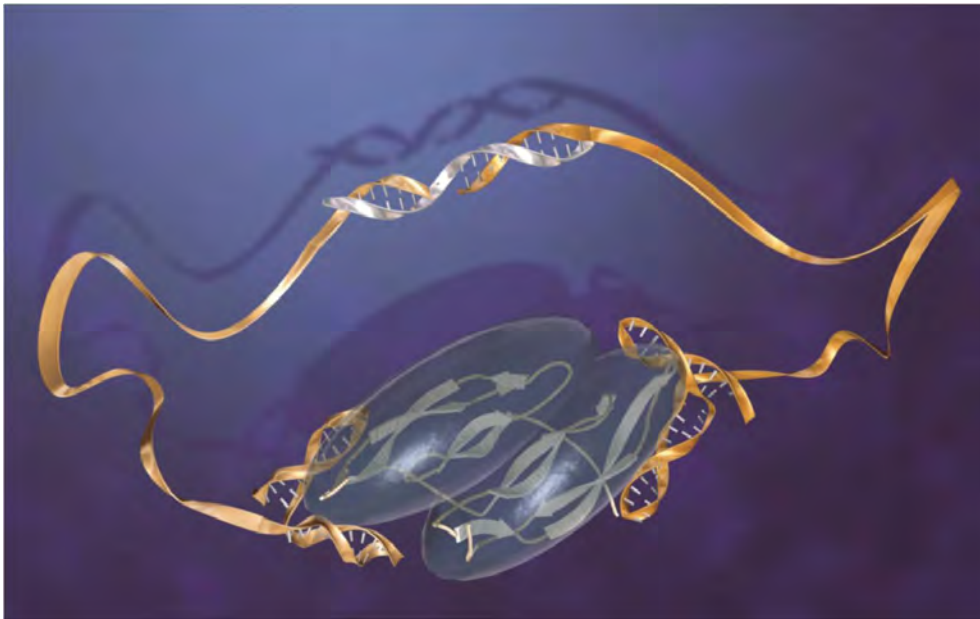




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## Multiplex protein analysis by proximity ligation assay with microarray analysis



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## Summary

The aim of this study was to investigate five biomarkers for ischemic heart disease in the same reaction volume. The five biomarkers were supposed to be investigated simultaneously in the same reaction volume with the use of a Proximity Ligation Assay (PLA) combined with a specially designed microarray platform for high-performance nucleic acid analysis.

Antibodies are excellent tools in protein analysis (immunoassays). One of the most widely known and used immunoassays are Enzyme-Linked ImmunoSorbent Assay (ELISA). ELISA is often used for diagnostics and quality control in various industries. There is one problem with these assays; they cannot analyze several proteins in parallel. Parallel assays save time and money, reduce the sample size but also creates problems due to possibilities for nonspecific adsorption and cross-reactions. These problems rapidly increase as more antibody pairs are added to the assays. This finally leads to an assay which performs and has a specificity of a single binder assay. The PLA is a immunoassay that might offer several advantages in parallel protein studies, since it has been shown to enhance analysis of individual or small numbers of serum biomarkers, infectious agents, protein DNA interactions, and protein-protein interactions in fixed tissue sections. The PLA takes advantage of the progress made in the genomic study field by translating protein information back into reporter molecules made of DNA. These reporter molecules can in turn be investigated by Polymerase Chain Reaction (PCR), Quantitative PCR (qPCR) and microarrays. In order to get a reporter molecule, e.g. a template for the PCR, two conjugates with individual tagged DNA strands have to bind to the same molecule of interest, hence the name proximity ligation. The PCR amplified products from the PLA can be sorted and analyzed on a specially designed microarray. The microarray used here was designed in house and it has dual tagged probes that are complementary to the PCR amplified DNA products. This microarray can discriminate signals from several proteins in parallel and the dual tag system reduces the signals from cross-reacting antibodies.

I started the study by making the conjugates (which serve as probes), against vascular endothelial growth factor (VEGF) and interleukin-10 (IL-10). I successfully conjugated polyclonal goat anti-VEGF and anti-IL-10 antibodies with the different individually tagged oligos (proximity arms) with the use of sulfo-SMCC, dithiothreitol (DTT) or by using biotinylated antibodies and streptavidin-oligos. A native polyacrylamide gel electrophoresis (native PAGE) was run and DNA silver staining was performed to verify the efficiency of the coupling. The proximity ligation assay was performed with microtiter plate systems and with magnetic bead format to compare them. The microtiter plate based PLAs performed well and worked like a positive control since the magnetic beads are required for multiplex protein analysis. The beads were used as solid supports in place of the microtiter wells to enable scalable multiplex protein analyses. The bead format allowed beads with separate capturing antibodies to be combined in individual assays without cross-reactivity from neighboring capturing antibodies for different proteins, and competition is avoided by segregating antibody capture to distinct beads. The results showed that the microtiter plate system based PLAs performed better than the magnetic bead PLAs. In order to enhance the performance of the magnetic bead PLAs new conjugates were made. These conjugates together with an extra oligo, called backbone, made it possible to do a rolling circle amplification (RCA) step after binding and ligation of conjugates. The RCA step increased the signal and increased the ratio between signal and background. In this study I showed that the proximity ligation could detect and measure proteins at very low concentrations. I was able to detect and quantify VEGF (vascular endothelial growth factor) in solutions containing only 10 fM VEGF. I also showed that it is possible to do a microarray analysis of the PCR amplified DNA products from proximity ligation (with and without RCA step) and that the microarray analysis can discriminate several different proteins and measure the concentration. The goal was not reached; no multiplex analysis was done due to the shortage of time because of problems in the beginning of the project and a lot of time consuming optimizations. The study was performed with single biomarker proteins so it is yet to be seen if this technique performs well during multiplex protein studies.

## Introduction

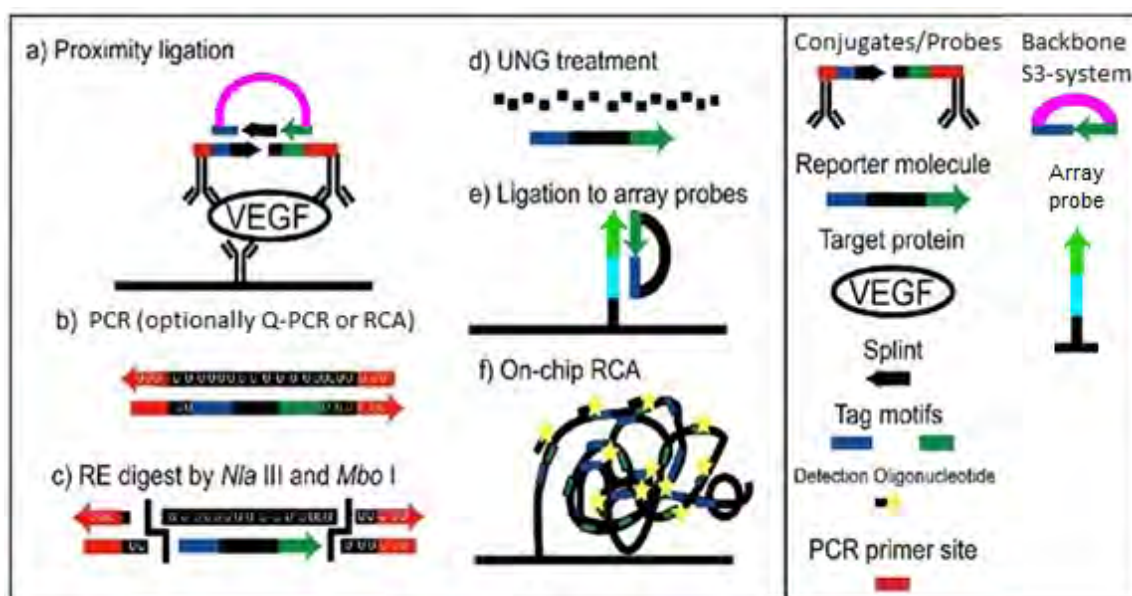
### Immunoassays

Antibodies are excellent tools in protein analysis (immunoassays). One of the most widely known and used immunoassays is Enzyme-Linked ImmunoSorbent Assay (ELISA). ELISA is often used for medicinal diagnostics and quality control in various industries. In most immunoassays a special variant is used, called sandwich-immunoassays. In sandwich-immunoassays two binders (antibodies) are used instead of one. This requires a set of polyclonal antibodies or antibodies derived from different animals. One of the antibodies is immobilized on a solid support and captures the protein of interest and the other one is conjugated to a reporter and used for detection [2]. Double-binder assays (e.g. sandwich-immunoassays) have increased specificity, since they require dual recognition of the protein of interest, one from the immobilizing or anchoring antibody and one from the detection antibody. The immunoassays are often scaled up with the use of microtiter plate systems [3]. Microtiter plates are a flat plate with multiple "wells" used as small test tubes which provide the solid support for the anchoring antibodies. These plates make it possible to test several proteins or other analytes at the same time, but still only one protein or analyte per well. To scale up these assays even further and make it possible to investigate more proteins or other analytes in parallel, two new forms of immunoassays have been developed: bead and microarray formats. Magnetic beads come in several sizes and with different surface functionalities, for use in a wide variety of applications. Some beads are pre-coupled with a biomolecule (ligand). The ligand can be an antibody, protein or antigen, DNA/RNA oligonucleotide or any other molecule with an affinity for the desired target [2]. When added to a sample, the magnetic beads bind to the desired target. This interaction relies on the specific affinity of the ligand on the surface of the beads. The beads respond to a magnetic field, allowing bound material to be rapidly and efficiently separated from the rest of the sample. Unbound material is simply removed by aspiration, and the bead-bound target washed by the use of the magnet. The target is then released from the beads for use in other applications. Alternatively, the target can be used directly while attached to the beads. A microarray is a multiplex lab-on-a-chip. It is a two dimensional arrays on a solid substrate usually a glass slide or silicon thin-film cell that assays large amounts of biological material using high-throughput screening methods [9]. A parallel assay saves time and money and reduces the sample size but it also creates problems due to possibilities of nonspecific adsorption and cross-reactions [3]. These problems rapidly increase as more antibody pairs are added to the assays. This finally leads to an assay with performance and specificity that asymptotically becomes similar to a single-binder assay. However it is possible create a parallel assay with good performance for some ten proteins if the antibody pairs are carefully chosen [2,3].

### Proximity ligation assay

Earlier a lot of the attention has been directed towards techniques designed to study DNA and RNA which has resulted in very powerful techniques and methods. Proximity ligation assay (PLA) took advantage of this progress by translating protein information back into reporter molecules made of nucleic acids (DNA). These reporter molecules can in turn be investigated by Polymerase Chain Reaction (PCR), Quantitative Polymerase Chain Reaction (qPCR), microarray and other powerful and accurate nucleic acid analysis techniques. Proximity Ligation Assay (PLA) stands for a new approach to protein detection and measurement. PLA offers several advantages in parallel protein studies, since it has been shown to enhance analysis of individual [6] or small numbers of serum biomarkers [7], infectious agents [8], protein DNA interactions [9], and protein-protein interactions in fixed tissue sections [14]. The target proteins (the proteins under investigation) are subjected to, detected and immobilized by an antibody which is attached to a solid surface or bead (anchoring antibody), to improve the assay (Figure 1a) [6]. After the binding between the protein and the first antibody (anchoring antibody), the conjugates are added (Figure 1a). The conjugates consist of a polyclonal antibody pair against the protein under investigation. These antibodies are conjugated to single DNA strands (proximity arms, Figure 1). All antibody pairs have their own unique DNA strands (proximity

arms); each proximity arm is equipped with a tag sequence (complementary to the microarray probes or other detection oligonucleotides) and a PCR primer site. When the conjugates bind to the same protein or protein complex, their increased proximity makes it possible for a connector oligonucleotide also known as splint to hybridize to both oligonucleotide arms which makes it possible for a ligase to ligate the two proximity arms together into one single DNA strand. It is also possible to use a third conjugate to increase the specificity [13]. The third conjugate has a proximity arm that works like the splint; it connects the other proximity arms together and the actual splint (includes the third tag sequence and is complementary to the third conjugate) fill up the space between the two original proximity arms. A third probe requires two ligations and gives a ligation product with three tags [13]. The ligation products can be amplified by PCR or qPCR (Figure 1b) and rolling circle amplification (RCA) if an additional longer oligo called backbone are used during the ligation step (Figure 1a) [4,7,14].



**Figure 1**

Schematic overview of the proximity ligation assay with the use of a capturing antibody and microarray analysis. a) The antigen VEGF is immobilized by the capturing antibody and the antibody-oligonucleotide conjugates (antibody against the studied protein conjugated to a short oligonucleotide called proximity arm) are added into the mixture. An oligonucleotide called splint is added that is complementary to the free ends on both oligonucleotides and joins them together. This is followed by washing and ligation with a DNA ligase. Each proximity arm is equipped with a tag sequence (blue and green) and a PCR primer site (red). The tag sequences and the sequence between them on the proximity arms are designed so that the lower single-stranded DNA sequence in figure 1c does not contain any thymidine. The backbone oligo binds to the proximity arms close to the splint and becomes circularized and this makes rolling circle amplification possible when the S3-system is used. b) The new oligonucleotide that is formed by ligation (joining) of the proximity arms is amplified by PCR or alternatively qPCR with dUTP instead of dTTP. c) The PCR amplified products are cleaved by the restriction enzymes *Mbo* I and *Nla* III at sites present in the oligonucleotide conjugates. d) The digested uracil-containing PCR products are degraded by uracil-DNA glycosylase (UNG) in order to get the tag-containing DNA single-stranded. The single-stranded dual tag (the blue and the green tag) sequence does not contain any thymidine or uracil. e) The remaining single-stranded reporter oligonucleotides from the UNG-treatment are hybridized to the microarray probes which are complementary to both tags and the ends of the reporter oligonucleotide are ligated together. f) The hybridized and ligated reporter molecules are amplified with rolling circle amplification (RCA). The RCA product is visualized by detection oligonucleotides with fluorophores, which are hybridized to the RCA product.

PLA can be the solution to the problems with nonspecific adsorption and cross-reactions mentioned above. The background signals from nonspecific adsorption and cross-reactions are reduced because of the strict requirement of very close binding between the conjugates to give rise to detectable signal [4]. Another advantage with PLA is that it can distinguish between specific conjugate pairs (detection reagents) in a multiplex analysis by limiting the opportunities for ligation. For example a conjugate pair against VEGF (vascular endothelial growth factor) can only be ligated together and not to a probe against Interleukin-8 (IL-8) because of the highly specific antibodies and a conjugate against VEGF cannot be ligated together with a conjugate against IL-8 [4].

## Oligo systems

An oligo system consists of two proximity arms, primers for PCR and one splint (connector oligo, Figure 1). There is one exception and that is the S3- oligo system that has an extra oligo called the backbone [13]. The proximity arms are always used in pairs since they have one free 3' or 5'- end and one modified end for attachment to an antibody [6]. The sequence closest to the antibody is the primer site (universal) followed by the tag sequence (Figure 1). The sequences closest to the free 3' or 5' ends are called connector sequences and they are universal since only one splint is used for all proximity arms [4,6,7]. EINAR, BIOVIC and S3 are three different oligo systems (used in this project) [4,6,7]. In the BIOVIC and S3 system the oligos are conjugated to the antibodies in the same way. The antibodies used in these systems are biotinylated and the DNA oligos have streptavidin attached in the 3' or 5' end [5,6,10]. These conjugates are made just before use. In the EINAR system the antibodies are activated with the use of sulfo-SMCC (sulfo-succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) and the oligos are reduced with dithiothreitol (DTT) [7]. Sulfo-SMCC is a water-soluble, non-cleavable and membrane impermeable crosslinker. It contains an amine-reactive *N*-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. NHS esters react with primary amines at pH 7-9 to form stable amide bonds. The EINAR conjugates can be stored for a longer period of time. The EINAR system is designed for PLA with microarray analysis [4]. The BIOVIC system does not contain any tag sequences and is used as a control since it is conjugated in a different way. The S3 system with the extra backbone oligo is a newly developed system. The backbone oligo binds to the proximity arms close to the splint and becomes circularized and this makes rolling circle amplification possible after the proximity ligation (Figure 1a) [4]. The backbone sequence also includes primer sites which enable normal PCR or qPCR with the newly synthesized DNA from the RCA as template. The rolling circle amplification makes it possible to amplify the signals from proteins at very low concentrations and it reduces the background signals [6,14].

## Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a technique based on the polymerase chain reaction (PCR). This technique enables simultaneous detection and quantification (as absolute number of copies) of a specific sequence in a DNA sample, for example the DNA strand formed during PLA. It follows the principle of PCR but the DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. This is made possible by using a fluorescence-detecting thermocycler and fluorescent dyes that intercalate in double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. SYBR green is a dye that intercalates with double-stranded DNA. This means that an increase in DNA product during PCR leads to an increase in fluorescence intensity, which is measured at each cycle allowing DNA concentrations to be quantified. [15]

First the PCR reaction is prepared as usual with the addition of the SYBR green dye. The reaction is then run in a fluorescence-detecting thermocycler, and the levels of fluorescence are measured with a detector after each cycle. This means that an increase in DNA product during PCR leads to an increase in fluorescence intensity, which is measured at each cycle allowing DNA concentrations to be quantified. With reference to a standard dilution, the double-stranded DNA concentration in the PCR can be determined. The relative concentration of DNA present during the exponential phase of the

reaction is determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background is then determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold ( $C_t$ ). The relative amounts of DNA can be calculated since the quantity of DNA doubles every cycle during the exponential phase. For example a sample whose  $C_t$  is 4 cycles earlier than another's has 16 ( $2^4$ ) times more template. The actual amounts of DNA are determined by a comparison between the results and a standard curve produced by qPCR of a serial dilution of a known amount of DNA. [15]

Quantitative polymerase chain reaction is used to determine the number of cycles that should be run in the PCR (figure 1b) [4,7]. The maximum number of cycles is when the sample with the highest concentration reaches saturation (the plateau of the curve). A qPCR is said to be saturated when an increase in the number of cycles does not give an increase in signal [15]. It is important that none of the samples reaches saturation in order to get good results from the microarray. All samples that reach saturation will give the same signal (the highest signal possible).

## Microarray analysis

The PCR amplified products from the PLA can be sorted on and be analyzed on microarrays [4]. The PLA microarray designed in the Ericsson laboratory has dual tagged probes that are complementary to the tag sequences on the oligonucleotide-arms on the PLA conjugates [4]. The individual spots on the microarray have different dual tag sequence setups (e.g. the blue and the green tags in figure 1e) that enable discrimination of different conjugate pairs and their amplified single-stranded PLA product (Figure 1) [4]. This also eliminates the background from cross-reactive antibodies since both tag sequences are required for hybridization and later ligation (figure 1) [4,7]. The PCR-amplified products from the PLA are cleaved by the restriction enzymes; *Mbo* I and *Nla* III to get the actual reporter oligonucleotides for the microarray (Figure 1c). The sequence is double-stranded but it has to be single-stranded for the hybridization to the microarray. This is solved by not having any thymidine in single stranded dual tag sequence that is used for the microarray and performing the PCR with dUTP instead of dTTP. This makes it possible to degrade the uracil containing strand with uracil-DNA glycosylase (UNG) resulting in an intact single-stranded sequence (Figure 1b, c and d). The remaining single-stranded reporter oligonucleotides are hybridized to the microarray probes which are complementary to both tags and the ends of the reporter oligonucleotide are ligated together (Figure 1e). The hybridized and ligated reporter molecules are amplified with rolling circle amplification (RCA) using Phi29 DNA polymerase. The RCA product is visualized by detection oligonucleotide hybridization (Figure 1f) [4].

## Biomarkers

Biomarkers are proteins or other molecules used as indicators for a certain biological state such as ischemic heart disease [7]. Ischemic heart disease is a disease characterized by reduced blood supply to the heart muscle, usually due to coronary artery disease (atherosclerosis of the coronary arteries). Its risk increases with age, smoking, hypercholesterolaemia (high cholesterol levels), diabetes, hypertension (high blood pressure) and is more common in men and those who have close relatives with ischemic heart disease [16].

## Aims

The aim of this project was to combine PLA and a microarray in order to investigate five biomarkers for ischemic heart disease in the same reaction volume. The five biomarkers were supposed to be investigated simultaneously in the same reaction volume with the use of PLA combined with the specially designed microarray platform for high-performance nucleic acid analysis. In order to get this assay to work some optimization had to be done and several oligo systems (different proximity arms, connector oligonucleotides) had to be tested during the PLA.

## Results

### Protein detection with proximity ligation and microarray analysis

#### Conjugation and proximity ligation

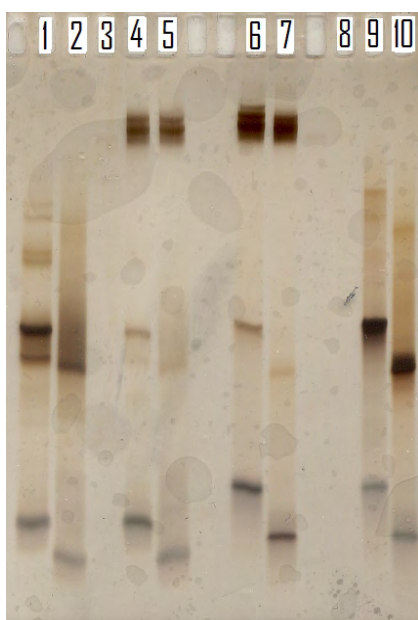
The key components in a successful PLA are high affinity antibodies and well working oligo systems. I tested three different oligo systems together with high affinity antibodies directed against the vascular endothelial growth factor (VEGF). The EINAR oligo system (UDA-system, Microarray system) was the first system that I tested since it is developed for microarray analysis. I conjugated anti- VEGF antibodies to oligonucleotide arms belonging to an EINAR sub-system called UDA-A. These oligonucleotide arms are equipped with tag sequences A1 and A2 (hence the name UDA-A; the UDA-B oligonucleotide arms are equipped with tag sequences B1 and B2 and so on) that are complementary to a specific individual probe sequence on the microarray. The anti-VEGF antibodies and EINAR oligonucleotide arms were conjugated with use of sulfo-SMCC. To test the efficiency of the conjugation, the conjugates were run on a native PAGE gel and were then silver stained. I expected a single band in each conjugate well that would be slightly higher than the bands in the antibody controls wells. The conjugate bands should be higher because of their increase in size and volume, since the oligonucleotides add molecular weight and volume to the antibodies. Larger proteins with more volume migrate slower than a smaller protein. A native PAGE gel separate proteins according to their conformation, volume and molecular weight since the native PAGE gel is run in non-denaturing conditions. A SDS PAGE gel separate proteins according to their length of polypeptide chain or molecular weight since SDS denature native proteins into polypeptides and it binds to polypeptides in a constant weight ratio which give the polypeptide chains an identical charge per mass unit. The conjugates were visible on a native PAGE gel (PhastGel gradient 8 %- 25 % polyacrylamide) with silver staining. Although the antibody control was missing (Lane 3 and 8 in figure 2), two bands could be seen in the conjugates wells (Figure 2). The lower faster migrating bands in the conjugates wells (Lane 4, 5, 6 and 7 in figure 2) are the unbound antibodies and the slightly higher slower migrating bands are the conjugates. Several bands could be seen in the pure oligonucleotide lanes (Lane 1, 2, 9 and 10 in figure 2) due to intramolecular secondary structures (e.g. hairpins) within the oligonucleotides since they are not denatured. Thus, the conjugation was successful. These conjugates were subsequently used in several PLA experiments.

The PLA was performed with both magnetic beads (Dynabeads<sup>®</sup> MyOne<sup>™</sup> Streptavidin T1, Invitrogen) and a microtiter platesystem (Robostrips from Roboscreen) as support for the anchoring antibody (figure 1a). The beads were prepared by coupling biotinylated anti-VEGF antibodies to the streptavidin-coated beads and the microtiter plates were coated with polyclonal anti-VEGF antibodies. The following proximity ligation and qPCR steps were performed simultaneously. The magnetic beads were tested since they are crucial in the multiplex analysis and the microtiter platesystem was used as a control since it is the original procedure which is reliable and works well [6].

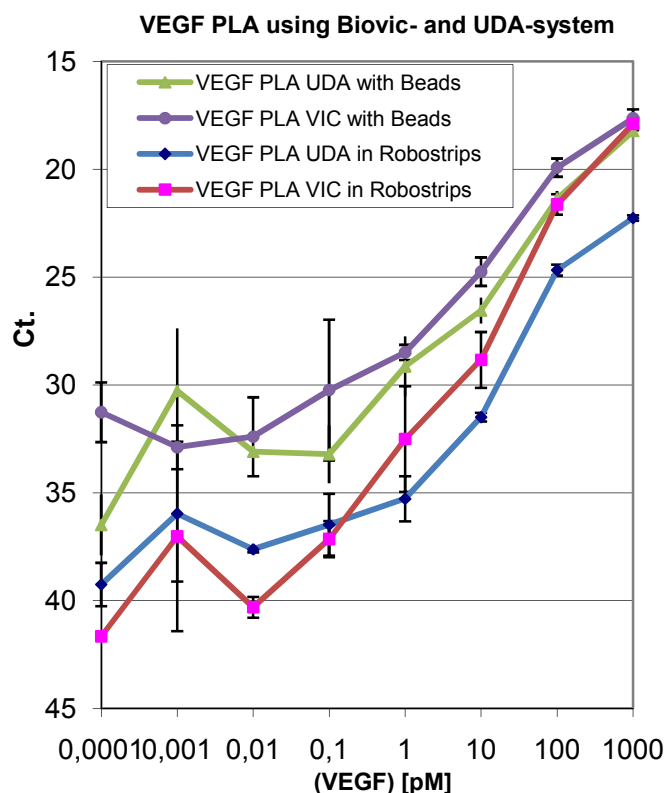
I tested the reliability of the UDA conjugates by running several PLAs with varying concentrations of VEGF in my samples. The PLA was performed with both magnetic beads and the microtiter platesystem. After the initial proximity ligation step (figure 1a) I analyzed the samples with qPCR (figure 1b). I expected equally linear standard curves from the bead based PLA and the microtiter based PLA and that they should be linear from 1 nM down to 1fM (the green and the blue curve in figure 3). The results from the qPCR showed standard curves that were linear from 1 nM down to 0.1 pM (Figure 3). The curves were not linear from 0.1 pM down to 0.1 fM and they had slightly high standard deviations. There were also high background signals from the 1 fM sample. The standard deviations were higher in the bead based PLAs (the green curve in figure 3) and they were also less reliable (the results vary from time to time) than the microtiter platesystem based PLA (the blue curve in Figure 3). However the EINAR- anti-VEGF conjugates turned out to be quite unreliable, some batches appeared fine on the native PAGE but did not perform well in the PLA experiments. The qPCR

result from the faulty EINAR- anti-VEGF conjugates had very high standard deviations even in the higher concentrations. A probable cause to this unreliability could be that something was wrong with the coupling between the anti-VEGF antibody and the EINAR oligonucleotide arms. Because of this unreliability another oligo system had to be tested. The results showed that it was possible to detect VEGF in solutions containing only 0.1 pM VEGF. The bead based PLA did not work as well as the microtiter platesystem based PLA (Figure 3). The standard deviations were higher and the signals were lower but I continued with the magnetic beads since they are crucial to the multiplex investigation.

The BIOVIC oligo system was tested and compared with the EINAR oligo system. This system act as a positive control system since it is reliable, known to work well and it is conjugated in a different way [6]. The EINAR conjugates and BIOVIC conjugates worked equally well but the BIOVIC conjugates were more reliable (Not batch dependent, data not shown). The qPCR results from the BIOVIC conjugate PLA were also more linear down to 10 fM VEGF than the EINAR conjugate PLA (the purple curve and the pink curve in Figure 3) which would be good in the subsequent microarray analysis since it reduces background signals from the PLA. But I continued with the EINAR oligo system since the EINAR oligonucleotide arms contain a dual tag sequence.



**Figure 2**  
Analysis of conjugates with native PAGE gel with silver staining. Lane 1 was loaded with UDA-B1 (Pure oligo). Lane 2 was loaded with UDA-B2 (pure oligo). Lane 3 was loaded with pure IL-10 antibody. Lane 4 was loaded with anti-IL10-UDA-B1 conjugate. Lane 5 was loaded with anti-IL-10-UDA-B2 conjugate. Lane 6 was loaded anti-VEGF-UDA-A1 conjugate. Lane 7 was loaded with anti-VEGF-UDA-A2 conjugate. Lane 8 was loaded with pure VEGF antibody. Lane 9 was loaded with UDA-A1 (Pure oligo). Lane 10 was loaded with UDA-A2 (pure oligo).

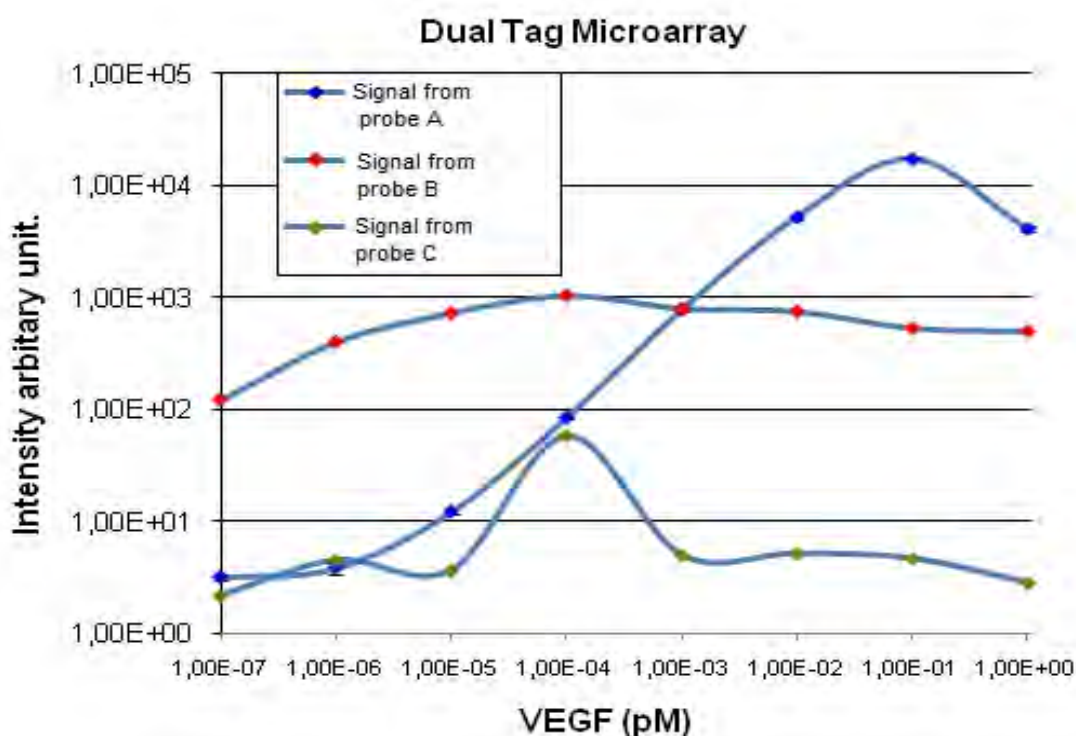


**Figure 3**  
Comparison of qPCR results from bead based PLA and microtiter platesystem based PLA with UDA (EINAR) - and VIC (BIOVIC) oligo systems. The green line (dots) is the result from bead based PLA with UDA system. The purple line (dots) is the result from bead based PLA with VIC system. The blue line (dots) is the result from microtiter platesystem based PLA with UDA system. The pink line (dots) is the result from microtiter platesystem based PLA with VIC system.



## Microarray

The microarray analysis was performed with UNG-treated PCR products from PLAs investigating VEGF concentrations with anti-VEGF- UDA-A conjugates (EINAR subsystem A oligonucleotide arms). The oligonucleotide arms in these conjugates contain the dual tag sequence complementary to the probes in the A spots on the microarray (Figure 4). Before the actual ligation of the anti-VEGF- UDA-A conjugates (Figure 1a) and PCR step (figure 1b), the PLA magnetic bead solutions were divided in to two groups (a triplicate of each concentration VEGF per group). The first group was analyzed with qPCR (figure 1b) to check at which cycle the sample with the highest concentration reached saturation (the plateau). The result from the qPCR decided how many cycles should be run in the standard PCR (figure 1b) for the other group. It is important that none of the samples reaches saturation (the plateau) in order to get good results from the microarray. Based on the qPCR, the PCR was cycled 25 times. After restriction enzyme digestion with *Mbol* and *NlaIII* (figure 1c), the uracil-containing PCR products were degraded with UNG (figure 1d). The UNG-treated PCR products were successfully hybridized on the microarray and the ligation between the free 5' and 3' ends of the UNG-treated PCR products were also succesfull (figure 1e) followed by successful rolling circle amplification (RCA, figure 1f). However, detection oligonucleotide hybridization was a half success (Figure 4). The detection oligonucleotides hybridized well to the RCA products but they also hybridized to UDA-B systems microarray probes (the probes that contain the complementary sequences to tag B1 and B2) which would have led to increased background signals in a multiplex analysis (Figure 4). This would only be a problem if the proteins under investigation have lower concentrations than 10 fM. The signals from the C probes was the expected and wanted background signals except for the spots corresponding to 1.00E -04 pM VEGF (Figure 4).

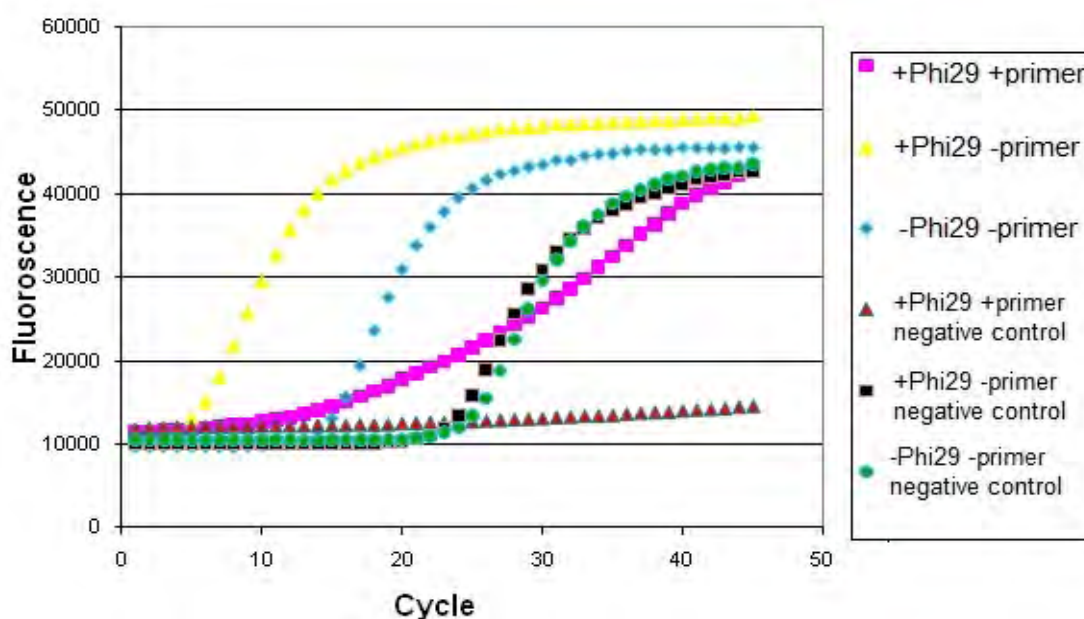


**Figure 4**

Dual Tag Microarray analysis of VEGF PLAs using anti-VEGF- UDA-A conjugates. The oligonucleotide arms in these conjugates contain the dual tag sequence complementary to the probes in the A spots on the microarray. The blue dots are the results from microarray probes that are complementary to UDA-A subsystem PCR products. The red dots are results from microarray probes that are complementary to UDA-B subsystem PCR products. The yellow dots are results from microarray probes that are complementary to UDA-C subsystem PCR products.

## The S3 oligo system

Even though the EINAR conjugates and BIOVIC conjugates worked in the PLA, the S3 oligo system had to be tested to see if the background could be reduced and the signals increased. The difference between the S3-system and the other two systems is that the S3 system includes an extra oligo called backbone. The splint connects the oligos in the conjugates (Figure 1a) and the backbone also binds to the proximity arms and therefore can be ligated to the splint and form circular DNA (Figure 1a). The circular DNA then can be amplified (RCA) with Phi29 DNA polymerase. The backbone sequence also includes primer sites which enable normal PCR or qPCR with the newly synthesized DNA from the RCA as template. The S3 conjugates are also conjugates between biotinylated antibodies and streptavidin-oligos. Therefore, these conjugates are supposed to be equally reliable as the BIOVIC conjugates. The PLA with the S3 oligo system was performed with RCA and without a RCA step (figure 1b, results figure 5) to compare the effect of RCA with respect to noise, signal increase and overall performance. Figure 5 shows qPCR amplifications plots from a PLA experiment using the S3-system to investigate samples that had the same concentration of VEGF. The yellow triangles were the result from the PLA with RCA and the blue diamonds were the result from the PLA without RCA. Two different ligases were also tested in the same experiment, T4 DNA ligase and Ampligase (Heat stable). T4 DNA ligase and Ampligase were also tested together with the BIOVIC system. The ligation step (figure 1a) during this PLA was performed with different temperatures since the Ampligase is thermostable which means that it could ligate under more stringent conditions. There was no difference in performance between T4 DNA ligase and Ampligase in the BIOVIC PLA but these ligases were compared again in the S3-system PLA because the S3-system requires two ligations and the Ampligase gave slightly better results (data not shown). The result from the S3-system PLA showed that the RCA increased the signal in the qPCR and increased the ratio between signal and background (Figure 5, yellow triangles= PLA with RCA, blue diamond's= PLA without RCA). The standard deviations were also lower (data not shown).



**Figure 5**

qPCR amplification plots from a PLA experiment using the S3-system. The pink squares are the amplification plot from the combined RCA and qPCR when Phi29 (for the RCA) and PCR-primers were added from the beginning. The yellow triangles are the amplification plot from the combined RCA and qPCR when Phi29 were added from the beginning and the PCR-primers were added after the pre-incubation step. The blue diamonds are the amplification plot from the combined RCA and qPCR when no Phi29 were added from the beginning and the PCR-primers were added after the pre-incubation step. The other amplification plots are the respective negative controls (the targeted protein (VEGF) was excluded).

## **Combined rolling circle amplification and polymerase chain reaction**

I tested the possibility to combine the RCA step with the PCR step into one step (figure 1b) in order to save time and limit the room for error. This was done by adding the Phi29 DNA polymerase into the PCR mixture (figure 1b) and adding a pre-incubation step for 1 hour at 37°C (for the actual RCA before the normal PCR cycles) to the normal PCR program. The Phi29 DNA polymerase amplifies the circular DNA from the PLA during the incubation hour. When the PCR cycles starts the PCR primers hybridize to newly synthesized DNA from the RCA and then the Taq polymerase extend the primers as in a "normal PCR or qPCR". This was a positive or negative experiment (is it possible to combine RCA and PCR or qPCR or not?). Thus, all samples had the same concentration of VEGF (0.1 nM). The result was negative (pink squares, figure 5); RCA and PCR could not be combined. The amplification curves in the qPCR were linear instead of sigmoid. The linear curves gave a hint that the PCR was not working. It turned out that Phi29 DNA polymerase is not only a polymerase but also a very good exonuclease (3'=>5'exonuclease) and probably destroys all the primers [5]. To test this, the primers were excluded from the RCA-PCR mixture and were added after the RCA pre-incubation step (the Phi29 was deactivated by heating, described as "-primer" in figure 5). The experiment was a success, the RCA and PCR could be combined if the primers were protected (special protected 3'-modified primers were ordered and used in later experiments, data not shown) or excluded in the beginning (described as "-primer" in figure 5). The signal was significantly lower in the RCA-PCR samples without Phi29 DNA polymerase, then the RCA-PCR samples with Phi29 DNA polymerase (-Phi29 or +Phi29 in figure 5). This experiment showed once again that RCA increase the signal (Figure 5).

## Discussion

The results from the conjugation clearly showed that it was possible to conjugate high affinity anti-VEGF antibodies with the EINAR-systems (UDA-A, Microarray system) oligonucleotide arms. But the results from the PLA showed that the EINAR conjugates were quite unreliable compared to the BIOVIC-systems and S3-systems conjugates. The cause of this unreliability could be that it was hard to get the same concentration and know the exact concentration of each conjugate in a conjugate pair (e.g. anti-VEGF- UDA-A1 and anti-VEGF- UDA-A2) and this could be seen on the native PAGE gel with silver staining (uneven band thickness, figure 2). Uneven concentration of conjugates ultimately leads to that one of the conjugates out-competes the other. One solution to the EINAR-system conjugate unreliability problem could be to use the same conjugation technique as with the BIOVIC-system and the S3-system. PLAs performed with the BIOVIC-system and the S3-system gave nice linear titration curves after qPCR, which means that they would perform well on the microarray and since the PLA was performed on samples with very low concentrations ranging from 1 nM to 10 fM, this shows that PLA is a very good diagnostic tool for the future. Especially the S3-system PLA which performed very well, especially when the extra RCA step was performed which increased the signal and increased the ratio between the true signal and background, which is important during investigation of samples with very low protein concentrations. I also showed that an extra RCA step could be combined with PCR if protected primers were used. This will save working hours in the lab and probably reduce the standard deviations. The S3-system could be used in future multiplex PLAs with microarray analysis if the backbone is redesigned so that it includes the dual tag sequence between the PCR primer sites.

The microtiter plate system was compared with the magnetic bead format and the result from these experiments showed that the microtiter plate system based PLAs performed better than the magnetic bead based PLAs. This was sad since the microtiter plate system was actually a positive control. The microtiter plate system was used as a control because it performs well but the magnetic beads are required for multiplex analysis. The microtiter plate system cannot be used in multiplex analysis because of the shortage of space for the binding of capturing antibody. The combined binding surface of the beads is much larger than that of the microtiter plate systems. Also the problems with capturing antibodies out-competing each other is solved by using beads, since it's possible to add the same amount of every kind of bead into the reaction volume. The performance problem of the beads probably could be solved by using a better washing system than the filter membrane-bottom microtiter plate (MSHVN4510, Millipore) and a vacuum manifold (Millipore) used here. The target protein- bead conjugation and proximity ligation took place in the wells of the filter membrane-bottom microtiter plate. The beads were washed after target protein-bead conjugation, after the addition of conjugates and after the ligation step by sucking away the liquid with vacuum using the vacuum manifold from Millipore. The problem is that a lot of the beads got caught in the filter. This wouldn't be a problem if the beads were evenly distributed on the filter between the wells but they were not, a lot of beads were caught in some wells and almost no beads in others.

The microarray analysis was successful since it showed a nice linear standard curve of the VEGF PLAs (Figure 4) but one major problem was also shown. The detection oligonucleotides for the UDA-A systems RCA product hybridized with the microarray probes on the UDA-B microarray features. This would lead to increased background for the target proteins that are analyzed with antibodies conjugated to the UDA-B sub-system in a multiplex analysis. The problem must be solved before any multiplex analysis can be made using the UDA-B sub system. The problem can be solved by performing the microarray steps under more stringent conditions or redesign UDA-B sub-systems oligos and the UDA-B features oligonucleotide probes on the microarray.

The goal was not reached; no multiplex analysis was done due to the shortage of time because of problems in the beginning of the project and a lot of time consuming optimizations. But I am sure that there will be well-performing multiplex protein analysis with the use of PLA and microarray analysis in the future since it works so well for single protein analysis. In fact a group at Stanford University has already identified a biomarker panel with the use of a proximity ligation assay that improves accuracy

of pancreatic cancer diagnosis [1]. The microarray analysis is however crucial to the ischemic heart disease panel since the concentration of the biomarkers are so low.

After this investigation was concluded a high throughput protein biomarker discovery tool has been developed. The new tool is based on multiplexed proximity ligation assays (PLA) with microfluidic high capacity qPCR analysis instead of microarray analysis. The tool's platform consisted of four 24-plex panels profiling up to 74 supposed biomarkers. Only 1  $\mu$ l of sample was required to each well on the 24-plex panel. All conjugates were then added to each well of the platform. The system used either matched monoclonal antibody pairs or single batches of affinity purified polyclonal antibodies to generate conjugates with unique oligonucleotide arms similar to the dual tag-sequence. The tags on the oligonucleotide arms were unique PCR primer sites for each conjugate, the unique PCR primer sites were flanked with universal primer sites. After proximity ligation the reporter sequences were pre-amplified with PCR using the universal PCR primersites. Multiplex PLA thereby converted multiple target analytes into PCR amplicons that were individually quantified using microfluidic high capacity qPCR in nano liter volumes (it functions like a qPCR microarray). The quantification of each specific analyte was performed using only one qPCR primer pair for each analyte, for example VEGF reverse and VEGF forward. The assay showed excellent specificity, even in multiplex. [11]

Another report showed that the dynamic range (the ratio between the largest and smallest possible values of a changeable quantity) of PLA could be improved by 2 orders of magnitude and the sensitivity could be improved by a factor of 1.57. That was achieved by using asymmetric splint hybridization to reduce the possibility of target-independent ligation which gives the background signal. The standard PLA splint consisted of 20 bases which hybridize symmetrically, this was varied to an asymmetric splint with a total of 15 bases. The results of this model suggested that weakening the affinity of one side of the splint to one oligonucleotide arm would significantly reduce background (target-independent ligation) without greatly affecting the true signal from target-dependent ligation. These predictions were shown to be accurate. This novel, asymmetric PLA approach should impact any previously developed PLA method by reducing target-independent ligation events, thus generally improving the sensitivity and dynamic range of the assay. [10]

## Materials and methods

### Conjugation of the probes

Three different oligo systems were used: EINAR-, BIOVIC- and S3-system. The BIOVIC and S3 systems are conjugated in the same way. The antibodies used in these systems are biotinylated and the DNA oligos have streptavidin attached in the 3' or 5' end. In the EINAR system the antibodies are activated with the use of sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate from PIERCE) and the oligos are reduced with dithiothreitol (DTT). Sulfo-SMCC is a water-soluble, non-cleavable and membrane impermeable crosslinker. It contains an amine-reactive *N*-hydroxysuccinimide (NHS ester) and a sulfhydryl-reactive maleimide group. NHS esters react with primary amines at pH 7-9 to form stable amide bonds. All antibodies are listed in table 1. All oligonucleotides are shown in table 2 and they were manufactured by Solulink.

**Table 1.** Antibodies used for conjugation and later PLAs.

| <b>Antibodies used</b>      | <b>Modifications</b> | <b>Manufacturer</b> |
|-----------------------------|----------------------|---------------------|
| Polyclonal goat anti-VEGF   | Biotinylated         | R&D systems         |
| Polyclonal goat anti-VEGF   | none                 | R&D systems         |
| Polyclonal goat anti- IL-10 | Biotinylated         | R&D systems         |
| Polyclonal goat anti- IL-10 | none                 | R&D systems         |

**Table 2. Oligonucleotides**

| System Name |                               | Sequence <sup>1</sup>   | Modifications  |
|-------------|-------------------------------|---|----------------|
| EINAR (UDA) | Forward primer                | CGATTTCGAGAACGTGACTGC   |                |
| EINAR (UDA) | Reverse primer                | GCGAAACATGGTCCGGTATC  |                |
| EINAR (UDA) | System A                      |   |                |
| EINAR (UDA) | Oligonucleotide arm A Free 3' | TTTTGCGAAACATGGTCCGGTATCGATCCCGCTTTCCTGTC<br>TTCGTTCTGCCCTTTTCGTG           | 5' -SH         |
| EINAR (UDA) | Oligonucleotide arm A Free 5' | TCGTGTCTTTTCCTTGTTCCTTCATGGCAGTCACGTTCT<br>CGAATCGTTTT                      | 5' -P & 3' -SH |
| EINAR (UDA) | System B                      |   |                |
| EINAR (UDA) | Oligonucleotide arm B Free 3' | TTTTGCGAAACATGGTCCGGTATCGATCGTGTCTCCTCTGC<br>GTCGTTCTGCCCTTTTCGTG           | 5' -SH         |
| EINAR (UDA) | Oligonucleotide arm B Free 5' | TCGTGTCTTCTGTCTTGGGTGCCATGGCAGTCACGTTCT<br>CGAATCGTTTT                      | 5' -P & 3' -SH |
| EINAR (UDA) | System C                      |   |                |
| EINAR (UDA) | Oligonucleotide arm C Free 3' | TTTTGCGAAACATGGTCCGGTATCGATCCGTGCTTGTTCGG<br>TTCGTTCTGCCCTTTTCGTG           | 5' -SH         |
| EINAR (UDA) | Oligonucleotide arm C Free 5' | TCGTGTCTTCTCGTTTGGGTTCTCATGGCAGTCACGTTCT<br>CGAATCGTTTT                     | 5' -P & 3' -SH |
| EINAR (UDA) | Splint                        | TTTAAAGACACGACACGAAAAAGTTT  |                |
| EINAR (UDA) | MicroArray Tag A2-A1          | TCTCTCTCTCTCTCTCTCTCTTCCTTGTTCCTTCCTTCCTTC<br>GCCTGTCT                      | Amine          |
| EINAR (UDA) | MicroArray Tag B2-B1          | TCTCTCTCTCTCTCTCTCTCTGTCTTGGGTGCCGTCTCCTTCT<br>GCG                          | Amine          |
| EINAR (UDA) | MicroArray Tag C2-C1          | TCTCTCTCTCTCTCTCTCTCTCGTTTGGGTTCTCGTGTCTTCTTC<br>CGT                        | Amine          |
| BIOVIC      | Forward primer                | GGGAATCAAGGTAACGGACTTTAG  |                |
| BIOVIC      | Reverse primer                | CATCGCCCTTGACTACGA  |                |
| BIOVIC      | Oligonucleotide arm Free 3'   | CGCATCGCCCTTGACTACGACTGACGAACCGCTTGCCTGA<br>CTGATCGCTAAATCGTG               | Streptavidin   |
| BIOVIC      | Oligonucleotide arm Free 5'   | TCGTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCCTCTTG<br>AAAAATTCGGCCTCGGTGA          | Streptavidin   |
| BIOVIC      | Splint                        | TACTTAGACACGACGATTTAGTTT  |                |
| S3          | Oligonucleotide arm A1        | TTTTTTTTTCTACGACTGAGATCCCGCTTTGCCTGTCTGTTCG<br>CTTTTTTCGTGCC                | Streptavidin   |
| S3          | Oligonucleotide arm A2        | TTTTTTTTTTCGTGTCTTTTGTCCGTTTCCTTGTTCCTTCCTCA<br>TGCTCTTG                    | Streptavidin   |
| S3          | Backbone                      | GATCTCAGTCGTAGTGCAGTCACGTTCTCGAATCGTTTTGCG<br>AAACATGGTCCGGTATCTTCAAGAGCATG |                |
| S3          | Spli                          | AAAGACACGAAAGGACGACACAAGCAGAGAAGGGCAGAAAA<br>AG nt                          |                |
| S3          | Specific primer A rev         | TCGATCCCCTTTGCCTGTC   |                |
| S3          | Specific primer A fwd         | GCCATGAGGGGAAACAAGGA  |                |

<sup>1</sup>the colored part of the sequences were the tag sequences.

## **Conjugation with sulfo-SMCC and dithiothreitol (EINAR system)**

To conjugate anti-VEGF and anti-IL-10 non-biotinylated antibodies (Table 1) to oligos UDA-A1,2 and UDA-B1,2 (Table 2), the anti-VEGF and anti-IL-10 antibodies were dialyzed against 5 L 1XPBS (5 L H<sub>2</sub>O, 40 g NaCl, 1 g KCl, 7.2 g Na<sub>2</sub>HPO<sub>4</sub>, 1.2 g KH<sub>2</sub>PO<sub>4</sub>) with the use of PIERCE dialysis cups (cut off 7kDA). The antibodies were concentrated with Millipore YM-10 columns to approximately 2 µg/µl (20 µg (135 pmoles) were used from the beginning). The antibodies were then activated by addition of 1 µl freshly prepared 4 mM sulfo-SMCC (PIERCE, final concentration 0.4 mM) in DMSO (dimethylsulfoxid) per 10 µl antibody solution. The antibodies were then incubated for 2 hours at room temperature. The oligos were reduced by mixing 3 µl of 100 µM oligo stock (350 pmoles) with 12 µl of 100 mM dithiothreitol (freshly prepared using 4.0 mg of dithiothreitol in 259 µl of 1XPBS, 5 mM EDTA (ethylene diamine tetraacetic acid)). The oligos were incubated for 1 hour at 37°C. In order to stop the reactions and to get rid of the remaining sulfo-SMCC and DTT, the oligos and antibodies were run through three microspin G50 columns (Amersham) that had been calibrated with 1XPBS and 5 mM EDTA before use. After the removal of sulfo-SMCC and DTT, the oligos were mixed with their respective antibody and dialyzed against 5 L 1XPBS overnight at 4°C. Later a native PAGE with silver staining was performed to validate the conjugation. The conjugate samples were analyzed on a precast PhastGel (GE healthcare) with a polyacrylamide gradient ranging from 8 % up to 25 %. The gel was run in a Phastsystem (GE Healthcare) with native buffer strips that contained 0.25 M Tris and 0.8 M L-alanine (pH 8.8). The gel was silver stained with PlusOne, silver staining kit (GE Healthcare). The native PAGE gel and silver staining was performed according to GE Healthcare's recommendations and protocols. After the validation the probes were preserved by addition of NaN<sub>3</sub> in 1XPBS to a final concentration of 0.05 %.

## **Conjugation with biotinylated antibodies and streptavidin-oligos**

The biotin-streptavidin conjugations were made fresh every time in 1XPBS with 0.1 % BSA. The conjugation mix consisted of 50 nM biotinylated antibodies (R&D systems) and 50 nM streptavidin-oligo (Solulink). Each conjugate in a conjugate pair (e.g. anti-VEGF- UDA-A1 and anti-VEGF- UDA-A2 are a conjugate pair) were conjugated separately at room temperature for 1 hour. After the incubation the conjugates were mixed and diluted down to 1 nM in a biotin containing buffer (1 % BSA, 1 mM biotin, 100 µg/ml salmon sperm DNA (Invitrogen), 1XPBS, 0.05 % Tween) followed by 0.5 hour incubation at room temperature.

## ***Solid phase proximity ligation assay***

### **Microtiter plate systems (Robostrips from Roboscreen)**

The microtiter wells were coated overnight at 4°C with 50 µl (1 ng/µl) of the non-biotinylated polyclonal goat anti-X antibodies (R&D systems) depending on which protein was investigated in ELISA coating buffer (10 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.6). After the coating the wells were washed three times with 1XPBS and 0.05 % Tween 20 (Sigma). The washing steps during the PLA were done with a Columbus Pro Microplate strip-washer (Tecan Trading AG). The microtiter wells were then blocked with 200µl blocking buffer (1XPBS, 1 % BSA, 1 mM biotin, 100 µg/ml salmon sperm DNA (Invitrogen) and 0.05 % Tween 20) for 1 hour at room temperature. The blocking buffer was washed away. The antigen (the protein which the antibodies had been raised against) was added in an eight step titration series, 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM and a negative control lacking antigen in 50 µl blocking buffer. The strips were incubated for three hours at 37°C. After the antigen binding, the strips were washed six times with blocking buffer. The conjugates were added in a final concentration of 1 nM in a 50 µl reaction volume (blocking buffer). The strips were incubated for 1 hour at room temperature. Thereafter 50 µl of ligation mix was added providing a final concentration of 40 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 0.5 mM ATP (pH 7.8), 0.5 units T4 DNA ligase (Fermentas) and 100 nM splint (connector oligo (Table 2)), 100 nM backbone (only when the S3-system was used (Table 2)). Alternatively, the ligation was performed with Ampligase thermostable DNA ligase (Epicentre). The Ampligase mix consisted of 200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM MgCl<sub>2</sub>, 5



mM NAD, 0.1% Triton X-100, 100 nM Splint (connector oligo (Table 2)), 100 nM backbone (only when the S3-system was used (Table 2)), 3 units Ampligase. The strips were incubated for 1 hour at 37°C.

### **Proximity ligation assay with bead format**

The beads were prepared by coupling biotinylated antibodies (Table 1) to the streptavidin-coated beads (Dynabeads<sup>®</sup> MyOne<sup>™</sup> Streptavidin T1, Invitrogen) according to the manufacturer's protocols and recommendations. Seventy five µg/ml antibodies in a total volume of 100 µl were mixed with approximately 10<sup>9</sup> beads. The mix was incubated over night at 4°C while being end-over-end rotated. After the incubation the beads were washed 3 times with 1XPBS with 0.05 % Tween 20. Before use the beads were diluted 1/100 with blocking buffer (1XPBS, 1 % bovine serum albumin (BSA), 1 mM biotin, 100 µg/ml salmon sperm DNA (Invitrogen) and 0.05 % Tween 20). Five µl of the diluted beads were added to each 45µl sample e.g. VEGF (eight step titration series, 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM e.g. VEGF and a negative control in blocking buffer). The mixtures were then incubated 2 hours at room temperature during end-over-end rotation. The beads were then washed three times with 1XPBS and 0.05 % Tween 20 with the use of a filter membrane-bottom microtiter plate (MSHVN4510, Millipore) and a vacuum manifold (Millipore). After the washing 50 µl of probe mix (1 nM of each conjugate in blocking buffer) were added directly into the filter-plate. The plate was shaken (520 rpm) for 2 hours at room temperature. After the incubation the liquid was sucked out with a vacuum manifold (Millipore) and the filter-plate was washed six times with 1XPBS and 0.05 % Tween 20. Thereafter 50µl ligation mixture (the same ligation mixtures mentioned in the microtiter plate system section) was added directly into the filter-plate followed by 1 hour incubation at 37°C. After the ligation the filter-plate was washed three times with 1XPBS and 0.05 % Tween 20. Then 50 µl of PCR or qPCR (see PCR and qPCR section) mixture was added, the mixture was then transferred to optical PCR-tubes (Eppendorf).

### **Rolling circle amplification**

This step was only performed when the S3 oligo system was used. After the ligation and later washing of the ligated conjugates (with splint and backbone, table 2), 50 µl RCA mixture was added. The RCA mixture consisted of 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1 % (v/v) Tween 20, 1 µg/µl BSA, 100 µM dNTP and 0.1 units Phi29 DNA polymerase (Fermentas). After the addition of RCA mix the beads or microtiter strips were incubated for 1 hour at 37°C.

### **Polymerase chain reaction and quantitative-polymerase chain reaction**

The PCR mix consisted of 50 mM KAc, 3 mM MgAc, 20 mM Tris-acetate (pH 7.5 at 37°C), 1 mM DTT, 0.2 mM dNTP (dTTP replaced by dUTP), 100 nM of each primer (forward and reverse, table 2), 100 nM splint, 0.6 units Platinum Taq polymerase (Invitrogen). The ligation and PCR or qPCR were performed at the same time with only one mix (only when EINAR- and BIOVIC system was used) or separately. The ligation-PCR mixture consisted of 5 mM KAc, 3 mM MgAc, 20 mM Tris-HAc pH 7.5, 1 mM DTT, 0.2 mM dNTP, 100 nM of each primer (forward and reverse, table 2), 0.6 units Platinum Taq polymerase (Invitrogen), 0.5 mM ATP, 0.5 units T4 DNA Ligase (Fermentas) and 100 nM splint (connector oligo, table 2). The RCA-PCR mixture consisted of 5 mM KAc, 3 mM MgAc, 20 mM TrisAc (pH 7.5 at 37°C), 1 mM DTT, 0.2 mM dNTP, 100 nM of each primer (forward and reverse, table 2), 0.6 units Platinum Taq polymerase (Invitrogen), and 0.1 units Phi29 DNA polymerase (Fermentas). Two and a half µl 10XSyBR gold (0.5X, molecular probe, nucleic acid staining cyanine dye; Invitrogen) was added to the PCR mixture for the qPCR analysis. The qPCR was performed with the use of a Stratagene MX 3000P machine. The machine was programmed to start with a incubation step for 2 min at 95°C (heat-activation of Taq platinum) followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. When RCA and PCR were combined in the same reaction mixture, an additional incubation step for 1 hour at 37° (for the RCA) was added before the normal program. The PCR prior to microarray analysis was cycled 25 times.

## Microarray analysis

Before the microarray analysis the Taq platinum polymerase was deactivated by addition of 1 unit Proteinase K (Fermentas) to each of the 50 $\mu$ l PCR reactions and incubation for 1 hour at 37°C followed by Proteinase K deactivation incubation for 20 min at 95°C. Twenty-five  $\mu$ l of the Proteinase K-treated PCR products was digested with restriction enzymes, 10 U/ $\mu$ l NlaIII (New England Biolabs) and 10 U/ $\mu$ l MboI (Fermentas) at 37°C for 2 hours. The top strand of the restriction enzyme digested uracil containing PCR products were then degraded by addition of 1 U uracil-DNA glycosylase (Fermentas) to each reaction and incubation for 30 min at 37°C followed by heating to 95°C for 20 min.

Fifty  $\mu$ l ligation mixture was added to each reaction chamber (containing the microarray probes) on the microarray, containing 200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM MgCl<sub>2</sub>, 5 mM NAD, 0.1 % Triton X-100, 2  $\mu$ g/ $\mu$ l BSA, 0.1 U/ $\mu$ l Ampligase (Epicentre) and 10  $\mu$ l UNG-treated PCR products. The ligation reactions in the reaction chambers were then incubated for 1 hour at 55°C. The ligation mix was washed away by flushing the wells with 1XTNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20) to avoid contamination. The slide was then washed again with 1XTNT buffer followed by a second wash with 0.1XSSC (15 mM NaCl, 1.5 mM monosodium citrate). The slide was dried with the use of a centrifuge (Arrayit<sup>®</sup> microarray high-speed centrifuge). After the washing, 50  $\mu$ l RCA mixture was added to the reaction chambers. The RCA mixture consisted of 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1 % (v/v) Tween 20, 10 mM DTT, 1  $\mu$ g/ $\mu$ l BSA, 100  $\mu$ M dNTP, 10 units Phi29 DNA polymerase (Fermentas). The slide was then incubated for 30 min at 37°C. The slide was then washed with 1XTNT buffer followed by a second wash with 0.1XSSC.

The slide was dried with the use of a centrifuge (Arrayit<sup>®</sup> microarray high-speed centrifuge). Ten nM detection oligos (Table 2) in 50  $\mu$ l 1XTNT were then hybridized to the RCA products at 55°C for 1 hour. The slide was scanned with a GenePix 4000B microarray scanner and the picture was processed by GenePix Pro 6.0 software.

## Oligonucleotide microarray manufacturing

A Piezorray system (PerkinElmer Life and Analytical Sciences, Inc. MA, USA) was used to manufacture the microarrays. Sixteen subarrays were printed with 9 mm spacing along the x- and y-axis. A silicone rubber mask was used to separate the subarrays from each other during the enzymatic and hybridization reactions [12]. Each subarray was printed with oligonucleotides in triplicates. The microarrays were printed on Codelink slides from GE healthcare Europe, GmbH, Germany. The slides were then incubated in a humidity chamber (containing distilled water) followed by a 5 min blocking step in blocking solution at room temperature. The blocking solution contains 100 ml PBS, 20 % ethanol, 0.05 M NaBH<sub>4</sub>. The blocking solution was washed away with water and the slides were dried with the use of a centrifuge (Arrayit<sup>®</sup> microarray high-speed centrifuge).

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