

Cytokine detection by antibody-based proximity ligation

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Efficient and precise detection techniques, along with extensive repertoires of specific binding reagents, will be needed to meet the challenges of proteome analyses. The recently established proximity ligation mechanism enables sensitive high-capacity protein measurements by converting the detection of specific proteins to the analysis of DNA sequences. Proximity probes containing oligonucleotide extensions are designed to bind pairwise to target proteins and to form amplifiable tag sequences by ligation when brought in proximity. In our previous report, both the ligatable arms and the protein binders were DNA molecules. We now generalize the method by providing simple and convenient protocols to convert any polyclonal antibodies or matched pair of monoclonal antibodies to proximity probe sets through the attachment of oligonucleotide sequences. Sufficient reagent for >100,000 proximity ligation assays can be prepared from 1 μ g of antibody. The technique is applied to measure cytokines in a homogenous test format with femtomolar detection sensitivities in 1- μ l samples, and we exemplify its utility in situations when only minute sample amounts are available.

The availability of total genome sequence information provides an overview of the proteins potentially present in an organism. It must now be a high priority to devise reagent sets and analytic procedures that can provide insights into the role of all these proteins in molecular processes and pathological alterations thereof (1). We have recently established the proximity ligation assay, which is promising as a general mechanism for highly specific and sensitive detection of proteins, singly or in parallel, in solution or localized in tissue and cell samples (2).

In proximity ligation, binding of pairs of specific protein-binding reagents to the same target-protein molecule brings oligonucleotides attached to the binding reagents in proximity. Next, a mixture is added that contains all components required for ligation of the oligonucleotides and for amplification and detection of the ligation products. This mixture includes a connector oligonucleotide added in molar excess, which hybridizes to the ends of nearby DNA strands, guiding their prompt ligation (Fig. 1). Thereby, proximity ligation allows proteins to be represented as amplifiable information carrying DNA strands through a highly specific mechanism that depends on dual recognition of target molecules. Excellent sensitivity is ensured by the great increase in reactivity of ligatable ends on coincident target binding through increased relative concentration in combination with amplified DNA detection by real-time PCR, enabling the measurement of very few ligation products. Proximity ligation can also be performed by using a solid phase format and, due to its proximity-dependent signal, it has displayed higher sensitivity than another DNA-based protein detection assay, immuno-PCR (2, 3).

We have previously shown that proximity ligation by using pairs of protein-binding DNA aptamers provides advantages for protein analyses. These assays have proven of particular value under conditions where exceptional sensitivity, not offered by traditional techniques, is required (4–6). Unfortunately, only a limited number of suitable aptamers are currently available (7),

limiting the utility of the procedure. Here we describe simple and efficient protocols to prepare proximity probes using readily available batches of polyclonal antibodies or matched pairs of monoclonal antibodies. We further present means to predict assay performance and report the use of these reagents for sensitive cytokine detection in minute samples derived from serum, conditioned media, or tissue lysate.

Materials and Methods

Antibodies. Affinity-purified polyclonal antibodies and targets for IL-2 and IL-4 assays were from R & D Systems, whereas reagents for the vascular endothelial growth factor (VEGF) and homodimer of platelet-derived growth factor B chain (PDGF-BB) assays were from PeproTech (Rocky Hill, NJ). Monoclonal antiinsulin antibodies M1-1 and M2-2 and human insulin were generous gifts from Mercodia (Uppsala).

Covalent Antibody–Oligonucleotide Conjugation. Monoclonal insulin antibodies were incubated with a 30-fold excess of succinimidyl 4-[*p*-maleimidophenyl]butyrate (SMPB) (Pierce) freshly prepared in DMSO in a total volume of 35 μ l of PBS at room temperature (RT) for 30 min. Reactions were separated twice over a G-50 spin column to remove excess SMPB and eluted into 3 μ l of 10 $\times$ PBS plus EDTA. Oligonucleotides (B)5'-SH and (B)3'-SH (see Table 1, which is published as supporting information on the PNAS web site) in a 3:1 ratio to the amount of antibody were pretreated with 10 mM DTT for 30 min, diluted to 30 μ l, purified three times on G-50 microspin columns, and mixed with the activated antibody. Coupling proceeded for 2 h at RT or overnight at 8°C. Purification was performed as follows. The antibody and antibody–oligonucleotide conjugates were precipitated with an equal volume of saturated ammonium sulfate at RT for 1 h or +8°C overnight. The reaction mix was centrifuged at 6,500 $\times$ g for 30 min, and the supernatant was discarded. Free antibody was removed by applying the reconstituted sample to a 1-ml Q-Sepharose (Amersham Pharmacia Biosciences) column and washing with 6 ml of 50 mM Tris-HCl, pH 7.5/300 mM NaCl. The antibody–oligonucleotide conjugate and remaining free oligonucleotides were eluted with 6 ml of 50 mM Tris-HCl, pH 7.5/1.5 M NaCl into a tube preloaded with 1% BSA. The sample was concentrated on a YM-100 microcon concentrator (Millipore) with three consecutive additions of 2 ml of PBS with 5 mM EDTA, also removing remaining oligonucleotides. Coupling efficiencies were $\approx$ 1–10%, as judged by the amount of oligonucleotide present after purification of the conjugates. Gel analysis of radiolabeled and purified probes showed that >75% of all oligonucleotides were coupled to the

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Abbreviations: STV, streptavidin; RT, room temperature; PDGF-BB, homodimer of platelet-derived growth factor B chain; VEGF, vascular endothelial growth factor.

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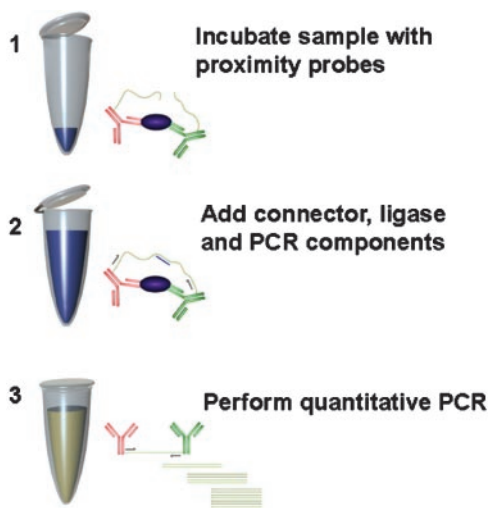


Fig. 1. The principal steps of the proximity ligation assay. Step 1, incubation of sample with proximity probe pair (≈ 1 h); step 2, addition of all components required for ligation and detection by quantitative PCR (≈ 5 min ligation time); step 3, quantitative PCR (≈ 2 h).

antibodies (data not shown). The retentate was collected and diluted in 0.2% BSA in $1\times$ PBS/5 mM EDTA/0.1% sodium azide and stored at $+8^\circ\text{C}$ or aliquoted and frozen at -20°C .

Proximity-Probe Construction Through Streptavidin (STV)–Biotin Linkage. Maleimide-derivatized STV (0.5 nmol) (Sigma) was coupled to 2 nmol of DTT-reduced oligonucleotides (A)3'-SH or (A)5'-SH in 50 μl of phosphate-buffered saline with 5 mM EDTA. Reduction was performed in a 50- μl volume of 50 mM freshly prepared DTT with 2% triethylamine for 10 min at RT. Excess DTT was removed by centrifugation through a prespun 3-ml gel filtration column with a 10% (wt/vol) slurry of G-50 (Amersham Pharmacia) at $1,500\times g$ for 1 min. The eluate was directly combined with the STV for 2 h at 37°C . The reaction was quenched by the addition of 0.5 μl of 0.5 M 2-mercaptoethanol.

Crude conjugates were purified from free DNA by protein precipitation with 1 volume of saturated ammonium sulfate at RT for 1 h, then centrifuged at $+4^\circ\text{C}$ at $13,000\times g$ for 30 min. The supernatant was removed, the material redissolved in phosphate-buffered saline with 5 mM EDTA, and the protein precipitation process was repeated once more. This was followed by DNA precipitation with 0.1 volume of 3 M NaAc, pH 4.6/10 mM $\text{Mg}(\text{Ac})_2$ /2 volumes of ice-cold 95% ethanol to remove free protein. After 2 h at RT, the sample were centrifuged at $13,000\times g$ for 30 min at $+4^\circ\text{C}$. The final product was dissolved in 50 μl of 10 mM Tris-HCl, pH 7.4, with 0.02% sodium azide and stored at $+4^\circ\text{C}$. The protein content of the purified product was estimated by the NanoOrange kit (N-6666, Molecular Probes) by using the included BSA standard and an Applied Biosystems 7700 fluorometer. Oligonucleotides were quantified by absorbance measurements at 260 nm with a NanoDrop spectrophotometer. Product purity was analyzed by separating the samples on an Agilent (Palo Alto, CA) Bioanalyzer 2100 with the mRNA pico kit. Oligonucleotide sequences are shown in Table 1.

Biotinylated antibodies were combined with the STV-oligonucleotide conjugates in 10- μl volumes with 50 nM of each component for 1 h at RT in phosphate-buffered saline with 5 mM EDTA (PBSE) with 0.1% BSA. These were then diluted to 1 nM in PBSE with 1% BSA and 0.02% sodium azide and stored separately at $+4^\circ\text{C}$ for the 3' and 5' proximity probes. Upon use, these solutions were further diluted to a concentration of 100 pM in PBS with 1% BSA/16 $\mu\text{g}/\text{ml}$ sheared polyA bulk nucleic acid

(Sigma), and with 1 mM free biotin added to quench any further biotin–STV interaction. 3' and 5' proximity probes were then combined and further diluted into 5- μl incubations with a 1- μl sample. Samples were prepared by diluting targets in PBS with 1% BSA. The IL-2 and IL-4 assays were performed by using 25 pM proximity probes and the VEGF assay with 5 pM probes.

Theoretical Calculations. The concentration of free probe $[P]$, free target $[T]$, and probe–protein complexes $[PT]$ at equilibrium was calculated based on the formula for calculating the dissociation constant (K_d), Eq. 1, and knowing the concentration of probes, target protein, and the K_d for the interaction.

$$K_d = \frac{[P][T]}{[PT]} \quad [1]$$

By multiplying the fraction of target-binding site 1 that is bound by probe 1 with the fraction of site 2 bound by probe 2, the fraction of target proteins simultaneously bound by the two proximity probes ($fT_{\text{bound}*2}$) is calculated according to Eq. 2.

$$fT_{\text{bound}*2} = \frac{[P_1T]_*[P_2T]}{[T][T]} \quad [2]$$

To correct for impurities, account must be taken of free oligonucleotides that do not contribute to signal but give rise to background and of free antibodies that could reduce signal but not affect the background. The situation can be described by using Eq. 3, where $fP_{1\text{conj}}$ and $fP_{2\text{conj}}$ denote the fraction of the two antibodies having oligonucleotides attached. To estimate the signal over background, consideration must also be given to the background caused by free oligonucleotides, which can be experimentally determined.

$$\%T_{\text{bound}*2} = fP_{1\text{conj}} \frac{[P_1T]_*[P_2T]}{[T][T]} * 100 \quad [3]$$

Because PDGF-BB is a homodimeric protein and both proximity probes are directed against the same epitope, only 50% of target proteins bound by two proximity probes can be expected to involve reagents with both free 5' and 3' ends and capable of generating a signal through ligation. For simplicity, ligation efficiency of such trimeric complexes is assumed to be 100%. Signal over background in Fig. 2 was calculated for 20 pM of probes (directed against PDGF and thrombin) or 5 pM or probes (directed against insulin), as used in the corresponding experiments.

Homogenous Proximity Ligation. Except where indicated, assays were performed by incubating samples with proximity probes in 5- μl incubations for 1 h, before addition of a 45- μl mix containing components required for probe ligation and real-time PCR as described (2). The mix contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl_2 , 0.4 units of T4 DNA ligase (Amersham Pharmacia Biosciences), 400 nM connector oligonucleotide, 80 μM ATP, 0.2 mM dNTPs, 0.5 μM primers, 200 nM probe for the 5' nuclease assay, and 1.5 units of platinum *Taq* DNA polymerase (Invitrogen). After a 5-min ligation reaction at RT, the reactions were transferred to the real-time PCR instrument for temperature cycling: 95°C for 2 min and then 95°C for 15 sec and 60°C for 60 sec, repeated 45 times (Applied Biosystems PRISM 7700 or 7000). Oligonucleotide sequences are shown in Table 1.

Cell Culture. Human 293T embryonic kidney cells were cultured in DMEM cell culture medium (Invitrogen) with 10% FBS (Invitrogen). For measurement of VEGF-A protein levels, 200,000 cells were seeded per well in six-well plates. After 24 h, cells were transferred to an airtight humidified chamber and

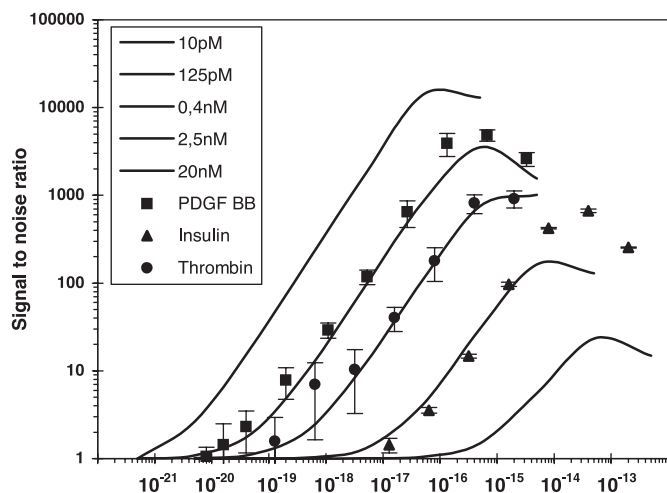


Fig. 2. Triplicate measurements of dilutions of PDGF-BB (squares), thrombin (filled circles), and insulin (triangles), with standard error of the mean, compared to theoretical standard curves for probes with K_d , from left to right, of 10 pM, 125 pM, 0.4 nM, 2.5 nM, and 20 nM, respectively (lines).

flushed with a 95% nitrogen/5% carbon dioxide gas mixture until the O_2 level reached 0.5%, as measured by a PacIII oxygen instrument (Dräger, Partille, Sweden). Cells were then incubated at 37°C for another 24 h in hypoxia. As a control, cells were maintained in normoxia (20.9% O_2). Conditioned media were collected and analyzed for VEGF levels.

VEGF ELISA. The R & D Systems DuoSet ELISA Development Kit was used according to the manufacturer's instructions and within the reported working range of 0.375–95 pM VEGF in 100- μ l samples.

Results

Preparation of Proximity Probes. We have established two procedures to convert small amounts of antibodies to proximity probes and used these for highly sensitive homogenous protein detection. Oligonucleotides with free 3' or 5' ends were attached directly to antibodies by using the bifunctional crosslinker succinimidyl 4-[p-maleimidophenyl]butyrate. Alternatively, oligonucleotides were first coupled to STV for subsequent binding to biotinylated antibodies. Covalent protein–oligonucleotide conjugates were purified from unreacted components before use, because DNA–protein conjugation is typically of low yield. In the case of the STV–oligonucleotide conjugates, purification was performed by using two selective precipitations, first of protein material by using ammonium sulfate and then nucleic acid using NaAc/ethanol. Quantitative analysis of the two purified conjugates revealed that on average around one oligonucleotide was attached per STV molecule [7.7 μ M STV with 6.8 μ M oligonucleotide-A(3'SH) and 2.2 μ M STV and 3.5 μ M oligonucleotide-B(5'SH)]. Microfluidic capillary electrophoresis performed on an Agilent BioAnalyzer revealed that 90% of all DNA was attached to STV for the purified 3'-STV and 80% for the 5'-STV (Fig. 5, which is published as supporting information on the PNAS web site). Because no chromatography steps are involved, it is easy to scale the process for batch purification of many different STV–oligonucleotide conjugates. Once prepared, batches of purified STV–oligonucleotide conjugates represent universal reagents that are simply combined with any biotinylated antibodies of the desired specificity to form proximity probes ready for use without further purification. The prepared proximity probes are then stable over several months of storage.

Correlation Between Probe-Affinity and Assay Sensitivity. The proportion of target proteins bound by a pair of proximity probes at equilibrium can be estimated if the concentration of reagents and the K_d for the interactions are known. By taking into account the background signal observed in the absence of target proteins, these calculations provide estimates of signal over background ratios for various target concentrations, representing theoretical standard curves. The background was empirically measured by varying the concentration of two ligatable oligonucleotide [(B)-3' and (B)-5'] in 5 μ l incubations, ligated and amplified with sequence-system B. As expected, increasing the concentration of one of the probes five times resulted in a 5-fold increase (4.57 ± 0.62) in background, whereas a 5-fold increase of both probes yielded an ≈ 25 -fold higher background (23.4 ± 3.2 -fold).

In Fig. 2, we display estimated standard curves, assuming probe–target interactions with the indicated dissociation constants. These estimates are compared with experimental results from detection of PDGF-BB, thrombin, and insulin. The PDGF-BB aptamers have a reported affinity of 129 ± 11 pM (8), whereas the thrombin aptamers are ≈ 1 nM (9, 10). The PDGF-BB and thrombin data using SELEX aptamers are from Fredriksson *et al.* (2). Proximity ligation signals increase linearly with increasing target up to a point where the probability of each target molecule being bound by two probes decreases. This point depends on the affinity of the particular probes used and their concentration. Also included are data generated by using two anti-insulin monoclonal antibodies that form a proximity probe pair after covalent succinimidyl 4-[p-maleimidophenyl]butyrate coupling of oligonucleotides directly to the antibodies ($K_d \approx 10$ nM; P. Lindstedt, personal communication). The proximity ligation assay for insulin has a sensitivity of 30 pM in 1- μ l samples, whereas the detection limit using these antibodies in a 25- μ l ELISA is 6 pM (standard assay; Mercodia, Uppsala, Sweden) or 0.42 pM (ultrasensitive assay, Mercodia). The PDGF-BB experimental data closely match the 125 pM theoretical standard curve, whereas the thrombin and insulin data fit the expected results for curves calculated for reagents with a K_d of 0.4 and 2.5 nM, respectively. Probe affinities and assay performance are thus strongly correlated, demonstrating that proximity ligation reactions can also be used to estimate affinities of biomolecular interactions. Moreover, the method could be used to study the dynamics of binding by revealing on and off rates (S.M.G., S.F., and U.L., unpublished data), as well as to characterize inhibitors of protein–protein interactions (S.F. and U.L., unpublished data).

Theoretical Setup of Proximity Ligation Assays and Reagent Purity. To determine whether the concentrations of proximity probes applied in the incubation should be adjusted on the basis of their affinities, we calculated the expected signal over background over a range of dissociation constants (see Table 2, which is published as supporting information on the PNAS web site). We found that with probes having K_d values between 0.1 and 10 nM, it is suitable to use a fixed low amount of both probes. However, enough probes should be used in the assay to generate a stable protein-independent background in a range where real-time PCR offers high precision. This is achieved with ≈ 50 –500 amplicons, corresponding to 5–25 pM of the proximity probes in a 5- μ l incubation volume, ligated and amplified in 50 μ l.

Reagent purity is of importance for assay performance. Impurities derived from proximity probe generation, such as free antibodies and free oligonucleotides, should be removed by purification. High levels of free antibody are expected to reduce the signal by blocking probe binding, but lower levels are not harmful because the assay operates below target saturating conditions. By contrast, free oligonucleotides as well as proximity probes with inactive protein binders reduce assay performance by raising the background (Table 3, which is published as

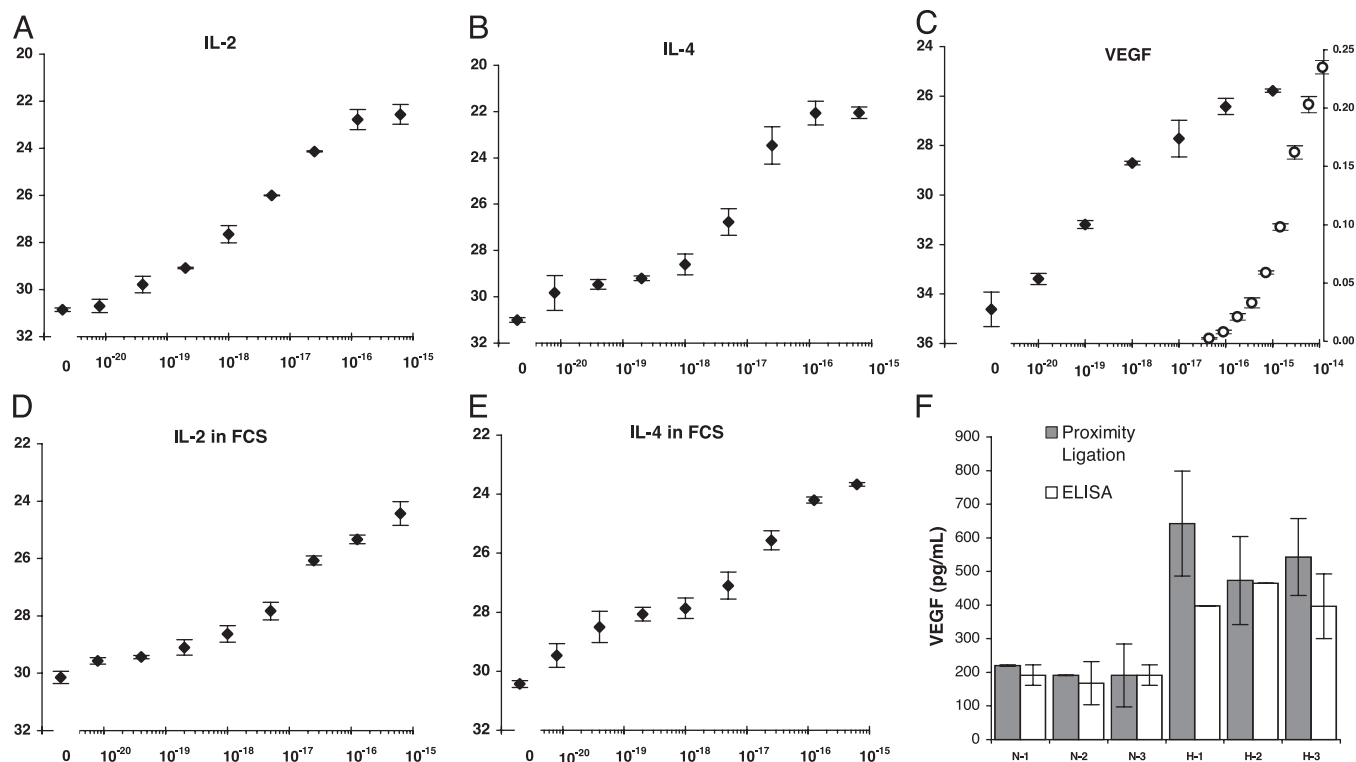


Fig. 3. Cytokine detection by polyclonal antibody-based proximity ligation. (A and B) Analyses of IL-2 and IL-4 in PBS. (D and E) Analyses were performed in the presence of 100% FCS. Comparison of proximity ligation- (diamonds) and ELISA-based (circles) detection of VEGF is shown. x axes display the molar amount of target protein present in 1- μ L samples for proximity ligation and 100- μ L samples for ELISA; y axes display the cycle threshold values from real-time PCR assays or absorbance at 450 nm for the ELISA. A cycle threshold value (C_T) of 30 corresponds to 250 ligation events. (F) Comparison between VEGF measurement by proximity ligation and ELISA. Triplicate samples of conditioned media of cultures grown under either normoxia (N 1–3) or hypoxia (H 1–3) culture conditions. Shown are triplicate measurements with standard deviations for A, B, D, and E and duplicates for C and F.

supporting information on the PNAS web site). It is also important that the oligonucleotides used in proximity probes are full length, and that their sequences have been selected to avoid secondary structures that could prevent hybridization of the connector oligonucleotide and formation of interprobe hybrids.

Affinity-Purified Polyclonal Antibodies. Because polyclonal antisera typically contain antibodies against different epitopes of the same antigen, we assumed proximity probe pairs could be formed by dividing such sera into two portions and coupling oligonucleotides with free 5' and 3' ends to the two portions. Accordingly, affinity-purified and biotinylated polyclonal antibodies directed against two monomeric proteins, IL-2 and IL-4, and one homodimeric protein, VEGF, were split in two aliquots each, which were combined with two preparations of STV conjugated to oligonucleotides with ligatable ends. As shown in Fig. 3, all assays displayed low femtomolar sensitivities in 1- μ L samples, thus detecting tens of zeptomol of protein. Because the polyclonal antibodies comprise a heterogeneous population of probes of various affinities, the proportionality of increasing signal with increasing target concentration is not as prominent as when monoclonal probes are used. The IL-2 and IL-4 assays were also performed in the presence of 100% FCS without influencing the limit of detection. Compared to a commercial ELISA, the VEGF assay detected 5,000-fold less protein molecules, and it exhibited a wider dynamic range. Cell culture supernatants of embryonic kidney cells, induced to overexpress VEGF by hypoxia and normoxic controls, were analyzed for VEGF content by proximity ligation and ELISA (Fig. 3f). The amount of VEGF was estimated, by reference to standard

curves, with a precision of 21% coefficient of variance for proximity ligation and 16% for ELISA.

A number of lysed glomeruli tissue samples from conditional PDGF-BB knockout mice and controls were analyzed by proximity ligation by using a polyclonal antibody-based assay and compared to previously obtained results using DNA aptamers (Fig. 4). Samples and aptamer-based data are from Bjarnegård *et al.* (6). Results using antibody- and aptamer-based proximity ligation assays correlate well. By contrast, the sensitivity of conventional ELISAs is insufficient to measure the low amounts of cytokines in some of these minute samples. In the antibody-based assay, the intercalating dye SYBR green I was substituted for the TaqMan probe, illustrating its utility as alternative reporter.

Although it is possible to predict the assay performance for directly conjugated proximity probes of known affinity, this is not true for reagents having oligonucleotides attached via STV conjugates. The latter reagents can form multivalent complexes due to the tetrameric structure of STV and the multiple biotins groups coupled per antibody (11, 12). Accordingly, each proximity probe can involve several antibodies and STV molecules, increasing the apparent affinity by cooperative effects.

Discussion

We show herein that the readily available protein-binding reagents, mono- and polyclonal antibodies, can be conveniently converted to proximity probes. Polyclonal reagents eliminate the need to select matched antibody pairs toward each target protein, while maintaining the element of dual recognition to ensure high specificity. The greater the affinity of the binding reagents, the higher the assay sensitivity, because the assays are

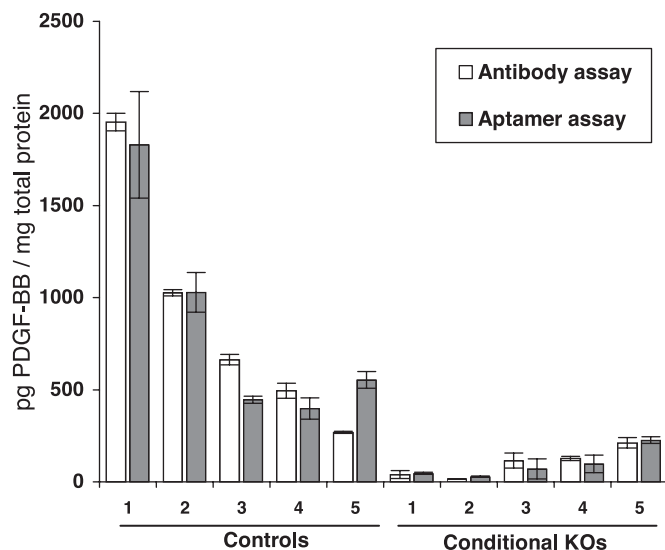


Fig. 4. Proximity ligation analysis of PDGF-BB from lysed mouse glomeruli tissues of controls (1–5) and conditional knock-outs (1–5) using DNA aptamers or polyclonal antibodies as affinity reagents.

performed below target-saturating conditions to minimize background. Antibodies subjected to *in vitro* maturation can reach exceptionally high affinities with K_d of tens of picomolar (13, 14) or even femtomolar (15). Any 10-fold increase in affinity of both members of a proximity probe pair is expected to result in a 100-fold increase in sensitivity (compare Fig. 2).

Homogenous proximity ligation is suitable for automation in high-throughput applications, because no washing steps are required but only two sequential additions to the incubation of

first the sample and then a ligation/PCR mixture. The high assay sensitivity allows 1- μ l sample aliquots to be monitored by proximity ligation, reducing sample consumption and enabling analysis of samples available only in very small amounts and previously not analyzable by traditional techniques, as illustrated by the mouse glomeruli assays shown herein. Also, 1,000-fold less antibody is used per assay compared to standard ELISAs, and because all assays perform favorably at similar reagent concentrations, new assays do not require extensive optimization.

The precision of proximity ligation is currently at the level of real-time PCR detection, but improved quantitative detection strategies for nucleic acids may offer a further increase in precision (16–18). Multiplexed detection is the goal for many technologies under development, especially for antibody-based microarrays. As more detection reactions are performed in parallel, the issue of antibody crossreactivity becomes an increasing problem limiting scalability (19). Proximity ligation offers a possible solution to this problem if unique ligation junctions are used for each cognate proximity probe pair. The proximity ligation mechanism is also being adapted for *in situ* detection of target proteins and for high-throughput protein–protein interaction studies (O. Söderberg, M.G., M. Emilsson, F. Bahram, L. G. Larsson, and U.L., unpublished work; S.M.G., S.F., and U.L., unpublished work). Finally, by encoding different proteins as amplifiable DNA tag sequences through proximity ligation, parallel analyses may be possible, allowing standard oligonucleotide arrays to be used for absolute or relative measurements of large sets of proteins.

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