Self-assembly of proximity probes for flexible and modular proximity ligation assays

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Proximity ligation assay (PLA) is a recently developed strategy for protein analysis in which antibody-based detection of a target protein via a DNA ligation reaction of oligonucleotides linked to the antibodies results in the formation of an amplifiable DNA strand suitable for analysis. Here we describe a faster and more cost-effective strategy to construct the antibody-based proximity ligation probes used in PLA that is based on the noncovalent interaction of biotinylated oligonucleotides with streptavidin followed by the interaction of this complex with biotinylated antibodies.

Proximity ligation assay (PLA) is a novel protein detection strategy based on the transformation of detected proteins into DNA sequences through a DNA ligation reaction (1–6). This conversion of protein into DNA strands is followed by DNA amplification and detection, for example, by quantitative PCR or rolling circle amplification (RCA). One further development of the basic proximity ligation strategy is the triple-specific proximity ligation assay (3PLA), in which three independent recognition events are required to give rise to a specific signal (3). 3PLA has been shown to detect as little as 100 molecules of the vascular endothelial growth factor (VEGF) in 1-µL samples, and very low concentrations were also detected of the biomarkers prostate-specific antigen (PSA) and troponin I (3).

In 3PLA, the binding of three proximity probes, identified as proximity probes 1, 2, and 3, results in the eventual formation of one amplifiable DNA sequence (Figure 1A). Initially, proximity probes 1 and 2 are blocked by oligonucleotides that hybridize to their ligatable ends, but upon binding of all three proximity probes to the same target molecule, the blocking oligonucleotides are displaced by proximity probe 3, which templates the ligation reaction of proximity probe 1, a cassette oligonucleotide, and proximity probe 2 to form a new DNA sequence that can be amplified and detected by real-time PCR. The blocking oligonucleotides ensure that the nontarget-mediated background signal is low. In the present study, all experiments were based on the 3PLA strategy.

In previous studies (2,3), the most commonly used protocol for conjugation of antibodies to oligonucleotides to produce the proximity probes uses the high-affinity interaction of biotin for streptavidin (K_D approximately 10^{-15} M) (7). In this covalent streptavidin-biotin (B-STV) conjugation approach, single-stranded oligonucleotides are covalently attached to streptavidin molecules via maleimide conjugation chemistry (2,8). The purified conjugates can then be added to any biotinylated antibody to form functional proximity probes against a range of different target molecules (3,5,6) with no need for further purification. However, the conjugation of many different oligonucleotides to streptavidin molecules in this approach is tedious and time-consuming due to the coupling and purification procedure.

In the present study, we compared the B-STV conjugation approach to a self-assembly protocol that is solely based on the noncovalent interaction of streptavidin to biotins (B-STV-B). In the B-STV coupling approach, the covalently linked oligonucleotide-streptavidin constructs are added to biotinylated antibody at a ratio of 2:1 and allowed to form proximity probes (Figure 1B, panel i). The antibody conjugates are diluted in PLA buffer and used in PLAs (Figure 1B, panel ii). For the B-STV-B self-assembly, biotinylated oligonucleotides are allowed to bind streptavidin molecules at a 1:1 ratio. Subsequently, biotinylated antibodies are added at a ratio of 1:2, and proximity probes are allowed to form.

For the B-STV method of proximity probe conjugation, DNA oligonucleotides covalently coupled to streptavidin [100 nM diluted in 1× phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA) buffer; Solulink, San Diego, CA, USA] were added to 100 nM recombinant streptavidin (Sigma-Aldrich, St. Louis, MO, USA) prepared in 1× PBS, 0.1% BSA buffer at a 1:2 ratio, incubated at 65°C for 1 h, and then allowed to cool down to room temperature. For conjugation to the antibody, 40 µL of this 100 nM streptavidin-oligonucleotide conjugate, 20 µL 100 nM biotinylated antibody (R&D Systems, Abingdon, UK) against either VEGF or tumor necrosis factor α (TNFα), and 40 µL 1× PBS, 0.1% BSA buffer were mixed and incubated at 37°C for 1 h and then at room temperature for 30 min.

For the B-STV-B method of proximity probe conjugation, 300 µL 100 nM biotinylated oligonucleotides (Integrated DNA Technologies, Coralville, IA, USA) and 300 µL 100 nM recombinant streptavidin, diluted in 1× PBS, were combined, incubated at 37°C for 1 h, and allowed to cool down to room temperature for 30 min. Eighty microliters streptavidin-oligonucleotide conjugates were then added to 20 µL 100 nM VEGF or TNFα biotinylated antibody solutions. The mixtures were incubated at 37°C for 1 h, followed by cooling to room temperature for 30 min. The B-STV and B-STV-B conjugates were diluted in PLA buffer [1× PBS, 1% BSA, 16 µg/mL sheared poly(A) bulk nucleic acid (Sigma-Aldrich), 1 mM free biotin, and 100 nM streptavidin] and either used directly in PLAs or stored at 4°C for up to 1 month.

For the standard (two-step) PLA, sets of proximity probes 1, 2, and 3 of B-STV or B-STV-B conjugates were diluted to 100 pM in PLA buffer containing 100

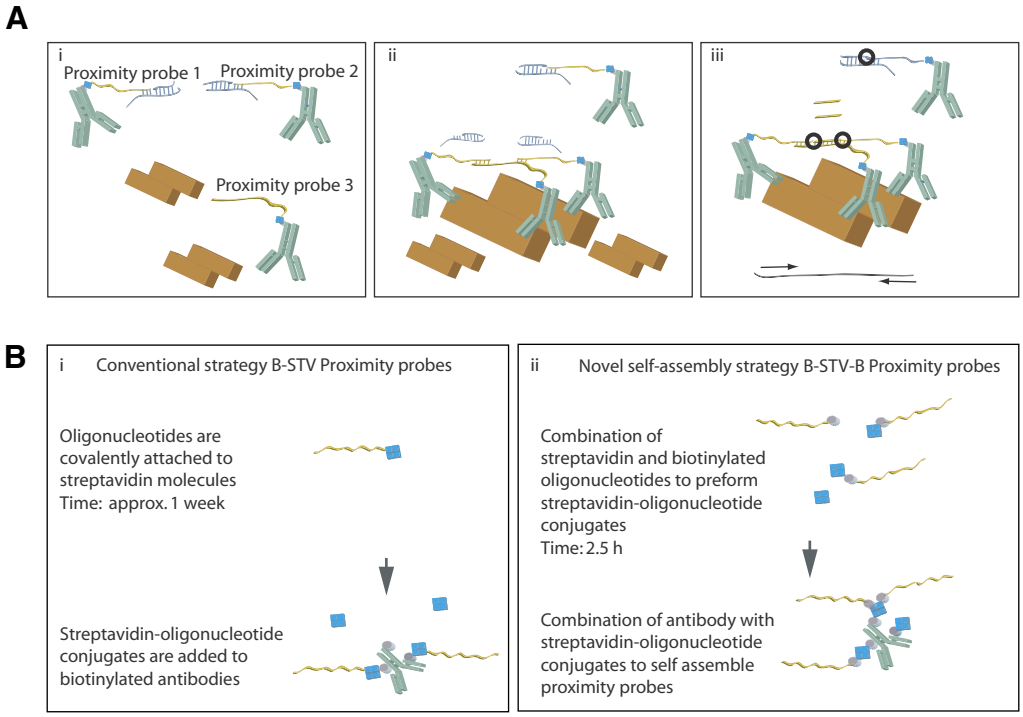

Figure 1. The triple-specific proximity ligation assay (3PLA) reaction and formation of proximity probes. (A) Overview of the 3PLA reaction. (panel i) Proximity probes and sample are combined in a reaction tube. (panel ii) Upon binding to the same target molecule, the proximity probes are brought close, and proximity probes 1 and 2 can hybridize to the third proximity probe. This hybridization replaces the blocking oligonucleotides that hybridize to the ligatable ends of the proximity probes. (panel iii) Reagents necessary for the ligation and PCR step are added, and the proximity probes 1 and 2 are ligated (black circle) together with an interposed cassette oligonucleotide, forming a new sequence (middle) that can be amplified and detected by real-time PCR (bottom). Unreacted proximity probes are blocked by ligation to the blocking oligonucleotides (top). (B) Overview comparing formation of proximity probes by the covalent streptavidin-biotin (B-STV) and noncovalent interaction of streptavidin to biotins (B-STV-B) strategies. (panel i) The B-STV strategy is composed of the dilution of covalent streptavidin-oligonucleotide conjugates with free streptavidin. Streptavidin-oligonucleotide conjugates are added to biotinylated antibodies and allowed to form streptavidin-biotin interactions. Dilution of the proximity probes in PLA buffer containing free biotin blocks the unreacted sites on streptavidin for the use in PLA. (panel ii) In the B-STV-B strategy, biotinylated oligonucleotides are added to streptavidin molecules in a ratio of 1:1 and allowed to form biotin-streptavidin interactions. These preformed biotin-streptavidin probes are in turn added to biotinylated antibody and again are allowed to form biotin-streptavidin interactions. The probes are then diluted in PLA buffer and used for the detection of proteins.

nM blocking oligonucleotides (Biomers, Ulm, Germany) and incubated at 37°C for 10 min. Five microliters proximity probe mixes were combined with 2.5 µL sample in a 96-optical well plate (Applied Biosystems, Foster City, CA, USA) and sealed tightly using an adhesive cover (Applied Biosystems). The optical well plate was quickly centrifuged in a plate centrifuge to collect all samples in the bottom of the wells and incubated at 37°C for 50 min. After the incubation time, 42.5 µL PCR-ligation mixture were added to a total volume of 50 µL (1× PCR buffer; Invitrogen, Inchinnan, UK), 2.5 mM $MgCl_2$, 0.4 U T4 DNA ligase (Fermentas, St. Leon-Rot, Germany), 40 nM cassette oligonucleotide (Eurogentec, Seraing, Belgium), 80 µM ATP, 0.2 mM each dNTP, 200 nM forward and reverse amplification primers (Biomers), 200 nM MGB™ TaqMan® probe (system 2 for the TNFα detection and system 3 for the VEGF detection in Table 1; Applied Biosystems), and 0.3 U Platinum *Taq* polymerase (Invitrogen), followed by a 5-min ligation at room temperature, and then transferred to a real-time PCR instrument (Mx3000P® and Mx3005P®; Stratagene, La Jolla, CA, USA) for temperature cycling (95°C for 2 min; 45 cycles of 95°C for 15 s; and 60°C for 60 s).

We used the B-STV and B-STV-B strategies to convert a polyclonal VEGF antibody batch into 3PLA proximity probes for the detection of recombinant VEGF serially diluted in PLA buffer. A good signal-to-noise ratio was observed for both B-STV- and B-STV-B-coupled reagents (Figure 2A). We observed an extended upper end of the dynamic range and a slightly increased background signal for the B-STV-B approach. The increased background signal can be explained by random attachment of free biotinylated oligonucleotide arms 1, 2, and 3 to the same streptavidin molecule during the incubation step. Streptavidin has been shown to aggregate under certain conditions (7), which might enhance the background signal effect of B-STV-B conjugates. In the B-STV conjugation, binding to the same streptavidin molecule does not occur, as the oligonucleotide is covalently attached. We have previously shown that the B-STV conjugation approach combined with 3PLA can yield very sensitive detection of as little as 100 molecules in a 1-µL sample (3). When forming proximity probes using the B-STV-B

Table 1. Oligonucleotide Sequences and Type of Modifications Used in 3PLAs for Both the Conventional B-STV Conjugation and the B-STV-B Self-Assembly Protocol

Name	Sequence	Modification
Proximity probe 1	5′-CGCATCGCCCTTGGACTACGACTGACGA ACCGCTTTGCCTGACTGATCGCTAAATCGTG-3′	5′ Biotin; 5′ Streptavidin
Proximity probe 2	5′-TCGTGTCTAAAGTCCGTTACCTTGATTC CCCTAACCCTCTTGAAAAATTCGGCATCGGTGA-3′	5′ Phosphate; 3′ Biotin; 5′ Phosphate; 3′ Streptavidin;
Proximity probe 3	5′-TAGCTAAGGCTTAGTTAGACACGAGCA TTATGGAGTGCAGGATCACGATTTAG-3′	3′ Biotin; 3′ Streptavidin
L9072 Proximity probe 1 containing a unique identification sequence	5′-CATCGCCCTTGGACTACGACATATCGTGTCACA GGTCGTTCTGATCGCTAAATCGTG-3′	5′ Biotin
L9073 Proximity probe 1 containing a unique identification sequence	5′-CATCGCCCTTGGACTACGACGACGCCGTTATG AGAGTCCACTGATCGCTAAATCGTG-3′	5′ Biotin
L9093 Proximity probe 1 containing a unique identification sequence	5′-CATCGCCCTTGGACTACGACTCCGTCTGTT GAGTTAGGCCCTGATCGCTAAATCGTG-3′	5′ Biotin
L9094 Proximity probe 1 containing a unique identification sequence	5′-CATCGCCCTTGGACTACGACATGATGTGCAAA GTGCCGTCCTGATCGCTAAATCGTG-3′	5′ Biotin
L8388 Primer fwd	5′-CATCGCCCTTGGACTACGA-3′	
L8389 Primer rev	5′-GGGAATCAAGGTAACGGACTTTAG-3′	
X 0555 Cassette oligonucleotide	5′-ATCCTGCACTCCATAATGC-3′	5′ Phosphate
MGB TaqMan probe system 2	5′-TGACGAACCGCTTTGCCTGA-3′	
MGB TaqMan probe system 3	5′-ATCCTGCACTCCATAATGC-3′	
Blocking oligonucleotide 1, top strand L9277	5′-CGACGGACTGCAATCCGTAATTCGATCT CAGTATTTTTTTTT-3′	
Blocking oligonucleotide 1, bottom strand L9278	5′-TTTTTTTTTTACTGAGATCGAATTACGG ATTGCAGTCCGTCGCACGATTAATTTTT-3′	
Blocking oligonucleotide 2, top strand L9279	5′-TTTTTTTTTGCTGCCTGACGTTAGGCAT TAAGCTAGAGTCAT-3′	
Blocking oligonucleotide 2, bottom strand L9581	5′-TTTTTTTTTCACGAATGACTCTAGCTTA ATGCCTAACGTCAGGCAGCTTTTTTTTT-3′	

3PLA, triple-specific proximity ligation assay; B-STV, covalent streptavidin-biotin; B-STV-B, noncovalent interaction of streptavidin to biotins.

assembly, the same high sensitivity is not achieved due to the increased background signal.

The advantages of B-STV-B coupling are the low cost and the rapid setup, which makes it especially attractive for the fast and easy custom design of PLAs with any biotinylated polyclonal antibody batch or matched monoclonal antibody set. We have tested the performance of sets of PLA probes containing different tag sequences in proximity probe 1. In this experiment, only proximity probe 1 was conjugated using the B-STV-B approach, while proximity probes 2 and 3 were conjugated using the B-STV protocol. We easily established functional sets of proximity probes containing four different proximity probe 1 sequences (Figure 2, B and C). The performance of these probes was comparable to the dynamic range of the B-STV-B approach. These results show that this can be a useful approach for the conjugation and evaluation of sets of proximity probes, for example, for the use in multiplexed proximity ligation-based protein detection.

PLAs are usually carried out as a two-step protocol by performing real-time PCR directly after the ligation step. This can be a disadvantage when analyzing large numbers of samples with limited real-time PCR capacity. We have established a three-step protocol in which the ligation step is separated from the real-time PCR readout. After the affinity interaction and ligation, the stable ligation product can be frozen or stored at 4°C for days. For the three-step PLAs, the incubation of the samples was performed as described previously. After the 50-min incubation time, 20 µL ligation mixture (1× PCR buffer, 0.4 U T4 DNA ligase, 100 nM cassette oligonucleotide, 80 µM ATP) were added, and the ligation was carried out for 5 min at room temperature. The ligase was then inactivated at 95°C for 10 min. At this stage, the mixture can be stored for days at 4°C or frozen at -20°C. For analysis, 43 µL PCR mixture [1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 200 nM primers (see Table 1), 200 nM MGB TaqMan probe, and 0.3 U Platinum *Taq* polymerase] were combined with 2.5 µL ligated PLA samples, and the plate was transferred to a real-time PCR instrument for temperature cycling. We compared 3PLA for detecting recombinant TNFα in PLA buffer with B-STV conjugates using either the three-step protocol resulting in stable ligation mixtures or the standard two-step PLA and obtained

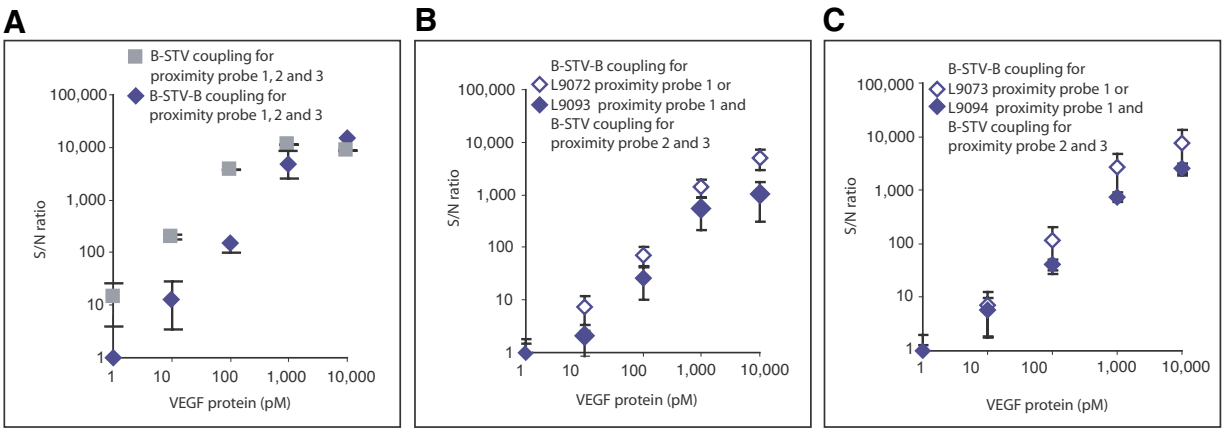

Figure 2. Comparison of triple-specific proximity ligation assay (3PLA) by probes formed with either the covalent streptavidin-biotin (B-STV) or noncovalent interaction of streptavidin to biotins (B-STV-B) strategies. (A) Vascular endothelial growth factor (VEGF) detection by the B-STV strategy is compared to a VEGF assay based on the B-STV-B strategy. (B and C) The B-STV-B strategy is used to form four different proximity probe 1 sequences, and each of these is combined with the same proximity probe 2 and 3 formed by the B-STV strategy. Error bars in all graphs indicate standard deviations from duplicate measurements. S/N, signal-to-noise ratio.

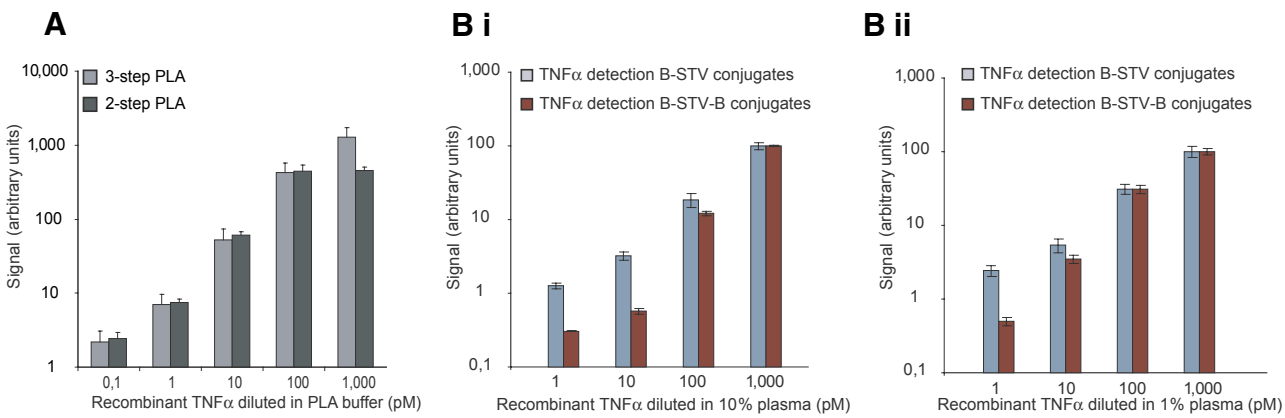

Figure 3. Comparison of three-step and two-step proximity litigation protocols. (A) Detection of recombinant tumor necrosis factor α (TNFα) serially diluted in proximity ligation assay (PLA) buffer by either a two-step triple-specific proximity ligation assay (3PLA), with a combined PCR and ligation reaction, or a three-step 3PLA, in which the ligation product was stored for a prolonged period before being transferred to the real-time PCR readout. (B) Detection of recombinant TNFα serially diluted in (panel i) 10% human plasma or (panel ii) 1% human plasma by proximity probes formed using either the covalent streptavidin-biotin (B-STV) or noncovalent interaction of streptavidin to biotins (B-STV-B) conjugation strategies. Error bars in all graphs indicate standard deviations from duplicate measurements.

similar results (Figure 3A). This simple approach of optimizing a separate ligation reaction independently from the real-time PCR allows the analysis of large numbers of samples in parallel without the immediate need for a real-time PCR readout and increases the flexibility of the PLA.

Finally we compared the performance of B-STV conjugation to B-STV-B-based proximity probes for detecting recombinant TNFα diluted in 10% (Figure 3B, panel i) and 1% (Figure 3B, panel ii) human plasma. We could show that both conjugations gave comparable results, demonstrating that B-STV-B assembly can be used for rapid custom design of PLA with any biotinylated antibody. The B-STV-B self-assembly conjugation strategy of proximity probes will be important for the future development of new PLA designs and for the customization of PLAs without the need for time-consuming and expensive covalent conjugation strategies.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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