluid or in tissue lysates by requiring recognition of target molecules by sets of three antibodies. An illustration of spPLA can be found in **Figure 1**.

Overview of the procedure

In spPLA¹¹, antibodies are first immobilized on a solid support, acting as capture agents for the proteins of interest (**Fig. 1a**). After protein capture, the solid phases are washed to remove matrix constituents that might contribute to background noise or interfere with binding reactions, enzymatic steps or recording of signals. Subsequently, captured proteins are detected using pairs of antibodies that have been conjugated to distinct oligonucleotides (**Fig. 1b**). Upon binding, washes are performed to remove unbound detection reagents to avoid target-independent reaction products. Oligonucleotides on pairs of PLA probes that have been brought in proximity owing to antigen recognition are then allowed to hybridize to a short connector oligonucleotide, so that the antibody-conjugated oligonucleotides can be joined by enzymatic ligation (**Fig. 1c**). Ligation products are then detected and quantified with qPCR or NGS, thus revealing protein concentrations according to the abundance of each specific ligation product (**Fig. 1d**).

spPLA is suitable for analyzing sample volumes from a few microliters to several milliliters. The unique requirement for recognition by three antibodies serves both to reduce background due to nonspecifically bound detection reagents and to minimize cross-reactions for irrelevant, even closely related molecules. The assay can be used to investigate a single protein species, and it can also be adapted for parallel detection of multiple proteins without an increasing risk of nonspecific reaction products associated with multiplexing. This is possible because the reagents can be designed such that only the correct combinations of PLA probes can give rise to ligated reporter strands¹². Finally, reporter-strand detection via next-generation sequencing provides a digital readout for greater

assay precision compared with assay readouts where intensities or threshold cycles are used as measures of protein amounts. An overview of the described protocol is shown in **Figure 2**.

Applications of the method

spPLA has been used to detect many different proteins in serum or plasma. Assay sensitivity is typically in the low-picomolar range, most often resulting in an improved sensitivity of detection compared with sandwich immunoassays, sometimes by as much as three orders of magnitude using the same affinity-purified polyclonal antibody preparation¹¹.

By using spPLA, concentrations of the protein GDF-15 have been measured in clinical samples with very good reproducibility in an investigation lending further support to the role of this protein as a marker for coronary artery disease¹². A multiplex version of spPLA has been used to simultaneously detect up to 36 proteins in order to identify diagnostic biomarkers for colorectal cancer¹³ and cardiovascular disease¹². In addition, up to 48 proteins have been analyzed using multiplex spPLA for the identification of biomarkers for chronic pain and Alzheimer's disease (A.-L. Lind, D.W., E. Freyhult, M. Gustafsson, T. Gordh, U. Landegren *et al.*, unpublished data).

The assay has also been used to detect amyloid- β (A β) protofibrils, efficiently discriminating between monomeric and aggregated forms of the protein and achieving a 25-fold improved sensitivity compared with a sandwich ELISA using the same monoclonal antibody¹⁴. Furthermore, spPLA can detect complex targets such as a form of microvesicles referred to as prostasomes¹⁵, by using a variant of the assay in which a combination of four antibodies

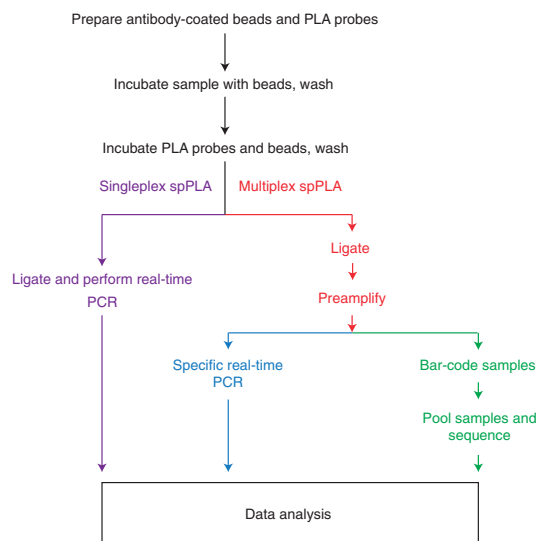


Figure 2 | Overview of the spPLA protocol. The initial three steps of the protocol are in common for both the singleplex and multiplex version of the protocol. When a single protein is detected, ligation and PCR detection are performed simultaneously, after the incubation of beads with PLA probes (purple). When multiple proteins are detected, ligation is performed separately, followed by universal PCR preamplification (red). Preamplified products are either prepared for sequencing (green) or for readout using real-time PCR (blue).

is used as a PLA probe. In this way, very low concentrations of prostasomes were detected in blood of patients with different stages of prostate cancer, thereby identifying these structures as promising diagnostic biomarkers that can potentially distinguish patients with different histological grades of malignancy. spPLA has furthermore been used to detect biosecurity agents¹⁶; interacting proteins¹⁷; and, via an inhibition format, low concentrations of low-molecular-weight molecules¹⁸.

Proximity-based immunoassays can be used when specific and sensitive detection is required. The assay can be multiplexed and miniaturized to allow for high-throughput biomarker validation in large sample cohorts, and it is expected to be of value for diagnostic applications and in drug development. Suitably modified assay formats could also render the assays useful for diagnostics at the point of care.

Comparison with other methods

Mass spectrometry is by far the most powerful method for analyzing thousands of proteins in a single run, but it remains limited in sensitivity for highly complex proteomes such as that of blood plasma¹⁹. The problems can be mitigated by sample depletion and fractionation before analysis, as well as by integration of affinity enrichment²⁰ with selective reaction monitoring²¹. Combinations of the above have resulted in very sensitive mass spectrometry assays, but at the expense of multiplexing and sample throughput²². PLA belongs to a class of next-generation immunoassays with increased sensitivity, specificity and/or suitability for multiplexing. Compared with standard colorimetric or fluorescent sandwich immunoassays, spPLA has improved sensitivity as a result of successful background suppression, owing to the use of PCR for amplified detection as well as to the requirement for multiple recognition events in order to generate detection signals.

Compared with other highly sensitive DNA-based assays such as immuno-PCR⁴ or bio-barcode assays²³, an advantage of spPLA is the requirement for multiple recognitions to generate amplifiable reporters, and the potential for multiplexing without compromising the sensitivity or specificity of detection. We have demonstrated experimentally that unspecific background increases only minimally when many proteins are detected in parallel, thus allowing assay performance to be maintained with multiplexing, in contrast to the use of sandwich assays¹². Although the homogeneous solution-phase proximity ligation or extension assays have a simple workflow because there are no washes, spPLA can offer a number of advantages. The opportunity to perform washes of molecules captured on the solid support means that excess affinity reagents and sample components that interfere with detection can be removed. It also means that sample volumes larger than the 1 or 2 μ l used in the homogeneous assays can be used, something that can be of value for enhancing detection sensitivity, dynamic ranges and avoiding stochastic noise. Finally, the spPLA goes beyond the more standard sandwich assays and homogeneous proximity assays with the requirement that three different affinity reagents all recognize each target molecule, which adds specificity and the ability to distinguish closely similar molecules.

As discussed, spPLA can also be used to specifically investigate classes of molecules that may be inaccessible to analysis by other methods such as aggregated¹⁴ or interacting proteins¹⁷, and large particulate structures such as microvesicles or exosomes¹⁵.

Limitations

As with any immunoassay, the performance of spPLA is highly dependent on the quality of the affinity reagents. Despite the fact that the assay design allows the influence of cross-reactive events to be minimized, optimal assay performance mandates the use of specific binders of high affinity. In addition, given the requirement for multiple antigen recognition, sets of compatible monoclonal binders or polyclonal binders capable of recognizing several epitopes of the antigen without steric hindrance must be used.

Typical spPLA coefficients of variation are approximately 20–25%, limiting precise quantification. A large part of this variation is the result of the PCR step, which can be highly variable at low target copy numbers. Other amplification methods such as RCA have been used, enabling linear signal amplification and digital quantification by counting RCA products, thereby resulting in improved assay precision¹⁶.

Experimental design

General considerations. spPLA is designed to achieve a high degree of specificity of protein detection by using three independent target-recognition events. Accordingly, the assay is well suited for analysis of proteins, ranging from small cytokine molecules to larger proteins, present at low concentrations in complex biological matrices. spPLA is also suitable for detecting targets such as protein complexes, proteins bearing post-translational modifications, and intact viruses and bacteria. It is also possible to reconfigure the assay architecture to detect more complicated targets, such as microvesicles or exosomes, by requiring four or more target recognizing events¹⁵. This protocol describes a triple-binder spPLA for the detection of proteins individually and in multiplex.

There are two main options with respect to the choice of solid support for spPLA: microtiter plates and microparticles. Both perform equally well for detecting single protein species; however, the microparticle-based protocol has better reaction kinetics and is more suitable for multiplex assays.

For a typical spPLA protocol, we recommend using solid phases with hydrophobic surfaces; however, hydrophilic surfaces may be suitable in specific cases. When you are using polyclonal antisera both for capture and detection, antibodies should be immobilized with an empirically adjusted antibody density in order to avoid blocking excessive numbers of epitopes by the capture antibodies. Unspecific background signals tend to be proportional to the total surface area used. It is thus important to maintain a balance between efficient capture and surface area. As spPLA uses antibodies coupled to DNA oligonucleotides, it is important that blocking agents include antisera from the same species as the detection antibodies, as well as oligonucleotide-blocking compounds such as sheared salmon sperm DNA.

The incubation time for the initial target capture or the subsequent detection depends on the affinity of antibody-antigen interaction. In a typical case, incubation for 1–2 h at room temperature (RT, 20–25 °C) is sufficient. Longer incubation times may result in increased background, whereas shorter incubation time may result in signal reduction and increased assay variation. It is usually sufficient to remove excess reagents from previous steps by two washes with PBS-T. However, different targets or biological matrices may require modified washing procedures.

The individual and multiplex spPLA protocols described herein use two different DNA ligases. These ligases have different

temperature optima and sequence preferences and may differ in efficiency and specificity. Therefore, deviations from the protocol we describe should be properly validated. In addition, reduced ligation times may result in loss of signals owing to incomplete ligation, whereas longer ligation incubation time at high temperature (e.g., 50 °C) can result in loss of signals from dissociation of antigen and antibodies. Enzyme solutions should be freshly prepared before use.

Affinity reagents. As mentioned above, sets of affinity reagents for any target protein should be capable of recognizing at least three nonoverlapping epitopes. In practice, we have a good success rate using affinity-purified polyclonal antibodies raised against whole proteins, as these typically include specificities directed against several epitopes. Alternatively, sets of three monoclonal reagents that bind distinct epitopes of the protein are also suitable, or combinations of monoclonal and polyclonal reagents. In spPLA, antibodies must be modified, for example, by biotinylation for binding to streptavidin-oligonucleotide conjugates or by direct covalent conjugation to oligonucleotides. There is a risk, however, that the antigen-binding ability of an affinity reagent is disrupted by the conjugation. We therefore recommend that new conjugates be validated by analyzing dilutions of known amounts of the target protein. This is also helpful for estimating absolute amounts of target proteins in unknown samples by reference to results from analysis of the standard curve.

Sample types. spPLA performs robustly in various sample types, such as plasma, serum, whole blood, cerebrospinal fluid, brain homogenates, and tissue and cell lysates. Typically, 5- μ l sample aliquots are used for each assay. To eliminate potential matrix interference, it may be helpful to perform an extra matrix depletion step by first removing irrelevant substances binding to the solid phase through a first incubation with microparticles without capture antibodies, or samples can be simply diluted tenfold before incubation with the capture supports.

Detection of ligation products. DNA ligation products can be detected by real-time PCR, either using specific TaqMan probes or nonspecific intercalating dyes such as SYBR Green. Ligation products can also be enumerated using NGS Technologies. To compensate for wide differences in the amounts of different targeted proteins for NGS readout, it can be helpful to adjust the concentrations of detection reagents so that less ligation products are generated for more abundant proteins.

Design of oligonucleotides. The reagents required for the detection of a protein by spPLA include five or six different oligonucleotides: two oligonucleotides for each pair of PLA probes, one ligation template, two PCR primers, and, if desired, also one TaqMan probe. If the ligation products are to be detected by NGS, sample tags and index motifs required by the particular sequencer must be introduced. Oligonucleotides should have minimal complementarity to extraneous sequences that may be present in the investigated sample. We recommend using BLAST or other *in silico* means to exclude the presence of such sequences. Furthermore, oligonucleotides should be of high purity and quality. Longer oligonucleotides can be ordered from commercial vendors that offer HPLC or PAGE purification.

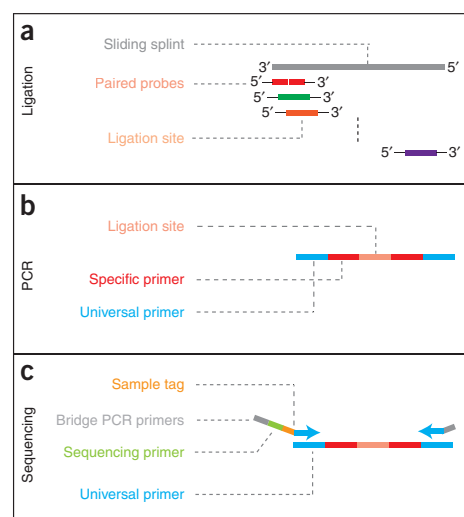


Figure 3 | Oligonucleotide design. (a) The sliding splint design. (b) Positioning of different sequence elements on the ligated PLA arms. (c) Design of oligonucleotides used for the preparation of libraries for NGS.

Newly designed oligonucleotides for PLA probes or PCR primers should be tested before use to assess ligation and amplification efficiency and specificity. Each oligonucleotide category is described in greater detail below and graphically explained in **Figure 3**.

- **Oligonucleotides for PLA probes.** Typically, the oligonucleotides coupled to antibodies are single-stranded DNA molecules between 50 and 60 nt in length, with a balanced GC-to-AT ratio. The oligonucleotides include two general functional motifs: sequence elements that allow the oligonucleotide to be joined via enzymatic ligation by hybridizing to a common connector oligonucleotide, and PCR primer-binding sites. In the case of multiplex PLA, each oligonucleotide includes two PCR primer sites in tandem: one universal site that is common to all PLA probe pairs and one specific site for each cognate PLA probe pair. The specific primer sites can also be used to identify correct ligation products after sequencing and are usually 20 nt long. Individual PLA oligonucleotides should be tested *in silico* to ensure minimum secondary structure by DNA-folding analysis tools (e.g., Mfold). A pair or set of PLA oligonucleotides should be tested to ensure minimum nonspecific hybridization and self-templated ligations by using multiple sequence alignment tools (e.g., ClustalW). To avoid contamination of oligonucleotides before conjugation, we recommend ordering the members of each pair of PLA oligonucleotides from two different commercial vendors. The oligonucleotides that participate in the ligation reaction via their 5' ends must be phosphorylated.
- **Ligation template.** The ligation template is designed to preferentially permit hybridization to oligonucleotides on PLA probes that have been brought in proximity. Each member of a PLA probe pair has a 10-nt-long hybridization sequence at its free ends, bringing the length of the hybridization template to 20 nt. This design is amenable to ligation with either Ampligase or T4 DNA ligase. In multiplex PLA, we have made use of a

‘sliding-splint’ design, allowing only the correct pairs of PLA probe oligonucleotides to be ligated, although all pairs can hybridize to a single ligation template. In this manner, only correct pairs of oligonucleotides are correctly positioned to be ligated, as the junction of each oligonucleotide pairs are all offset by at least one nucleotide from any other pair (Fig. 3). With a hybridization sequence of each PLA probe pair member of 10 nt in length, a ligation template that is n nucleotides long ($n > 20$) can template ligation for $n-20$ unique oligonucleotide pairs. It is important to avoid nonspecific PCR amplification owing to extension of the PLA oligonucleotides by the ligation template. We have used two different approaches to avoid this risk: in the singleplex spPLA protocol, the ligation template is extended with a three-nucleotide-long noncomplementary sequence at each end, whereas in the multiplex PLA protocol T residues in the connector oligonucleotide are replaced by U residues. This allows the degradation of the ligation template before PCR using the enzyme uracil-DNA glycosylase (UDG). By replacing thymidine triphosphate with deoxyuridine triphosphate during PCR, this enzymatic step also serves to remove any PCR contaminants. To ensure that only the correct combinations of PLA probes can give rise to detectable reaction products in multiplex spPLA, we use the above sliding-splint design for the ligation template. Besides this ‘constrained ligation’, primer pairs used for real-time PCR detection of protein-specific DNA reporters are also selected so that only the cognate pairs of PLA probes can give rise to amplification products. When reaction products are detected through NGS, any noncognate pair of tags that may be recorded can be discounted as acts of cross-reactivity.

- **PCR primers.** Primer design for spPLA follows guidelines for other PCR-based assays²⁴. Specific PCR primers in multiplex PLA should be tested *in silico* and *in vitro* for minimum interactions.
- **TaqMan probe.** TaqMan probe design for spPLA follows guidelines for other real-time PCR-based assays (e.g., <http://www.premierbiosoft.com/>).
- **Sequencing oligonucleotides.** Ligation products from spPLA can be identified using any NGS instrument, and besides protein-specific motifs the reporter strands can be modified to also include sample tags, added after the spPLA reaction to permit pooling of ligation products from many different analyzed samples. We have used two sets of oligonucleotides to prepare a sequencing library: one set of index primers that include motifs required by the particular sequencer and a sample index, and PLA primers used to label ligation products by PCR. In addition, one set of enrichment primers composed of sequencer-specific primers to enrich indexed ligation products by PCR is used.

PLA probe conjugations. A number of different protocols are available for coupling antibodies to oligonucleotides. Briefly, we have mainly used two approaches: biotin-streptavidin-mediated coupling¹¹ and covalent conjugation by the use of hetero-bifunctional linkers¹². Streptavidin-linked oligonucleotides, as well as antibody-DNA conjugates and commercial conjugation kits, can also be purchased from commercial vendors.

Controls and calibration curves. We recommend the inclusion of positive and negative controls with every experiment. Calibration curves of dilutions of recombinant antigens in buffer or a nonhuman-derived relevant matrix such as chicken serum or plasma can serve as positive controls. For most assays, the dilution series should cover concentrations starting from 1 or 10 nM to low-fM concentrations. The same diluent without the protein of interest serves as a negative control. To exclude template-independent amplification by the PCR primers, PCR can be attempted in the absence of added template or ligase.

To avoid saturation during universal PCR amplification after multiplex spPLA, and for normalization purposes, PLA reagents for an added protein control should be included, such as mouse IgG for the analysis of human samples, with the same concentration of the control added to each sample. The concentration of such spiked-in internal controls should be within the dynamic range of spPLA. For example, mouse IgG was spiked in every sample at a concentration of 10 pM in our previous studies of human protein biomarkers¹².

Raw data acquisition. Real-time PCR data are exported as values of threshold cycle (C_t) using each instrument’s built-in software. For further analysis, we recommend the conversion of the reverse and logarithmic C_t scale to a more convenient linear scale. This can be done using the following equation: $M_a = (1 + E)^{(C_{t1} - C_{ta})}$, where M_a is the number of PCR amplicons, E is the efficiency of replication in each PCR cycle, C_{t1} the number of PCR cycles required to bring an individual amplicon to the threshold for fluorescence detection and C_{ta} is the C_t value of each sample. The formula can be used without the intention to convert C_t values to actual numbers of molecules, and thus values for E and C_{t1} may be set arbitrarily without affecting the analysis. If exact conversion is required, then experimentally deduced values of E and C_{t1} should be used.

Reads obtained from DNA sequencing are first sorted according to the presence of sample indices, and then mapped to a reference library consisting of all the expected ligation products, counting the occurrence of each ligation product as a measure of the amounts of specific proteins present in this sample. Depending on the performance of the sequencing technology used, numbers of allowed mismatches in the alignment of obtained reads can be varied to obtain the best results. Only uniquely mapped reads are used for subsequent analysis.

Data analysis and quantification. Absolute concentrations of target proteins can be inferred using calibration curves. Calibration curves are constructed by plotting numbers of detected amplicons as converted from C_t values versus the concentration of each protein standard by regression analysis. We recommend plotting data using log/log axes. If samples have been diluted, the concentration calculated from calibration curves should be multiplied by the dilution factor.

For multiplex PLA, data of each individual sample should be normalized against its spike-in control value. We use numbers of starting amplicons directly for further analysis.

MATERIALS

REAGENTS

• Samples (see INTRODUCTION)

• DNA size ladder, 50 bp (Thermo Scientific, cat. no. SM0373)

• AccuStart Taq DNA polymerase (contains Taq polymerase, 5 U μl^{-1} , 10 $\times$ PCR buffer and 50 mM magnesium chloride; cat. no. 95061-05K, Quanta Biosciences)

• Ampligase DNA ligase, 5 U μl^{-1} (Epicentre Biotechnology, cat. no. A3210K)

• Ampligase 10 $\times$ reaction buffer (Epicentre Biotechnology, cat. no. A1905B)

• Antibodies, unmodified, selected according to the protein(s) targeted for analysis (for a list of validated polyclonal antibodies see **Supplementary Table 1**, and for validated monoclonal antibodies see refs. 8,14)

• Antibodies, biotinylated, selected according to the protein(s) targeted for analysis (for a list of validated biotinylated antibodies see **Supplementary Table 1**)

• ATP, 100 mM solution (Thermo Scientific, cat. no. R0441)

• BSA, 10 mg ml^{-1} (New England Biolabs, cat. no. B9001S)

• D-Biotin (Life Technologies, cat. no. B-1595)

• DMSO (Sigma-Aldrich, cat. no. 41648)

• Dynabeads MyOne streptavidin T1 beads, 10 mg ml^{-1} (Life Technologies, cat. no. 65602)

• dNTP set of 100 mM solutions, including deoxyuridine triphosphate in place of thymidine triphosphate (Thermo Scientific, cat. no. V0195)

• EDTA (EDTA disodium salt dihydrate; Sigma-Aldrich, cat. no. E5134-500G)

• DTT (dithiothreitol; Sigma-Aldrich, cat. no. D0632)

• GFX columns (GE Healthcare, cat. no. 27-9602-01)

• Goat IgG (Sigma-Aldrich, cat. no. I5256-50MG)

• β -Nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich, cat. no. N8535)

• Magnesium acetate

• Oligonucleotides (for different oligonucleotide designs see **Supplementary Tables 2–5** and the INTRODUCTION). Streptavidin-conjugated oligonucleotides were ordered from Solulink

• PLA probes (see INTRODUCTION)

• Potassium acetate

• Potassium chloride (Merck, cat. no. 1.04936.1000)

• Potassium dihydrogen phosphate (Merck, cat. no. 1.04873.1000)

• Salmon sperm DNA (Life Technologies, cat. no. 15632-011)

• Sodium chloride (Merck, cat. no. 1.06404.1000)

• Disodium hydrogen phosphate dihydrate (Merck, cat. no. 1.06580.1000)

• Sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC; Thermo Scientific, cat. no. 22322)

• SYBR Green I nucleic acid gel stain, 10,000 $\times$ concentrate in DMSO (Life Technologies, cat. no. S-7563)

• T4 DNA ligase, 1 U μl^{-1} (Thermo Scientific, cat. no. EL0016)

• Tween-20 (Sigma-Aldrich, cat. no. P9416)

• Uracil-DNA glycosylase (UDG), 1 U μl^{-1} (Thermo Scientific, cat. no. EN0362)

• UltraPure dithiothreitol (Life Technologies, cat. no. 15508-013)

• Zeba Spin desalting columns, 7 K MWCO, 0.5 ml (Thermo Scientific, cat. no. 89882)

• Zeba Spin desalting plates, 7 K MWCO (Thermo Scientific, cat. no. 89808)

• Trizma base

EQUIPMENT

• Beaker, 5 liters

• PCR strip, eight wells (e.g., Applied Biosystems, cat. no. 4316567)

• PCR plate, 96 wells (e.g., Sarstedt, cat. no. 72.1978.202; Thermo Scientific, cat. no. 732-4828)

• 2720 thermal cycler (Applied Biosystems, cat. no. 4359659)

• 7900 HT fast real-time PCR system (Applied Biosystems, cat. no. 4329001)

• Cap strips (8 caps per strip; e.g., Applied Biosystems, cat. no. 4323032)

• DynaMag-2 magnet (Life Technologies, cat. no. 12321D), or similar magnetic stand for microcentrifuge tubes

• DynaMag-96 side, 96-well-plate magnet (Life Technologies, cat. no. 12331D)

• E-gel agarose gel electrophoresis system (Life Technologies, cat. no. G6500)

• E-gel precasted agarose gel (Life Technologies, cat. no. G402002)

• Hydra 96 system (Robbins Scientific)

• Icebox

• Magnetic stir bar

• MicroAmp optical 384-well reaction plate with bar code (Applied Biosystems, cat. no. 4309849)

• Multichannel pipette (Eppendorf International)

• M $\times$ 3000P qPCR system (Agilent Technologies)

• Plate centrifuge (Eppendorf International)

• Rotator (Grant Bio PTR-30 360-degree vertical multifunction rotator, or similar)

• Self-adhesive PCR film (Sarstedt, cat. no. 95.1994)

• Slide-A-Lyzer MINI dialysis devices, 7 K MWCO (Thermo Scientific, cat. no. 69562)

• Vortex mixer

REAGENT SETUP

PBS buffer, 1 $\times$, pH 7.4 PBS buffer contains 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate. Adjust the pH to 7.4. Prepare the buffer before starting the experiment and store it at RT (20–25 °C) for up to 6 months.

PBS/0.1% (wt/vol) BSA, 1 $\times$ (storage buffer) Mix 1 $\times$ PBS (pH 7.4) with 1 mg ml^{-1} BSA. Prepare the buffer before starting the experiment; store it at 4 °C for up to 6 months.

PBS-T (washing buffer) Mix 1 $\times$ PBS (pH 7.4) with 0.05% (vol/vol) Tween-20. Prepare the buffer before the experiment; store it at RT for up to 6 months.

PLA buffer PLA buffer contains 1 $\times$ PBS, 1 mM D-biotin, 1 mg ml^{-1} BSA, 0.05% (vol/vol) Tween-20, 100 nM goat IgG, 0.1 mg ml^{-1} salmon sperm DNA and 5 mM EDTA. Prepare the buffer before starting the experiment. Store it at 4 °C for up to 2 months or at –20 °C for up to 12 months.

EDTA, 0.5 M, pH 8.0 Dissolve 0.5 M EDTA in high-purity water. Adjust the pH to 8.0 with NaOH. Sterilize the solution by autoclaving and store it at RT for up to 6 months.

Tris-HCl buffer, 1 M, pH 8.4 Dissolve 122 g of Trizma base in 1 liter of high-purity water and adjust the pH to 8.4. Prepare the buffer before starting the experiment and store it at RT for up to 6 months.

Ampligase 10 $\times$ (reaction buffer) Ampligase 10 $\times$ (reaction buffer) contains 200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM MgCl_2 , 5 mM NAD and 0.1% (vol/vol) Triton X-100. Store the buffer at –20 °C for up to 6 months.

Special PCR buffer Special PCR buffer contains 50 mM potassium acetate, 20 mM Trizma acetate and 3 mM magnesium acetate (pH 7.6). Store the buffer at –20 °C for up to 6 months.

Conjugation buffer Mix 1 $\times$ PBS with 5 mM EDTA (pH 7.4). Store the buffer at RT for up to 6 months.

PROCEDURE

Immobilization of antibodies on microparticles ● TIMING 1.5 h

1| Carefully resuspend microparticles in the stock tube to a homogeneous suspension. Transfer 100 μl of beads (10 mg ml^{-1}) into a 1.5-ml microcentrifuge tube for immobilization of the capture antibody. This will suffice for 200 spPLA reactions.

2| Place the tube on a magnetic stand for 30 s or until all beads form a pellet on the side of the tube toward the magnet and until the liquid appears clear in color. Remove and discard the supernatant.

PROTOCOL

3| Wash the microparticles by removing the tube from the magnet and resuspending it with 500 µl of washing buffer. Place the tube on the magnet for 30 s and discard the washing buffer. Repeat this step once.

4| Dilute the biotinylated antibody to 50 nM in storage buffer.

5| Add 200 µl of 50 nM biotinylated antibody into the tube containing the microparticles, and then incubate it on an end-over-end rotator at a speed of 16–20 r.p.m. for 1 h at RT.

▲ **CRITICAL STEP** When polyclonal antibodies are used as capture agents, signals decrease when the amount of antibody exceeds approximately one-tenth of the bead total capacity stated by the manufacturer. We believe that this is due to excessive epitope occupancy by the immobilized antibody, resulting in reduced epitope availability for the detection antibodies. We thus recommend using the suggested antibody coating density. In case that capture antibody concentration needs to be adjusted, we recommend titrations of capture antibody using the suggested amount of beads. We recommend the use of a positive and negative control to assess at which concentration the signal-to-noise to ratio is maximized.

6| Place the tube on a magnet for 30 s and discard the supernatant.

7| Wash the microparticles with 500 µl of washing buffer twice as described in Step 3.

8| After the last wash of microparticles in Step 7, resuspend the microparticles in 200 µl of storage buffer.

9| For preparing microparticles for the multiplex PLA, repeat Steps 1–8 separately for each additional antibody.

■ **PAUSE POINT** Functionalized beads can be stored at 4 °C for up to 6 months.

Conjugation of oligonucleotides on antibody ● **TIMING 10 h**

10| Dilute 20 µg of antibody in 10 µl of 1× PBS.

11| Prepare a fresh solution of 4 mM sulfo-SMCC in DMSO.

▲ **CRITICAL STEP** Sulfo-SMCC solution should always be freshly prepared before antibody functionalization.

12| Add 1 µl of dissolved sulfo-SMCC from Step 11 to the antibody from Step 10. Mix gently and incubate at RT for 2 h.

13| Freshly dissolve DTT in conjugation buffer to a final concentration of 100 mM.

▲ **CRITICAL STEP** DTT solution should always be freshly prepared before oligonucleotide reduction.

14| Mix 4 µl of the thiol-modified oligonucleotides (100 µM in water) with 11 µl of conjugation buffer and 15 µl of dissolved DTT from Step 13. Let the reduction proceed for 1 h at 37 °C.

▲ **CRITICAL STEP** The antibody activation and oligonucleotide reduction reactions should finish at the same time. To achieve this, initiate the reduction after ~1 h of activation.

15| Separately buffer-exchange the antibody and the oligonucleotides to conjugation buffer using Zeba columns or plates according to the manufacturer's recommendations.

16| Mix the collected antibody with the oligonucleotide.

17| Place the product of Step 16 into a 7-kD cutoff dialysis cup. Dialyze the product overnight at 4 °C against 5 liters of 1× PBS with constant stirring.

▲ **CRITICAL STEP** Dialysis should be performed immediately after mixing the activated antibody and oligonucleotides reduced by treatment with DTT.

18| Collect the conjugates from the dialysis cup and adjust the volume to 100 µl with storage buffer.

▲ **CRITICAL STEP** Antibodies that will undergo conjugation should be in buffer without free amine groups such as Tris, or carrier proteins such as BSA.

■ **PAUSE POINT** The conjugates can be kept at 4 °C for up to 6 months.

Sample preparation ● TIMING 30 min

19| Dilute samples such as plasma, serum or whole blood tenfold in PLA buffer. Each spPLA reaction requires 45 µl of diluted sample.

20| Prepare a dilution series of recombinant protein in PLA buffer as described in the INTRODUCTION. We recommend using recombinant protein dilution series in tenfold increments ranging from low-fM concentrations to 1 or 10 nM.

Capturing target protein on microparticles ● TIMING 2 h

21| Vortex the antibody-coated microparticles prepared in Steps 1–9 to achieve a homogeneous suspension.

22| For one singleplex PLA reaction, pipette 1 µl of the antibody-coated microparticle suspension. For one multiplex PLA reaction, pipette 0.1 µl of each bead suspension functionalized with different capture antibodies.

▲ CRITICAL STEP For multiplex PLA, a master mix of all beads is usually prepared to avoid the necessity of pipetting 0.1 µl. However, beads can be further diluted to allow reliable pipetting.

23| Remove the storage buffer by placing the beads on a magnet, and then add 5 µl of PLA buffer. Resuspend the beads by vortexing. Adjust the volumes for different numbers of assays.

24| Mix 5 µl of beads prepared in Step 23 with 45 µl of each sample prepared in Step 19, or with a protein standard prepared in Step 20 in a well of a microtiter plate or PCR strip. Seal the wells and vortex the plate to ensure that microparticles are homogeneously suspended.

25| Incubate the reactions on an end-over-end rotator at a speed of 16–20 r.p.m. for 1.5 h at RT.

Probe mix preparation ● TIMING 10 min–1 h

26| Calculate the amount of probe mix required for all PLA reactions. For each PLA reaction, 50 µl of a 250 pM probe mix is required.

▲ CRITICAL STEP Different preparations may be required depending on the nature of the conjugates. Covalently linked conjugates that have been prepared in advance can simply be diluted to the desired concentration. When streptavidin-coupled oligonucleotides are coupled to biotinylated antibodies, Steps 27–29 can be used.

27| Separately prepare the concentrated PLA probes (50 nM). For one spPLA reaction, combine the following:

Reagents	Starting concentration	Amount (µl)	Final concentration
Biotinylated antibody	1 µM	0.0125	50 nM
SLC1 or SLC2	100 nM	0.125	50 nM
PBS/0.1% (wt/vol) BSA	—	0.1125	—
Total volume	—	0.25	—

28| Incubate the concentrated probes for 1 h at RT.

29| Dilute the two concentrated probe mixes 100-fold in PLA buffer and incubate them for 5 min at RT.

▲ CRITICAL STEP When streptavidin-conjugated oligonucleotides (SLC1 and SLC2) are coupled to biotinylated antibodies, each PLA probe must be prepared and incubated in a separate tube. This is to avoid SLC1 and SLC2 binding to the same antibody, something that would give rise to increased assay background. The two PLA probes must be kept separate until they have been incubated with PLA buffer (containing free biotin) that blocks biotin-binding sites on SLC1 and SLC2.

■ PAUSE POINT We recommend preparing probes before every experiment. Alternatively, probes can be stored at 4 °C for time periods not exceeding a week.

30| Mix the two probes in one microcentrifuge tube. The final concentration of each probe should be 250 pM. For the multiplex PLA, mix covalent conjugates in one microcentrifuge tube to a final concentration of 250 pM for each probe.

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Detection of captured proteins ● TIMING 2 h

31| After incubation at Step 25, wash the beads twice with 100 µl of washing buffer: spin down the plate or strip in a plate centrifuge (1,000g for 5 s at RT) to collect all of the liquid at the bottom of the wells. Put the plate or strip on a 96-well plate magnet for 30 s until all the beads form a pellet by the side of the tubes. Discard the supernatant and add 100 µl of washing buffer into each well with a multichannel pipette. Let the plate or strip stand on the magnet for 30 s before removing the liquid. Repeat this step once.

32| Add 50 µl of probe mix prepared in Step 30 into each well. Vortex samples to obtain a homogeneous solution in each well.

33| Incubate the reactions on an end-over-end rotator with a speed of 16–20 r.p.m. for 1.5 h at RT.

▲ **CRITICAL STEP** Do not disrupt the magnetically attracted beads during washing. Completely remove the washing buffer before the addition of the probe mix. Do not leave the beads without liquid over prolonged periods of time.

34| If you are performing singleplex spPLA, follow option A. If you are performing multiplex spPLA and specific real-time PCR, follow option B. If you are performing multiplex spPLA and sequencing, follow option C.

(A) Ligation and qPCR for singleplex spPLA ● TIMING 2 h

(i) Prepare the ligation and PCR mix as described below. For each PLA reaction, 50 µl of ligation and PCR mix is needed.

Mix containing TaqMan probe (first table) or SYBR Green I (second table) can be used for the qPCR.

TaqMan probe mix:

Reagents	Starting concentration	Amount (µl)	Final concentration
PCR buffer	10×	5	1×
MgCl ₂	50 mM	2.5	2.5 mM
TaqMan probe	10 µM	1.1	0.22 µM
Biofwd	10 µM	0.5	0.1 µM
Biorev	10 µM	0.5	0.1 µM
Biosplint	10 µM	0.5	0.1 µM
ATP	100 mM	0.04	0.08 mM
dN(A,C,G,U)TP	25 mM	0.4	0.2 mM
Taq polymerase	5 U µl ⁻¹	0.3	0.03 U µl ⁻¹
T4 DNA ligase	1 U µl ⁻¹	0.5	0.01 U µl ⁻¹
UDG	1 U µl ⁻¹	0.1	0.002 U µl ⁻¹
H ₂ O	—	39.06	—
Total volume		50	

SYBR Green I mix:

Reagents	Starting concentration	Amount (µl)	Final concentration
PCR buffer	10×	5	1×
MgCl ₂	50 mM	2.5	2.5 mM
SYBR Green I in DMSO	10×	2.5	0.5×
Biofwd	10 µM	0.5	0.1 µM
Biorev	10 µM	0.5	0.1 µM
Biosplint	10 µM	0.5	0.1 µM
ATP	100 mM	0.04	0.08 mM
dN(A,C,G,U)TP	25 mM	0.4	0.2 mM

(continued)

SYBR Green I mix: (continued)

Reagents	Starting concentration	Amount (μL)	Final concentration
Taq polymerase	5 U μL^{-1}	0.3	0.03 U μL^{-1}
T4 DNA ligase	1 U μL^{-1}	0.5	0.01 U μL^{-1}
UDG	1 U μL^{-1}	0.1	0.002 U μL^{-1}
H ₂ O	—	37.16	—
Total volume	—	50	—

- (ii) After the incubation in Step 33, wash the beads twice with 100 μL of washing buffer as described in Step 31.
- (iii) Add 50 μL of ligation and PCR mix prepared in Step 34A(i) into each well.
- (iv) Incubate the plate or strip for 5 min at RT, and then perform the qPCR program as follows.

Cycle

1	2 min at 95 °C	
2–41	15 s at 95 °C	1 min at 60 °C

▲ **CRITICAL STEP** When a large number of reactions are performed, we recommend adding the reaction mix using a multichannel or multidispensing pipette. This can help mitigate ligation variation due to differences in incubation times.

- (v) After qPCR, export C_t values and analyze as described in the INTRODUCTION.

? TROUBLESHOOTING

(B) Multiplex spPLA readout by specific real-time PCR ● TIMING 5 h 20 min

- (i) *Ligation for multiplex spPLA*. Prepare ligation mix for one reaction as follows:

Reagents	Starting concentration	Amount (μL)	Final concentration
Ampligase reaction buffer	10×	5	1×
Ampligase	5 U μL^{-1}	0.5	0.05 U μL^{-1}
Connector oligonucleotide (sliding splint)	10 μM	0.5	100 nM
H ₂ O	—	44	—
Total volume	—	50	—

- (ii) After probing, wash the sample from Step 33 as described in Step 31.
- (iii) Add the ligation mix prepared in Step 34B(i) and incubate it at 50 °C for 10 min in a thermal cycler.
- (iv) Universal PCR preamplification for specific PCR readout. Prepare the universal PCR mix:

Reagents	Starting concentration	Amount (μL)	Final concentration
PCR buffer	10×	5	1×
MgCl ₂	50 mM	3	3 mM
dN(A,C,G,U)TP	25 mM	0.4	200 nM
Universal primer1	10 μM	0.5	100 nM
Universal primer2	10 μM	0.5	100 nM
Taq polymerase	5 U μL^{-1}	0.3	0.03 U μL^{-1}
UDG	1 U μL^{-1}	0.1	0.002 U μL^{-1}
H ₂ O	—	40.2	—
Total volume	—	50	—

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- (v) After ligation (Step 34B(iii)), wash the sample again as described in Step 31.
 (vi) Add the universal PCR mix from Step 34B(iv) and perform the universal PCR as follows:

Cycle			
1	10 min at 95 °C		
2–16	15 s at 95 °C	1 min at 62 °C	1 min at 72 °C

- (vii) *Specific PCR*. Mix specific primer pairs in water at a concentration of 500 nM and transfer 2 µl into a 384-well plate.
 (viii) Prepare the specific PCR mix for one reaction as follows:

Reagents	Starting concentration	Amount (µl)	Final concentration
PCR buffer	10×	1	1.25×
MgCl ₂	50 mM	0.4	2.5 mM
dN(A,C,G,U)TP	25 mM	0.08	250 nM
SYBR Green I in DMSO	10×	0.5	0.625×
Taq polymerase	5 U µl ⁻¹	0.06	0.0375 U µl ⁻¹
Universal PCR products	1×	0.2	0.025×
H ₂ O	—	5.76	—
Total volume	—	8	—

- (ix) Aliquot 8 µl of the PCR mix from Step 34B(viii) to the each well of the 384-well plate containing the specific primers from Step 34B(vi).
 (x) Perform the specific PCR as follows:

Cycle		
1	2 min at 95 °C	
2–41	15 sec at 95 °C	1 min at 60 °C

- (xi) Obtain the C_t values for each sample. Analyze the samples as described in the INTRODUCTION (Raw data acquisition, Data analysis and quantification sections).

? TROUBLESHOOTING

(C) Multiplex spPLA readout by sequencing ● TIMING 3 h 20 min

- (i) Perform ligation as described in Step 34B(i–iii).
 (ii) *Sequencing library preparation*. Prepare universal PCR mix for sequencing as described below:

Reagents	Starting concentration	Amount (µl)	Final concentration
Special PCR buffer	10×	5	1×
dN(A,C,G,U)TP	25 mM	0.4	200 nM
Universal primer1	10 µM	0.5	100 nM
Universal primer2	10 µM	0.5	100 nM
Taq polymerase	5 U µl ⁻¹	0.3	0.03 U µl ⁻¹
UDG	1 U µl ⁻¹	0.1	0.002 U µl ⁻¹
H ₂ O	—	43.2	—
Total volume	—	50	—

- (iii) Add the universal PCR mix for sequencing from Step 34C(ii) to the ligation (Step 34C(i)) and perform universal PCR using the following conditions:

Cycles			
1	10 min at 95 °C		
2–36	15 s at 95 °C	1 min at 62 °C	1 min at 72 °C

- (iv) Prepare indexing mix as follows:

Reagents	Starting concentration	Amount (μl)	Final concentration
Products from Step 34C(iii)	—	24	—
dN(A,C,G,U)TP	25 mM	0.4	200 nM
Index primer_1	100 μM	0.25	500 nM
Index primer_2	100 μM	0.25	500 nM
Taq polymerase	5 U μl ⁻¹	0.3	0.03 U μl ⁻¹
Special PCR buffer	10×	2.5	1×
H ₂ O	—	22.3	—
Total volume	—	50	—

- (v) Introduce sample index by incubating the mix from Step 34C(iii) at 95 °C for 10 min, 95 °C for 15 s, 62 °C for 1 min and 72 °C for 1 min.

- (vi) Prepare enrichment mix for the appropriate number of reactions, as follows:

Reagents	Starting concentration	Amount (μl)	Final concentration
Products from Step 34C(v)	—	10	—
dN(A,C,G,U)TP	25 mM	0.4	200 nM
Enrichment primer_1	100 μM	0.25	500 nM
Enrichment primer_2	100 μM	0.25	500 nM
Taq polymerase	5 U μl ⁻¹	0.3	0.03 U μl ⁻¹
Special PCR buffer	10×	4	1×
H ₂ O	—	34.8	—
Total volume	—	50	—

- (vii) Enrich indexed DNA products by incubating the mix from Step 34C(vi) as follows:

Cycles			
1	95 °C for 10 min		
2–4	95 °C for 15 s	65 °C for 30 s	72 °C for 30 s

- (viii) Purify indexed DNA by running all reactions in GFX columns.
 (ix) Select DNA fragments of 199 bp from the purified indexed DNA products by using a 2% (wt/vol) size-selection E-gel with a 50-bp DNA size ladder.
 (x) Pool DNA products and proceed with sequencing.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible cause	Suggested solution
Low signals	Antibodies do not bind	If you are using polyclonal antibodies raised against a small region of the protein, the epitope may not be accessible when the protein is naturally folded. Try denaturing the sample so that epitopes become exposed
	Low concentration of antibodies	Perform PLA probe titrations
	Low conjugation efficiency	Perform conjugation again and validate conjugated fraction using SDS-PAGE For biotin-streptavidin conjugation, check the quality of the biotinylated antibody
	5' free PLA oligonucleotide is not phosphorylated	Phosphorylate oligonucleotides using polynucleotide kinase
	Polymerase has lost activity	Use a new batch of polymerase Always keep polymerase at –20 °C
	Ligase has lost activity	Use new batch of ligase Always keep ligase at –20 °C
	Polymerase was not heat-activated	Heat-activate polymerase according to the manufacturer's instructions
	Oligonucleotide design is incorrect	When you are using oligonucleotides not described in this protocol, assess the performance of each design by performing ligations and amplifications in solution without any antibodies. Compare results with those obtained using sequences provided in this protocol
	Limited epitope availability	Try different combinations of monoclonal antibodies. Choose polyclonal antibodies raised against a substantial part of the target protein
	Ligation template serves as a primer during PCR, leading to false amplification products	Make sure that the ligation template can be degraded after ligation or is blocked for polymerase-based extension
High background	Oligonucleotide contamination from the manufacturer	Order the oligonucleotides belonging to the same PLA pair of oligonucleotides from different vendors
	Oligonucleotide contamination during conjugation	Take special care so that PLA oligonucleotides belonging to the same pair cannot be conjugated to the same antibody
	Contamination with PCR products	Take special care when handling amplified ligation products. Follow general guidelines for preventing contamination in PCR assays
	Oligonucleotide design is incorrect	When you are using oligonucleotides not described in this protocol, assess the performance of each design by performing ligations and amplifications in solution without any antibodies. Compare results with sequences provided in this protocol
High assay variation	Beads dry out during incubation	Make sure that beads are not left without buffer for long periods of time
	Suboptimal washing	Perform the washes as described. Make sure not to disturb the beads during washes
	Suboptimal PCR	PCRs have been optimized for the set of oligonucleotides provided in the protocol. If different primers are used, make sure that all PCRs are carefully optimized

● TIMING

Steps 1–9, immobilization of antibodies on microparticles: 1.5 h
Steps 10–18, conjugation of oligonucleotides on antibody: 10 h
Steps 19 and 20, sample preparation: 30 min
Steps 21–25, capturing target protein on microparticles: 2 h
Steps 26–30, probe mix preparation: 10 min–1 h
Steps 31–33, detection of captured proteins: 2 h
Step 34A, ligation and qPCR for singleplex spPLA: 2 h
Step 34B(i–iii), ligation for multiplex PLA: 20 min
Step 34B(iv–vi), universal PCR: 2 h
Step 34B(vii–xi), specific PCR: 3 h
Step 34C(i–iii), ligation for multiplex PLA: 20 min
Step 34C(iv–x), sequencing library preparation: 3 h

ANTICIPATED RESULTS

The results of an spPLA should be similar whether a protein is analyzed individually or in a multiplex assay. In **Figure 4**, the spPLA results for the detection of p53 is shown for both singleplex and multiplex protein detection. Results of the spiked-in mouse IgG control, added in all samples in equal concentrations, are also shown. When the experiment returns results with low signals, high background or high variability among replicates, please refer to **Table 1** for Troubleshooting.

Direct comparison of C_t values between different PCR runs is not really advisable. In addition, compared with singleplex PLA in which ligation products are detected directly with real-time PCR, multiplex PLA uses a preamplification step after ligation and before specific PCR or sequencing-based detection of each ligation product. Thus, we do not recommend comparing the absolute C_t values or sequencing reads for the same protein concentrations resulting from different PLA assay formats.

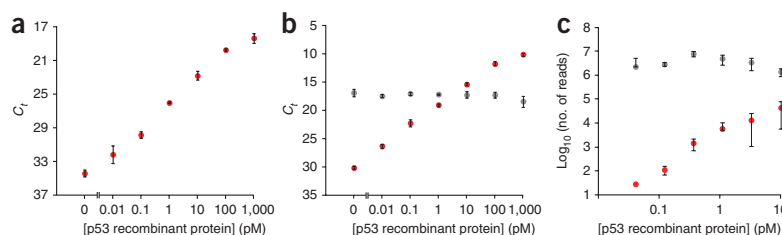


Figure 4 | Performance of spPLA for the detection of p53. (a) Singleplex detection of p53. (b,c) p53 detected in parallel with 35 additional proteins present at equimolar concentrations (red circles). In addition, detection of the spiked-in mouse IgG control is also shown (gray circles). Mouse IgG was spiked in at a constant concentration of 10 pM (b) or 100 pM (c). The x axes show p53 concentration in pM. The y axes show threshold cycle values of real-time PCR assays (b) or the number of reads from sequencing readouts (c). Error bars indicate s.d. calculated from duplicated reactions.

Note: Supplementary information is available in the [online version of the paper](#).

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COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the [online version of the paper](#).

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1. Nong, R.Y., Gu, J., Darmanis, S., Kamali-Moghaddam, M. & Landegren, U. DNA-assisted protein detection technologies. *Expert Rev. Proteomics* **9**, 21–32 (2012).
2. Fredriksson, S. *et al.* Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20**, 473–477 (2002).
3. Lundberg, M., Eriksson, A., Tran, B., Assarsson, E. & Fredriksson, S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. *Nucleic Acids Res.* **39**, e102 (2011).
4. Sano, T., Smith, C.L. & Cantor, C.R. Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. *Science* **258**, 120–122 (1992).
5. Schweitzer, B. *et al.* Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. *Proc. Natl. Acad. Sci. USA* **97**, 10113–10119 (2000).
6. Soderberg, O. *et al.* Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Meth.* **3**, 995–1000 (2006).
7. Gullberg, M. *et al.* Cytokine detection by antibody-based proximity ligation. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 8420–8424 (2004).

8. Schallmeiner, E. *et al.* Sensitive protein detection via triple-binder proximity ligation assays. *Nat. Meth.* **4**, 135–137 (2007).
9. Lundberg, M. *et al.* Multiplexed homogeneous proximity ligation assays for high-throughput protein biomarker research in serological material. *Mol. Cell Proteomics* **10**, M110 004978 (2011).
10. McGregor, L.M., Gorin, D.J., Dumelin, C.E. & Liu, D.R. Interaction-dependent PCR: identification of ligand-target pairs from libraries of ligands and libraries of targets in a single solution-phase experiment. *J. Am. Chem. Soc.* **132**, 15522–15524 (2010).
11. Darmanis, S. *et al.* Sensitive plasma protein analysis by microparticle-based proximity ligation assays. *Mol. Cell Proteomics* **9**, 327–335 (2010).
12. Darmanis, S. *et al.* ProteinSeq: high-performance proteomic analyses by proximity ligation and next generation sequencing. *PLoS ONE* **6**, e25583 (2011).
13. Wallin, U. *et al.* Growth differentiation factor 15: a prognostic marker for recurrence in colorectal cancer. *Br. J. Cancer* **104**, 1619–1627 (2011).
14. Kamali-Moghaddam, M. *et al.* Sensitive detection of A β protofibrils by proximity ligation—relevance for Alzheimer’s disease. *BMC Neurosci.* **11**, 124 (2010).
15. Tavoosidana, G. *et al.* Multiple recognition assay reveals prostasomes as promising plasma biomarkers for prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 8809–8814 (2011).
16. Goransson, J. *et al.* Rapid identification of bio-molecules applied for detection of biosecurity agents using rolling circle amplification. *PLoS ONE* **7**, e31068 (2012).
17. Hammond, M., Nong, R.Y., Ericsson, O., Pardali, K. & Landegren, U. Profiling cellular protein complexes by proximity ligation with dual tag microarray readout. *PLoS ONE* **7**, e40405 (2012).
18. Cheng, S. *et al.* Sensitive detection of small molecules by competitive immunomagnetic-proximity ligation assay. *Anal. Chem.* **84**, 2129–2132 (2012).
19. Anderson, N.L. & Anderson, N.G. The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell Proteomics* **1**, 845–867 (2002).
20. Anderson, N.L. *et al.* Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J. Proteome Res.* **3**, 235–244 (2004).
21. Lange, V., Picotti, P., Domon, B. & Aebersold, R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* **4**, 222 (2008).
22. Nicol, G.R. *et al.* Use of an immunoaffinity-mass spectrometry-based approach for the quantification of protein biomarkers from serum samples of lung cancer patients. *Mol. Cell Proteomics* **7**, 1974–1982 (2008).
23. Nam, J.M., Thaxton, C.S. & Mirkin, C.A. Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science* **301**, 1884–1886 (2003).
24. Hyndman, D.L. & Mitsuhashi, M. PCR primer design. *Methods Mol. Biol.* **226**, 81–88 (2003).

Corrigendum: Solid-phase proximity ligation assays for individual or parallel protein analyses with readout via real-time PCR or sequencing

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In the version of this article initially published, the PCR table at Step 34C(iii) was incorrect and the sliding splint sequence in Supplementary Table 3 was also incorrect. The errors have been corrected in the HTML and PDF versions of the article and in the revised Supplementary Table 3.