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

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ABSTRACT

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. We describe how to construct assays that can offer improved detection specificity by virtue of the recognition by three antibodies, and enhanced sensitivity due 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 is approximately 5 hours long. The multiplex version of the assay takes approximately 8 or 7 hours depending on whether qPCR or sequencing is used as readout. The time for 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 between the proteins, as seen for example for the plasma proteome ¹.

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:

- An 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 levels 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 posttranslational modification.
- Problems of 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 co-variation of levels of different protein species.

Proximity ligation ² and proximity extension assays ³ (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 employ sets of affinity reagents 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, while any unreacted probes fail to give rise to detection signals.

Proximity-based detection reactions differ from immuno-PCR ⁴ or immune rolling circle amplification (RCA) techniques ⁵. 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. Thereby, individual detection reagents fail to be detected, thus avoiding background due to single nonspecifically bound reagents.

Proximity reactions have been designed for localized detection in cells or tissues of single proteins, of co-localized pairs of proteins, or of post-translationally modified proteins via *in situ* PLA ⁶. In these reactions, oligonucleotides attached to pairs of antibodies having bound target epitopes in 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, while 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 amplification reactions. PEA and *in situ* PLA assays are commercially available from Olink Bioscience (Uppsala, Sweden).

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 where 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.

In the following, we provide protocols for another form of PLA, namely solid phase PLA (spPLA)¹¹, which can offer highly specific and sensitive detection of single or multiple proteins in e.g. blood plasma, cerebrospinal fluid or in tissue lysates by virtue of a requirement for 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). Following 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 due 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 detected and quantified with qPCR or next-generation sequencing (NGS), thus revealing protein levels according to the abundance of each specific ligation product (fig 1d).

spPLA is suitable for analyzing sample volumes spanning 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 for investigating 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 since the reagents can be designed such that only the correct combinations of PLA probes can give rise to ligated

reporter strands ¹². Finally, reporter strand detection via next generation sequencing provides a digital readout for greater assay precision compared to 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 to sandwich immunoassays, sometimes by as much as three orders of magnitude using the same affinity-purified polyclonal antibody preparation ¹¹.

Using spPLA, levels 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 disease ¹². A multiplex version of spPLA has been used to simultaneously detect up to 36 proteins in order to identify diagnostic biomarkers for colorectal cancer ¹³ and cardiovascular disease ¹². In addition up to 48 proteins have been analyzed using multiplex spPLA for identification of biomarkers for chronic pain and Alzheimer's disease (Lind *et al.*, in preparation).

The assay has also been used to detect A β protofibrils, efficiently discriminating between monomeric and aggregated forms of the protein, and achieving a 25-fold improved sensitivity compared to a sandwich ELISA using the same monoclonal antibody ¹⁴. Furthermore, spPLA can detect complex targets such as a form of microvesicles referred to as prostasomes ¹⁵, using a variant of the assay where a combination of four antibodies is used as PLA probes. In this way, very low levels of prostasomes were detected in blood of patients with different stages of prostate cancer, identifying this class of structures as promising diagnostic biomarkers that potentially can serve to distinguish patients with different histological grades of the malignancy. spPLA has furthermore been used for detection of biosecurity agents ¹⁶, interacting proteins ¹⁷, and via an inhibition format to detect low levels of low molecular weight molecules ¹⁸.

Proximity based immunoassays can be used when specific and sensitive detection is required. The assay can be multiplexed and miniaturized to allow high-throughput biomarker validation in large sample cohorts, and they are expected to prove of value for diagnostic applications, and in e.g. 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 (MS), is the by far 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 prior to analysis as well as integration of affinity

enrichment²⁰ with selective reaction monitoring²¹. Combinations of the above have resulted in very sensitive MS assays but in 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 to standard colorimetric or fluorescent sandwich immunoassays, spPLA shows an improved sensitivity as a result of successful suppression of background, due to the requirement for multiple recognition events in order to generate detection signals as well as the use of PCR for amplified detection.

Compared to 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 sacrificing 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. While the homogenous solution phase proximity ligation or extension assays have a simple workflow since 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 sample component interfering with detection, and also excess affinity reagents can be removed. It also means that sample volumes larger than the 1 or 2 μ l used in the homogenous 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 as well as homogenous proximity assays by requiring that three different affinity reagents all recognize each target molecule, for added specificity and 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/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 permits 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 (CV) 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 rolling circle amplification (RCA) have been used, enabling linear signal amplification and digital quantification by counting RCA products, resulting in improved assay precision¹⁶.

EXPERIMENTAL DESIGN

General considerations. spPLA is designed to achieve a high degree of specificity of protein detection by employing 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 regarding the choice of solid support for spPLA, namely microtiter plates and microparticles. Both perform equally well for detecting single protein species, however the microparticle-based protocol exhibits 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 using polyclonal antisera both for capture and detection, antibodies should be immobilized with an empirically adjusted antibody density 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 employs 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 one to two hours at room temperature (RT, 20-25°C) is sufficient. Longer incubation times may result in an increase of background, whereas shorter incubation time may result in reduction of signals and increased assay variation.

Usually it is 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 preference 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 due to incomplete ligation, whereas longer ligation incubation time at

high temperature, e.g. 50°C, can result in loss of signals due to 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 non-overlapping epitopes. In practice, we have a good success rate using affinity purified polyclonal antibodies raised against whole proteins since 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 mono- and polyclonal reagents. In spPLA, antibodies must be modified such as 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 are 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, tissue and cell lysates. Typically 5 µl 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 ten-fold prior to 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 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 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. Newly designed oligonucleotides for PLA probes or PCR primers should be tested prior to 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 to 60 nucleotides 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 nucleotides long. Individual PLA oligonucleotides should be tested *in silico* to ensure minimum secondary structure by DNA-folding analysis tools (e.g. Mfold). A pair/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' end 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 ten-nucleotide long hybridization sequence at their free ends, bringing the length of the hybridization template to 20 nucleotides. 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, since the junction of each oligonucleotide pairs are all offset by at least one nucleotide from any other pair (Figure 3). With a hybridization sequence of each PLA probe pair member of ten nucleotides 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 due 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 non-complementary 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 prior to PCR using the enzyme uracil-DNA glycosylase (UDG). By replacing thymidine triphosphate (dTTP) with deoxyuridine triphosphate (dUTP) 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 crossreactivity.

- **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 realtime 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. Additionally, one set of enrichment primers composed of sequencer specific primers to enrich indexed ligation products by PCR.

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-bi-

functional linkers ¹². 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 non-human derived relevant matrix such as chicken serum or plasma can serve as positive controls. For most assays the dilution series should cover concentrations 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.

In order 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 control 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 biomarkers.

Raw data acquisition. Real-time PCR data are exported as values of threshold cycle (Ct) using each instrument's built-in software. For further analysis, we recommend the conversion of the reverse and logarithmic Ct scale to a more convenient linear scale. This can be done using the following equation $M_a = (1+E)^{(Ct_1 - Ct_a)}$, where M_a is the number of PCR amplicons, E is the efficiency of replication in each PCR cycle, Ct_1 the number of PCR cycles required to bring an individual amplicon to the threshold for fluorescence detection and Ct_a is the Ct value of each sample. The formula can be used without the intention to convert Ct values to actual number of molecules and thus values for E and Ct_1 may be set arbitrarily without affecting the analysis. If exact conversion is required then experimentally deduced values of E and Ct_1 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 Ct 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 **Sample types**)

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

AccuStart™ Taq DNA polymerase (contains Taq polymerase 5 U/μl, 10X PCR buffer and 50 mM magnesium chloride; cat. no 95061-05K, Quanta Biosciences)

Ampligase® DNA Ligase, 5U/μl (cat. no. A3210K, Epicentre biotechnology)

Ampligase® 10X Reaction Buffer (cat. no. A1905B, Epicentre biotechnology)

Antibodies, unmodified, selected according to the protein(s) targeted for analysis (for a list of validated polyclonal antibodies see **Supplementary Table 1**, for validated monoclonal antibodies see publications.^{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 (cat. no R0441, Thermo Scientific)

Bovine serum albumin (BSA), 10 mg/ml (cat. no B9001S, New England Biolabs)

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

Dimethyl sulfoxide (DMSO) (cat. no 41648, Sigma-Aldrich)

Dynabeads® MyOne™ Streptavidin T1 beads, 10 mg/ml (cat. no 65602, Life Technologies)

dNTP set of 100 mM solutions, including dUTP in place of dTTP (cat. no. V0195, Thermo Scientific)

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

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

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

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

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

Oligonucleotides (for different oligonucleotide designs see **Supplementary Table 2-5** and the section **Design of oligonucleotides**). Streptavidin conjugated oligonucleotides were ordered from Solulink.

PLA-probes (see **PLA probe conjugations**)

Potassium chloride (cat. no. 1.04936.1000, Merck)

Potassium dihydrogen phosphate (cat. No 1.04873.1000, Merck)

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

Sodium chloride (cat. no. 1.06404.1000, Merck)

di-Sodium hydrogen phosphate dihydrate (cat. no 1.06580.1000, Merck)

Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) (cat.no. 22322, Thermo Scientific)

SYBR® Green I nucleic acid gel stain - 10,000X concentrate in DMSO (cat no. S-7563, Life Technologies)

T4 DNA ligase, 1 U/μl (cat. no. EL0016, Thermo Scientific)

Tween20 (cat. no. P9416, Sigma-Aldrich)

Uracil-DNA glycosylase (UDG), 1 U/μl (cat. no EN0362, Thermo Scientific)

UltraPure™ Dithiothreitol (cat.no. 15508-013, Life technologies)

Zeba Spin Desalting Columns, 7K MWCO, 0.5mL, (cat.no. 89882, Thermo Scientific)

Zeba Spin Desalting Plates, 7K MWCO, (cat.no. 89808, Thermo Scientific)

Equipment

5L beaker

8-well PCR strip (e.g. 4316567, Applied Biosystems)

96-well PCR plate (e.g. Cat. no 72.1978.202, Sarstedt; 732-4828, Thermo Scientific)

2720 Thermal Cycler (cat.no. 4359659, Applied Biosystems)

7900 HT Fast Real-Time PCR System (cat.no. 4329001, Applied Biosystem)

Cap strips (8 caps/strip; eg. 4323032, Applied Biosystems)

DynaMag™-2 Magnet (cat no. 12321D, Life Technologies), or similar magnetic stand for micro-centrifuge tubes

DynaMag™-96 Side, 96-well plate magnet (cat no. 65602, Life Technologies)

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

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

Hydra 96 System (Robbins Scientific)

Icebox

Magnetic stir bar

MicroAmp® Optical 384-Well Reaction Plate with Barcode (cat. no. 4309849, Applied Biosystems)

Multichannel pipette (Eppendorf International)

Mx3000P qPCR Systems (Agilent Technologies)

Plate centrifuge (Eppendorf International)

Rotator (Grant Bio PTR-30 360 Degree Vertical Multi-Function Rotator, or similar)

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

Slide-A-Lyzer MINI Dialysis Devices, 7K MWCO (cat.no. 69562, Thermo Scientific)

Vortex mixer

Reagent setup

1x PBS buffer pH 7.4 NaCl 137 mM, KCl 2.7 mM and phosphate 10 mM. Adjust pH to 7.4. Prepare before starting the experiment and store at room temperature (RT, 20-25°C) for up to six months.

1x PBS/0.1% BSA (Storage buffer) 1x PBS (pH 7.4) with 1 mg/ml BSA. Prepare before starting the experiment and store at 4°C for up to six months.

PBS-T (Washing buffer) 1x PBS (pH 7.4) with 0.05% Tween-20. Prepare before starting the experiment and store at RT for up to six months.

PLA buffer 1xPBS, 1 mM D-biotin, 1 mg/ml BSA, 0.05% Tween 20, 100 nM goat IgG, 0.1 mg/ml salmon sperm DNA, and 5 mM EDTA. Prepare before starting the experiment and store at 4°C for up to two 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 by autoclaving and store at RT for up to six months.

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

Ampligase 10X Reaction Buffer: 200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM MgCl₂, 5 mM NAD, and 0.1% Triton® X-100. Store at -20°C for up to six months.

Special PCR buffer: 50 mM KAc, 20 mM Tris-HAc, 3 mM MgAc₂, pH 7.6. Store at -20°C for up to six months.

Conjugation buffer: 1xPBS with 5 mM EDTA , pH 7.4. Store at RT for up to six months.

PROCEDURE

Immobilization of antibodies on microparticles TIMING 1.5 h

1. Carefully resuspend microparticles in the stock tube to a homogenous suspension. Transfer 100 μ l of beads (10 mg/ml) 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 sec or until all beads form a pellet on the side of the tube towards the magnet and the liquid appears clear in color. Remove and discard the supernatant.
3. Wash the microparticles by removing the tube from the magnet and resuspending with 500 μ l washing buffer. Place the tube on the magnet for 30 sec and discard the washing buffer. Repeat this step once.
4. Dilute the biotinylated antibody to 50 nM in storage buffer.
5. Add 200 μ l of 50 nM biotinylated antibody into the tube containing the microparticles and incubate on an end-over-end rotator at a speed of 16-20 rpm for 1 h at RT (room temperature, 20-25 °C).

CRITICAL STEP: When polyclonal antibodies are used as capture agents, signals decrease when the amount of antibody exceeds approximately 1/10 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 sec and discard the supernatant.
7. Wash the microparticles with 500 μ l washing buffer twice as described in step 3.
8. After the last wash of step 7, resuspend the microparticles in 200 μ l 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 six months.

Conjugation of oligonucleotides on antibody TIMING 10 h

10. Dilute 20 µg of antibody in 10 µl 1xPBS.
11. Prepare a fresh solution of 4 mM sulfo-SMCC in DMSO.

CRITICAL STEP: Sulfo-SMCC solution should always be prepared fresh prior to 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 prepared fresh prior to 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.
15. 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 approximately 1 h of activation.

16. Separately buffer exchange the antibody and the oligonucleotides to conjugation buffer using Zeba columns or plates according to the manufacturer's recommendations.
17. Mix the collected antibody with the oligonucleotide.
18. Place the product of step 16 into a 7kD cutoff dialysis cup. Dialyze over-night (ON) at 4°C, against 5 L of 1xPBS with constant stirring.

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

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

PAUSEPOINT The conjugates can be kept at 4°C for up to 6 months.

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

Sample preparation TIMING 0.5 h

20. Dilute samples such as plasma, serum or whole blood 10-fold in PLA buffer. Each spPLA reaction requires 45 µl of diluted sample.
21. Prepare a dilution series of recombinant protein in PLA buffer as described in the section **controls and calibration curves**. We

recommend the use of recombinant protein dilutions ranging from low fM to 1 or 10 nM in 10-fold increments.

Capture target protein on the microparticles TIMING 2 h

21. Vortex the antibody-coated microparticles prepared in step 1-9 to achieve a homogeneous suspension.
22. For one singleplex PLA reaction, pipette 1 μ l of the antibody-coated microparticle suspension. For one multiplex PLA reaction, pipette 0.1 μ l 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 μ l. However beads can be further diluted to allow reliable pipetting.

23. Remove the storage buffer by placing on a magnet and add 5 μ l of PLA buffer. Resuspend beads by vortexing. Adjust the volumes for different numbers of assays.
24. Mix 5 μ l of beads prepared in step 24 with 45 μ l of each sample prepared in step 20, or with a protein standard prepared in step 21 in a well of a microtiter plate or PCR strip. Seal the wells and vortex to ensure that microparticles are homogeneously suspended.
25. Incubate the reactions on an end-over-end rotator at a speed of 16-20 rpm 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 μ l 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	Start concentration	Amount (μ l)	Final concentration
Biotinylated antibody	1 μ M	0.0125	50 nM
SLC1 or SLC2	100 nM	0.125	50 nM
PBS/BSA	-	0.1125	-

Total volume	-	0.25	-
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28. Incubate the concentrated probes for 1 h at RT.
29. Dilute the two concentrated probe mixes 100 fold in PLA buffer and incubate 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.

PAUSEPOINT We recommend preparation of probes prior to 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.

Detection of captured proteins TIMING 2 h

31. Following incubation at step 26, wash the beads twice with 100 µl washing buffer: spin down the plate or strip in a plate centrifuge (1000 g for 5 sec) to collect all liquid at the bottom of the wells. Put the plate or strip on a 96-well plate magnet for 30 sec until all the beads form a pellet by the side of the tubes. Discard the supernatant and add 100 µl washing buffer into each well with a multichannel pipette. Let the plate or strip stand on the magnet for 30 sec before removing the liquid. Repeat once.
32. Add 50 µl of probe mix prepared in step 30 into each well. Vortex samples to obtain a homogenous solution in each well.
33. Incubate the reactions on an end-over-end rotator with a speed of 16-20 rpm for 1.5 h at RT.

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

34. If performing singleplex spPLA follow option A. If performing multiplex spPLA and specific real-time PCR, follow option B. If 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	Start concentration	Amount (µl)	Final concentration
PCR buffer	10X	5	1X
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	0.3	0.03 U/µl
T4 DNA ligase	1 U/µl	0.5	0.01 U/µl
UDG	1 U/µl	0.1	0.002 U/µl
H ₂ O	-	39.06	-
Total volume		50	

Sybr Green I mix:

Reagents	Start concentration	Amount (µl)	Final concentration
PCR buffer	10X	5	1X
MgCl ₂	50 mM	2.5	2.5 mM
SYBR Green I in DMSO	10X	2.5	0.5X
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	0.3	0.03 U/μl
T4 DNA ligase	1 U/μl	0.5	0.01 U/μl
UDG	1 U/μl	0.1	0.002 U/μl
H ₂ O	-	37.16	-
Total volume	-	50	-

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

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

CRITICAL STEP: When a large number of reactions are performed, we recommend addition of the reaction mix using a multi-channel and possibly multi-dispensing pipet. This can help to mitigate ligation variation due to differences in incubation times.

- v) After qPCR, export Ct values and analyze as described in sections **raw data acquisition** and **data analysis and quantification**.

B) Multiplex spPLA read out by specific real-time PCRTIMING 20 min for steps i-iii; 2h for steps iv –vi; 3h for steps vii-Xi

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

Reagents	Start concentration	Amount (μl)	Final concentration
Ampligase Reaction Buffer	10X	5	1X
Ampligase	5U/μl	0.5	0.05 U/μl
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 i and incubate at 50°C for 10 minutes in a thermal cycler.

iv) Universal PCR pre-amplification for specific PCR readout. Prepare the universal PCR mix:

Reagents	Start concentration	Amount (μl)	Final concentration
PCR buffer	10X	5	1X
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	0.3	0.03 U/μl
UDG	1U/μl	0.1	0.002 U/μl
H ₂ O	-	40.2	-
Total volume	-	50	-

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

Cycle			
1	10 min at 95°C		
2-16	15 sec at 95°C	1 min at 62°C	1 min at 72°C

vii) Specific PCR Mix specific primers 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:

Reagents	Start concentration	Amount (μl)	Final concentration
PCR buffer	10X	1	1.25X
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	10X	0.5	0.625X
Taq polymerase	5 U/ μ l	0.06	0.0375 U/ μ l
Universal PCR products	1X	0.2	0.025X
H ₂ O	-	5.76	-
Total volume	-	8	-

iX) Aliquot 8 μ l of the PCR mix from step viii to the each well of the 384-well plate containing the specific primers from step 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 Ct values for each sample. Analyze as described in sections **raw data acquisition** and **data analysis and quantification**.

C) Multiplex spPLA read out by sequencing TIMING 20 min for steps i-iii; 3 h for steps iv-X.

i) Perform steps i-iii of option B (ligation).

ii) **Sequencing library preparation** Prepare universal PCR mix for sequencing as described below:

Reagents	Start concentration	Amount (μ l)	Final concentration
Special PCR buffer	10X	5	1X
dN[A,C,G,U]TP	25 mM	0.4	200 nM
Universal primer1	10 μ M	0.5	100 nM
Universal primer2	10 μ M	0.5	100 nM
Taq polymerase	5 U/ μ l	0.3	0.03 U/ μ l
UDG	1 U/ μ l	0.1	0.002 U/ μ l
H ₂ O	-	43.2	-
Total volume	-	50	-

iii) Add the universal PCR mix for sequencing from step I to the ligation (step i) and perform universal PCR using the following conditions:

Cycles			
1	10 min at 95°C	1 min at 62°C	1 min at 72°C
2-36	15 sec at 95°C		

iv) Prepare indexing mix as follows:

Reagents	Start concentration	Amount (μl)	Final concentration
Products from step 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	0.3	0.03 U/μl
Special PCR buffer	10X	2.5	1X
H ₂ O	-	22.3	-
Total volume	-	50	-

v) Introduce sample index by incubating the mix from step iii at 95°C for 10 min, 95°C for 15 sec, 62°C for 1 min and 72°C for 1 min.

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

Reagents	Start concentration	Amount (μl)	Final concentration
Products from step 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	0.3	0.03 U/μl
Special PCR buffer	10X	4	1X

H ₂ O	-	34.8	-
Total volume	-	50	-

vii) Enrich indexed DNA products by incubating the mix from step vi as follows:

Cycles			
1	95°C for 10 min		
2-4	95°C for 15 sec	65°C for 30 sec	72°C for 30 sec

viii) Purify indexed DNA by running all reactions in GFX_{TM} columns.

ix) Select DNA fragments of 199 bp from the purified indexed DNA products by using 2% size-selection E-gel with 50 bp DNA size ladder.

X) Pool DNA products and proceed with sequencing.

TIMING

Step 1-9: **Immobilization of antibodies on microparticles** 1.5 h

Step 10-18: **Conjugation of oligonucleotides on antibody** 10 h

Step 19-20: **Sample preparation** 30 min

Step 21-25: **Capture target protein on the microparticles** 2 h

Step 26-30: **Probe mix preparation** 10 min-1 h

Step 31-33: **Detection of captured proteins** 2 h

Step 34 option A: **Ligation and qPCR for singleplex spPLA** 2 h

Step 34 option B steps i-iii: **Ligation for multiplex PLA** 20 min

Step 34 option B steps iv-vi: **Universal PCR** 2 h

Step 34 option B steps vii-Xi: **Specific PCR** 3 h

Step 34 option C steps i-iii: **Ligation for multiplex PLA** 20 min

Step 34 option C step iv-X: Sequencing library preparation 3 h

Trouble shooting

See Table 1 for troubleshooting guidance.

Table 1: Troubleshooting

Problem	Possible cause	Suggested solution
Low signals	Antibodies do not bind	If using polyclonal antibodies raised against a small region of the

		protein, maybe the epitope is not 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 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 following the manufacturer's instructions
	Oligonucleotide design is not correct	When 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
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. Use guidelines for preventing contamination in PCR assays
	Oligonucleotide design is not correct	When 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
	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 imprecision	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	PCR reactions have been optimized for the set of oligonucleotides provided in the protocol. If different primers are used, make sure that all PCR reactions are carefully optimized

Anticipated results

The results of a spPLA should be similar whether a protein is analyzed individually or in a multiplex assay. In **Figure 4**, the results of spPLA for 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 experiment returns results with low signals, high background or high variability among replicates, please refer to Table1 for troubleshooting.

Direct comparison of Ct values between different PCR runs is not really advisable. In addition, compared to singleplex PLA in which ligation products are detected directly with real-time PCR, multiplex PLA employs a pre-amplification step following ligation and preceding specific PCR/sequencing based detection of each ligation product. We thus do not recommend comparing the absolute Ct values or sequencing reads for the same protein concentrations resulting in different PLA assay formats.

Author contribution

All authors contributed to writing the protocol and accepted the final version. Questions regarding the protocol should be addressed to UL.

Competing financial interest

UL is a founder of Olink Bioscience, commercializing reagents for PLA and PEA.

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Figure Legends

Figure 1. Principle of spPLA

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

Figure 1

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, following the incubation of beads with PLA probes (purple). When multiple proteins are detected, ligation is performed separately followed by universal PCR pre-amplification (red). Pre-amplified products are either prepared for sequencing (green) or read out using real-time PCR (blue).

Figure 2

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.

Figure 3

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 (grey circles). Mouse IgG was spiked in at a constant concentration of 10 pM (b) or 100 pM (c). The x-axes display p53 concentration in pM. The y-axes display threshold cycle values of real-time PCR assays (b), or number of reads from sequencing readout (c). Error bars indicate standard deviations calculated from duplicated reactions.

Figure 4

Supplementary Material

Supplementary table 1. List of validated antibodies.

Supplementary table 2. List of different oligonucleotides used in multiplex spPLA.

Supplementary table 3. List of the connector oligonucleotide and universal primers used in multiplex spPLA.

Supplementary table 4. List of oligonucleotides used for sequencing library preparation in multiplex spPLA.

Supplementary table 5. List of oligonucleotides used in singleplex spPLA.

Supplementary Material

Supplementary table 1. List of validated antibodies.

Protein	Species	Type	Purification	Supplier	Modification	Catalog Number
Human Artemin	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-2589
Human Artemin	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-2589
Human beta-NGF	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-256
Human beta-NGF	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-256
Human Cathepsin-B	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-953
Human Cathepsin-B	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-953
Human Cathepsin-S	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1183
Human Cathepsin-S	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-1183
Human CCL16/HCC-4	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-802
Human CCL16/HCC-4	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-802
Human CCL2/MCP-1	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-279
Human CCL2/MCP-1	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-279
Human CCL4/MIP-1 beta	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-271
Human CCL4/MIP-1 beta	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-271
Human CCL5/RANTES	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-278
Human CCL5/RANTES	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-278
Human CD40Ligand/TNFSF5	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-617
Human CD40Ligand/TNFSF5	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-617
Human Coagulation Factor III/Tissue Factor	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-2339
Human Coagulation Factor III/Tissue Factor	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-2339
Human CXCL5/ENA-78	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-254
Human CXCL5/ENA-78	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-254
Human CXCL8/IL-8	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-208
Human CXCL8/IL-8	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-208
Human Cystatin-B	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1408
Human Cystatin-B	Goat	Polyclonal	Affinity	R&D	Biotin	BAF-1408

			purified	Systems		
Human Cystatin-C	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1196
Human Cystatin-C	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-1196
Human E-selectin	Sheep	Polyclonal	Affinity purified	R&D Systems	None	AF-724
Human E-selectin	Sheep	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-724
Human EGF	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-236
Human EGF	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-236
Human Fas/TNFRSF6	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-326
Human Fas/TNFRSF6	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-326
Human Follistatin	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-669
Human Follistatin	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-669
Human GDF-15	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-957
Human GDF-15	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-957
Human Growth Hormone	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1067
Human Growth Hormone	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-1067
Human ICAM-1/CD54	Sheep	Polyclonal	Affinity purified	R&D Systems	None	AF-720
Human ICAM-1/CD54	Sheep	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-720
Human IL-1 alpha/IL-1F1	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-200
Human IL-1 alpha/IL-1F1	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-200
Human IL-10	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-217
Human IL-10	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-217
Human IL-17	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-317
Human IL-17	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-317
Human IL-4	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-204
Human IL-4	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-204
Human IL-6	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-206
Human IL-6	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-206
Human IL-7	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-207
Human IL-7	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-207
Human Kallikrein-3/PSA	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1344

Human Kallikrein-3/PSA	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-1344
Human Kallikrein-6/Neurosin	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-2008
Human Kallikrein-6/Neurosin	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-2008
Human P-Selectin/CD62P	Sheep	Polyclonal	Affinity purified	R&D Systems	None	AF-137
Human P-Selectin/CD62P	Sheep	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-137
Human p53	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-1355
Human p53	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-1355
Human TIMP-1	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-970
Human TIMP-1	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-970
Human TIMP-4	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-974
Human TIMP-4	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-974
Human TNF-alpha	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-210
Human TNF-alpha	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-210
Human VEGF 165	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-293
Human VEGF 165	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-293
Mouse IgG	Goat	Polyclonal	Affinity purified	R&D Systems	None	AF-007
Mouse IgG	Goat	Polyclonal	Affinity purified	R&D Systems	Biotin	BAF-007

Supplementary table 2. List of different oligonucleotides used in multiplex spPLA.

Name	3' free sequence	5' free sequence	Specific primer1	Specific primer 2
PRO BE T1	AAAAACGATTCGAGAACGTGACTG CCATGGCTATTATGATGTCTGAGG CCCTATCCCTG	AGTCTATATGCCCGATGCTGAATT AGTATCGATCGATACCGGACCAGG TTTCGCAAAAA	GCTATTAT GATGTCTG AGGC	GATACTAA TTCAGCAT CGGG
PRO BE T3	AAAAACGATTCGAGAACGTGACTG CCATGTACCTCTATTGATACGTGG GTATCCCTGAG	TCTATATGATGACCTCACATTGAC CATAAGGATCGATACCGGACCAGG TTTCGCAAAAA	TACCTCTA TTGATACG TGGG	CTTATGGT CAATGTGA GGTC
PRO BE T5	AAAAACGATTCGAGAACGTGACTG CCATGAATAGAATCCCTACGCCTA GCCCTGAGTCT	ATATGATGACGAGCTGAGTGAATC TTTCTCGATCGATACCGGACCAGG TTTCGCAAAAA	AATAGAAT CCCTACGC CTAG	GAGAAAGA TTCACTCA GCTC
PRO BE T6	AAAAACGATTCGAGAACGTGACTG CCATGCTTTCAAGTACCTTAGCTC GCCTGAGTCTA	TATGATGACTCCCGTTAGACTATC TGCTATGATCGATACCGGACCAGG TTTCGCAAAAA	CTTTCAAG TACCTTAG CTCG	ATAGCAGA TAGTCTAA CGGG
PRO BE T9	AAAAACGATTCGAGAACGTGACTG CCATGTAGTCAGGTTGGATGTCTA CATCCCTGAGT	CTATATGATGTAGATACCGATACA GTGCTCGATCGATACCGGACCAGG TTTCGCAAAAA	TAGTCAGG TTGGATGT CTAC	GAGCACTG TATCGGTA TCTA
PRO	AAAAACGATTCGAGAACGTGACTG	TGACTATCGTCACATACGCTACAG	AAGGTAAT	CTGTAACCT

BE T12	CCATGAAGGTAATCTACACTACGG GGTCTATATGA	TTACAGGATCGATACCGGACCAGG TTTCGCAAAAA	CTACACTA CGGG	GTAGCGTA TGTG
PRO BE T14	AAAAACGATTCGAGAACGTGACTG CCATGCAATCATATCTAACCGGCT GCTATATGATG	ACTATCGTACGCCCGTATGGTAAC ATTATCGATCGATACCGGACCAGG TTTCGCAAAAA	CAATCATA TCTAACCG GCTG	GATAATGT TACCATAC GGGC
PRO BE T15	AAAAACGATTCGAGAACGTGACTG CCATGACTATCCGTCACCTCAAGTA GTATATGATGA	CTATCGTACCCAGTAAGTATAACG AGCTGCGATCGATACCGGACCAGG TTTCGCAAAAA	ACTATCCG TCACTCAA GTAG	GCAGCTCG TTATACTT ACTG
PRO BE T16	AAAAACGATTCGAGAACGTGACTG CCATGCCGATACTTAAAGCGTAGT GATATGATGAC	TATCGTACCCACCGACGCTAATA GTTAAGGATCGATACCGGACCAGG TTTCGCAAAAA	CCGATACT TAAAGCGT AGTG	CTTAACTA TTAGCGTC GGTG
PRO BE T17	AAAAACGATTCGAGAACGTGACTG CCATGGCTTACTATTCTACTGCG GTATGATGACT	ATCGTACCCGCGACGTATAGATT CGTATGGATCGATACCGGACCAGG TTTCGCAAAAA	GCTTACTA TTCATACT GCCG	CATACGAA TCTATACG TCGC
PRO BE T18	AAAAACGATTCGAGAACGTGACTG CCATGCCTATCGCTCTATATCTGG GATGATGACTA	TCGTACCCTGTAGCACTGGACATA GATGAGGATCGATACCGGACCAGG TTTCGCAAAAA	CCTATCGC TCTATATC TGGG	CTCATCTA TGTCCAGT GCTA
PRO BE T20	AAAAACGATTCGAGAACGTGACTG CCATGCGCTCTTATACCACTGTAG AGATGACTATC	GTACCCTGCACGGTACTCAGTCAT AGTGATGATCGATACCGGACCAGG TTTCGCAAAAA	CGCTCTTA TACCACTG TAGA	ATCACTAT GACTGAGT ACCG
PRO BE T22	AAAAACGATTCGAGAACGTGACTG CCATGCGCGTATCTCTCTCTAGTA GTGACTATCGT	ACCCGCAACGAGATGACGTGACA TATTCGGATCGATACCGGACCAGG TTTCGCAAAAA	CGCGTATC TCTCTCTA GTAG	CGAATATG TCACGTCA TCTC
PRO BE T23	AAAAACGATTCGAGAACGTGACTG CCATGGCAGTCTATTCCACTCAAT GGACTATCGTA	CCCTGCAACTCCCGCATTATTATA CACGAGGATCGATACCGGACCAGG TTTCGCAAAAA	GCAGTCTA TTCCACTC AATG	CTCGTGTA TAATAATG CGGG
PRO BE T24	AAAAACGATTCGAGAACGTGACTG CCATGGTAACTGCTTCGTATCAAG GACTATCGTAC	CCTGCAACTGGCCCGTTCTCTACA GTATATGATCGATACCGGACCAGG TTTCGCAAAAA	GTAAGTGC TTCGTATC AAGG	ATATACTG TAGAGAAC GGGC
PRO BE T25	AAAAACGATTCGAGAACGTGACTG CCATGCGTCATCATTTACTCACAGT GCTATCGTACC	CTGCAACTGTCAGACGACTATCGC ATAATGGATCGATACCGGACCAGG TTTCGCAAAAA	CGTCATCA TTACTCAC AGTG	CATTATGC GATAGTCG TCTG
PRO BE T28	AAAAACGATTCGAGAACGTGACTG CCATGGCGTGTGATTCCTAGTAAT GCGTACCCTGC	AACTGTGCGCAGCTCCTCGCTAATA AGAAACGATCGATACCGGACCAGG TTTCGCAAAAA	GCGTGTGA TTCCTAGT AATG	GTTTCTTA TTAGCGAG GAGC
PRO BE T29	AAAAACGATTCGAGAACGTGACTG CCATGCCAGCTCGTACTATCGAAT AATCGTACCCT	GCAACTGTGCGCAGGTGTATAGAGT TCTGCTGATCGATACCGGACCAGG TTTCGCAAAAA	CCAGCTCG TACTATCG AATA	AGCAGAAC TCTATACA CCTG
PRO BE T30	AAAAACGATTCGAGAACGTGACTG CCATGCCGCACTCTCGTAATATAG AGTACCCTGCA	ACTGTGCGATCCGGTTATCCAAGT GTACTAGATCGATACCGGACCAGG TTTCGCAAAAA	CCGCACTC TCGTAATA TAGA	TAGTACAC TTGGATAA CCGG
PRO BE T31	AAAAACGATTCGAGAACGTGACTG CCATGTATTCTAGTTCTGGACACG GTACCCTGCAA	CTGTGCGATCCATACTCTTGAAGC GATGTGATCGATACCGGACCAGG TTTCGCAAAAA	TATTCTAG TTCTGGAC ACGG	GACATCGC TTCAAGAG TATG
PRO BE T33	AAAAACGATTCGAGAACGTGACTG CCATGGTACTTCTGAAACGTGATG GCCCTGCAACT	GTCGCATCTGCACGGTATTTAATC GGATGGGATCGATACCGGACCAGG TTTCGCAAAAA	GTAATTCT GAAACGTG ATGG	CCATCCGA TTAAATAC CGTG
PRO BE T34	AAAAACGATTCGAGAACGTGACTG CCATGCCATAGTATCCTGTAAGCG TCCTGCAACTG	TCGCATCTGTCTACATAGACGATA GGGACGGATCGATACCGGACCAGG TTTCGCAAAAA	CCATAGTA TCCTGTAA GCGT	CGTCCCTA TCGTCTAT GTAG
PRO BE T35	AAAAACGATTCGAGAACGTGACTG CCATGACTGCTCAATCCTAGATAC GCTGCAACTGT	CGCATCTGTAACACTCACGACATA TAGGTGATCGATACCGGACCAGG TTTCGCAAAAA	ACTGCTCA ATCCTAGA TACG	GACCTATA TGTCGTGA GTGT
PRO BE T37	AAAAACGATTCGAGAACGTGACTG CCATGAATAGACATCAGTAGCTCC GGCAACTGTCTG	CATCTGTAGCGCCTAGTCACCAAT ATACGAGATCGATACCGGACCAGG TTTCGCAAAAA	AATAGACA TCAGTAGC TCCG	TCGTATAT TGGTGACT AGGC
PRO BE T38	AAAAACGATTCGAGAACGTGACTG CCATGGTGACAGATTCTATGTGTG CCAACTGTCTGC	ATCTGTAGCACTTCATATAGTGAG AGGCGTGATCGATACCGGACCAGG TTTCGCAAAAA	GTGACAGA TTCTATGT GTGC	ACGCCCTCT CACTATAT GAAG
PRO	AAAAACGATTCGAGAACGTGACTG	CTGTAGCAGTCTCAGCCGAGTATA	CGGTCCTC	AGTCAATA

BE T40	CCATGCGGTCCTCTGCATCTATAA TACTGTCGCAT	TTGACTGATCGATACCGGACCAGG TTTCGCAAAAA	TGCATCTA TAAT	TACTCGGC TGAG
PRO BE T42	AAAAACGATTCGAGAACGTGACTG CCATGCCGCTGCATCTACTATAAG ATGTCGCATCT	GTAGCAGTCTCATTTGCAGTAAGT CGGTAGGATCGATACCGGACCAGG TTTCGCAAAAA	CCGCTGCA TCTACTAT AAGA	CTACCGAC TTACTGCA AATG
PRO BE T43	AAAAACGATTCGAGAACGTGACTG CCATGGCTCTAATGTTAAGTGCTC GGTCGCATCTG	TAGCAGTCTGCACGAGTGCTAAGA TTTGAGGATCGATACCGGACCAGG TTTCGCAAAAA	GCTCTAAT GTTAAGTG CTCG	CTCAAATC TTAGCACT CGTG
PRO BE T45	AAAAACGATTCGAGAACGTGACTG CCATGGTCGCATATCGTTCTACCT ACGCATCTGTA	GCAGTCTGCCAGGCAGATACGATT AGTACGGATCGATACCGGACCAGG TTTCGCAAAAA	GTCGCATA TCGTTCTA CCTA	CGTACTAA TCGTATCT GCCT
PRO BE T46	AAAAACGATTCGAGAACGTGACTG CCATGGGCGCATATCTGTCTATAC TGCATCTGTAG	CAGTCTGCCGCGACGATGCTTATA GACTCTGATCGATACCGGACCAGG TTTCGCAAAAA	GGCGCATA TCTGTCTA TACT	AGAGTCTA TAAGCATC GTCTG
PRO BE T47	AAAAACGATTCGAGAACGTGACTG CCATGACTCGTATATCATAAGCCC GCATCTGTAGC	AGTCTGCCGCGCAACGGATAGTTAG TCAGGTGATCGATACCGGACCAGG TTTCGCAAAAA	ACTCGTAT ATCATAAG CCCG	ACCTGACT AACTATCC GTTG
PRO BE T48	AAAAACGATTCGAGAACGTGACTG CCATGCAACTAAGTGCTACCGTCT AATCTGTAGCA	GTCTGCCGCGGACTGCGTATCATA TAGGCTGATCGATACCGGACCAGG TTTCGCAAAAA	CAACTAAG TGCTACCG TCTA	AGCCTATA TGATACGC AGTC
PRO BE T50	AAAAACGATTCGAGAACGTGACTG CCATGCTGATGCTTAACCTCGTATG CCTGTAGCAGT	CTGCCGCGCTGATACTACGACATC TAGGGCGATCGATACCGGACCAGG TTTCGCAAAAA	CTGATGCT TAACTCGT ATGC	GCCCTAGA TGTCGTAG TATC
PRO BE T53	AAAAACGATTCGAGAACGTGACTG CCATGCTCCTAGCTTATGACAGCA TTAGCAGTCTG	CCGCGCTTTTCACTATCTAACGAGC TTGGTCGATCGATACCGGACCAGG TTTCGCAAAAA	CTCCTAGC TTATGACA GCAT	GACCAAGC TCGTTAGA TAGT
PRO BE T56	AAAAACGATTCGAGAACGTGACTG CCATGTACAGCTATTGGAGACGAT GCAGTCTGCCG	CGCTTTTCGCACCCGTGCATCAATA GTAGTAGATCGATACCGGACCAGG TTTCGCAAAAA	TACAGCTA TTGGAGAC GATG	TACTACTA TTGATGCA CGGG
PRO BE T58	AAAAACGATTCGAGAACGTGACTG CCATGATGTATCCGAAGTCGTAGT GGTCTGCCGCG	CTTTCGCAGTGCTGGATGAGTACG TCTATTGATCGATACCGGACCAGG TTTCGCAAAAA	ATGTATCC GAAGTCGT AGTG	AATAGACG TACTCATC CAGC

Supplementary table 3. List of the connector oligonucleotide and universal primers used in multiplex spPLA.

Name	Sequence	
Sliding splint	CCTATCCCTGAGTCTATATGATGACTATCGTACCCTGCAACTGTCGCATCTGTAGCAG TCTGCCGCGCTTTTCGCAGTATT	
Universal primer 1	CGATTCGAGAACGTGACTGC	
Universal primer 2	GCGAAACCTGGTCCGGTATC	

Supplementary table 4. List of oligonucleotides used for sequencing library preparation in multiplex spPLA.

Name	Sequence
PCR Index 1 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCCGTGATGCGAAACCTGGTCCGGTATC
PCR Index 2 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCACATCGGCGAAACCTGGTCCGGTATC
PCR Index 3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA

forward primer	TCGCCTAAGCGAAACCTGGTCCGGTATC
PCR Index 4 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCTGGTCAGCGAAACCTGGTCCGGTATC
PCR Index 5 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCCACTGTGCGAAACCTGGTCCGGTATC
PCR Index 6 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCATTGGCGCGAAACCTGGTCCGGTATC
PCR Index 7 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCGATCTGGCGAAACCTGGTCCGGTATC
PCR Index 8 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCTCAAGTGCGAAACCTGGTCCGGTATC
PCR Index 9 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCCTGATCGCGAAACCTGGTCCGGTATC
PCR Index 10 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCAAGCTAGCGAAACCTGGTCCGGTATC
PCR Index 11 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCGTAGCCGCGAAACCTGGTCCGGTATC
PCR Index 12 forward primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGA TCTACAAGGCGAAACCTGGTCCGGTATC
PCR Index reverse primer	CAAGCAGAAGACGGCATACGAGATCGATTGAGAACGTGACTGC
Enrich forward primer	CAAGCAGAAGACGGCATACGAGAT
Enrich reverse primer	AATGATACGGCGACCACCGAGATCT

Supplementary table 5. List of oligonucleotides used in singleplex spPLA.

Name	Sequence
SLC1	CGCATCGCCCTTGGA CTACGACTGACGAACCGCTTTGCCTGACTGATCGCTAAATCGTG
SLC2	TCGTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCCTCTTGAAAAATTCGGCATCGGTGA
PCR Primer	CATCGCCCTTGGA CTACGA
PCR Primer	GGGAATCAAGGTAACGGACTTTAG
Splint	TACTTAGACACGACACGATTTAGTTT