UPTEC X11 036
Examensarbete 30 hp
November 2011

Optimization of proximity ligation assay based Western blotting

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UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 11 036	**Date of issue 2011-11**
Author **Johan Johansson**	
Title (English) **Optimization of proximity ligation assay based Western blotting**	
Title (Swedish)	
Abstract Many of today's methods for the detection of biomolecules suffer from a high limit of detection due to poor signal generation upon recognition of target. By applying and optimizing proximity ligation assay (PLA) in Western blotting (WB), the limit of detection has been lowered down to the picomolar range. In this report I have optimized the different parameters that affect the signal generation and explored possibilities to increase the ease of use, by merging protocol steps and performing signal generating reactions at room temperature.	
Keywords Western blotting, proximity ligation assay, signal amplification, specificity, optimization	
Supervisors **Åsa Hagner-McWhirter, GE Healthcare**	
Scientific reviewer **Anna Edman-Örlefors, GE Healthcare**	
Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **30**

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Populärvetenskaplig sammanfattning

I dag kan många sjukdomar botas redan innan de brutit ut. Genom att dra nytta av så kallade biomarkörer, molekyler som kan visa på en specifik sjukdom så kan man starta en behandling före symptom uppstår. Det är dock svårt att diagnostisera med hjälp av biomarkörer. Detta då det finns väldigt lite av dem, samt att det är svårt att specifikt detektera en viss sort av dem.

Genom att utnyttja immunförsvarets egna sätt att söka upp fientliga organismer i kroppen, med hjälp av antikroppar, kan en molekyl fås som binder till det man söker. För att åskådliggöra biomarkören måste något fästas på antikroppen som kan signalera, detta kan till exempel vara en självlysande molekyl. Oftast behövs det många sådana molekyler på samma ställe för att man ska kunna se signalen. Eftersom det finns lite av biomarkören, och antalet signalmolekyler som kan fästas på en antikropp är begränsat, blir det svårt att upptäcka en signal. Genom att utnyttja Western blotting (WB) metoden för att separera biomarkören från andra proteiner, och därefter använda proximity ligation assayens (PLA) signalförstärkning, kan man detektera väldigt små mängder av biomarkören.

I den här rapporten har jag optimerat protokollet för kombinationen av dessa metoder.

Examensarbete 30 hp
Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet juni 2011

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1. Introduction

Proteins are involved in almost every aspect of life. They are part of supplying cells with nutrients, protecting the body against infections, maintaining homeostasis or coordinating cellular response to environment changes and more. To produce proteins the cell interprets its genetic code, the DNA, located in the nucleus. The DNA consists of exons and introns. Exons are often called the coding part of the DNA since it is the genetic code in the exons that is used to produce proteins. The DNA is translated into messenger RNA (mRNA) which is processed; specific exons are chosen and fused together, this process is called alternative splicing. The mRNA is transported out from the nucleus and interpreted by the ribosome which links together amino acids corresponding to the sequence of the mRNA. After transcription the protein folds to its specific shape and post-translational modifications (PTMs) can be made (figure 1) [1].

The study of proteins, proteomics, is of great interest to scientists. By understanding protein expression under different conditions, part of the functions of the protein might be revealed. The information gained can be used in many applications such as diagnosis, curing diseases, bio-pesticides, reducing recovery time after surgery, processing waste products and more.

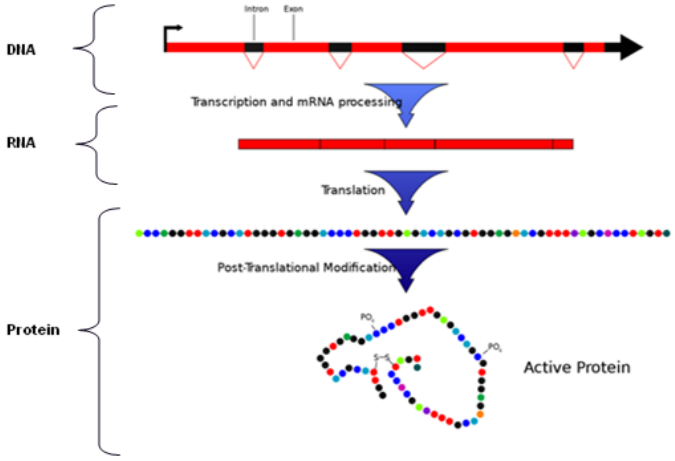

Figure 1. From DNA to protein. The DNA located in the nucleus is translated into mRNA and exons are fused together. The mRNA is then transported out of the nucleus to the ribosome that transcribes the mRNA to protein by joining amino acids together corresponding to the sequence of the mRNA. The protein is then folded into its specific shape and PTMs are made.

Shortage of protein, or its derivates, is causing many diseases. Type 1 diabetes mellitus is caused by a lack of insulin. Insulin is a 5.8 kDa peptide hormone that is secreted as a response to food intake by promoting the uptake and storage of glucose as glycogen in muscle, liver and fat cells [2]. Insulin is secreted by the cells in the islets of Langerhans and deficiency of it results in high glucose level in the blood stream and, if not treated, coma and eventually death.

In the example above, the protein is either there, or missing which results in a healthy or an unhealthy patient. But some diseases are caused by removal of parts of a protein or point mutations which exhibits in a non-functional protein. X-linked severe combined immunodeficiency (SCID-X1) results in a γc receptor deficiency by either a point mutation or a skip of exon 6 by a frame shift mutation of the genetic code for the receptor [3]. SCID-X1 leads to an early block in T and NK lymphocyte differentiation and is a lethal condition.

In a case, such as the insulin described above, a qualitative answer would be sufficient. Is the protein there or not? The applicable methods for a question as the one above are many, but the common procedure is to first separate the proteins in the sample and then detect the protein of interest. Gel electrophoresis is one of these methods, it separates the proteins according to their sizes and has been routinely used in laboratories for decades. Even when searching for a truncated protein, most of those methods can be applied if you compare your sample against a control. This because there will be a mass difference which is easily distinguished with today's methods. But if the only difference between a functional protein and a non-functional is one mutation, it might be hard to discriminate between the twos since they will share many protein properties *e.g.* molecular weight, epitopes and more. It gets even more complex since it is usually not enough to get a qualitative answer. Protein can be present under many conditions but expressed in different amounts. It is often those regulations that gives the key to the function of the protein, or the diagnose of the disease. Therefore one must apply methods that can quantify the amount of protein present in a sample.

The desire to quantify the contents of a sample is not limited to proteomics or diagnostics. When producing pharmapheutical substrates it is often regulated by laws to achieve a certain level of purity and concentration before a drug can be given to patients [4]. To quality secure the product workflow from start material to finished product, it is important to, during the process, always keep the different parameters constant or in a safe intervals. Therefore it is of great interest to analyze the product and impurities, both in between steps and as a finished product, with powerful detection and quantification methods.

1.1 Detection and quantification of proteins

To be able to distinguish different proteins from each other, many methods can be applied. Even though the applicable methods can differ much in throughput, dynamic range, limit of detection and reproducibility, the parameters they use to separate the sample is limited. Here one must look for differences in protein properties. Size is the most common parameter used

for separation of proteins, using methods such as gel electrophoresis, gel chromatography, HPLC and more. The sample is pushed trough a maze, consisting of *e.g.* cross-linked polymers or beads, by an applied force *e.g.* an electric field, gravity or centrifugal force. The mobility in the maze will be affected by the protein's size and thus a separation according to size will occur. Many other features of proteins can be exploited *e.g.* isoelectric point, charge, solvability, hydrophobicity and amino acid composition. It is also possible to combine methods based on these parameters to get a better separation.

To confirm that the separation was successful, the proteins must be detected. When separating proteins we looked at features that differ from one protein to another, but when detecting them we want to take advantage of the common characteristics to get a proportional signal to amount of detected protein. This is usually done by targeting the backbone of the protein or common amino acids with a dye. When choosing a method to visualize the proteins, it is desirable to have as broad dynamic range as possible, meaning you want to be able to detect both high and low abundant proteins at the same time. For quantification purposes, the signal should be proportional to the amount of protein you are detecting.

Another factor that is important is the limit of detection, which is the least amount of your target that you can detect. Standard methods that have been used for decades are Coomassie blue and silver staining. Both methods are convenient to use when searching for a qualitative answer for an abundant protein, but the capability to detect low amounts, or quantify the obtained data, is limited. Instead one can use methods based on fluorescence which still is convenient to use but has a much lower limit of detection. Fluorescence based detection is also quantifiable over a wide amount of protein. Sometimes it is desired to filter away the unwanted signals, meaning that it can be enough to just visualize the proteins you are interested in. By targeting specific proteins with antibodies conjugated with a reporter, only the targets, which the antibody binds to, will be detected. Methods that utilize antibodies to detect and generate a signal are called immunoassays. By only visualizing the desired protein, the demands on separation methods decreases since only the protein of interest will be detected. Therefore unwanted proteins will not give rise to a signal even if they are not distinguishable from each other. To successfully quantify the target, it is of importance to have a high specificity and sensitivity. Strong specific signal amplification is also desired to easily distinguish the true signal from the background and noise, especially for low amounts of target. Figure 2 shows an example of how a signal can look like for different amount of target.

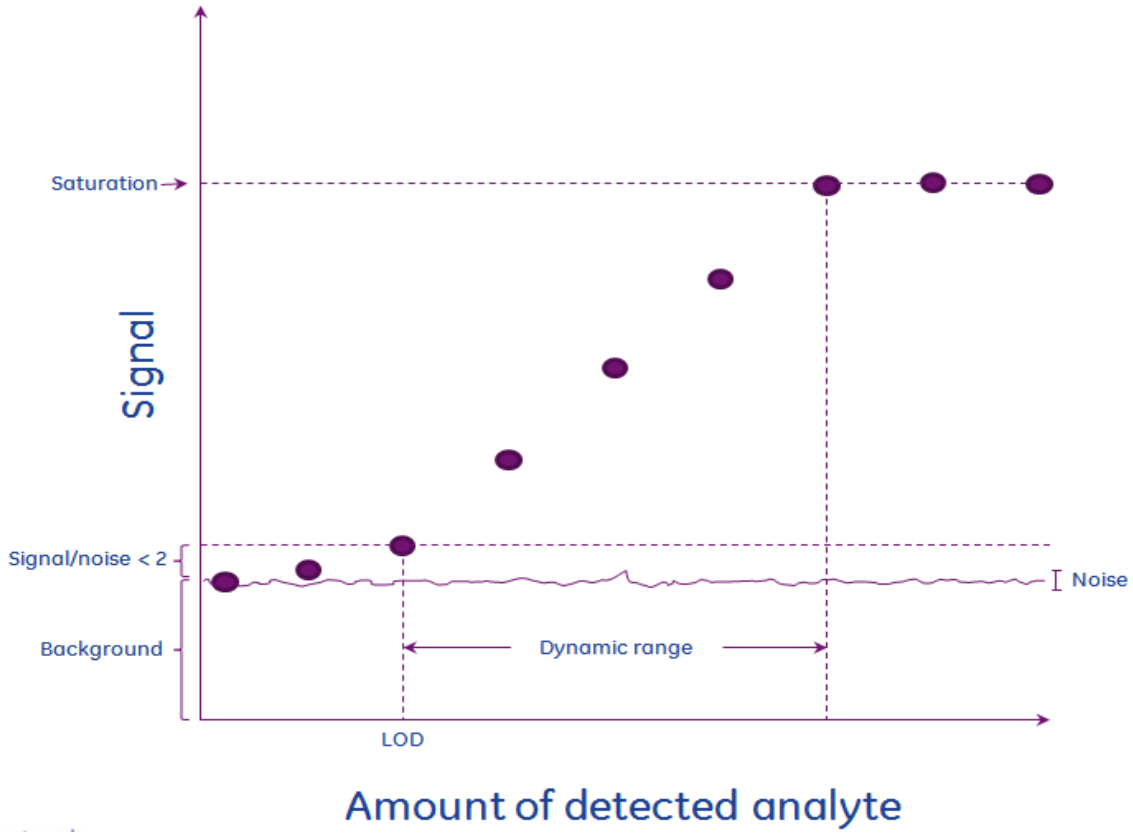

Figure 2. The characteristics of a detection curve. The limit of detection (LOD) is defined as the analyte concentration for which the signal is more than 2-3 standard deviations for the noise above the background. Saturation occurs when a higher analyte concentration does not give a higher signal due to incapability of the detection system to capture the signal. While the dynamic range is the range in between the LOD and saturation were the signal is linear proportional to the amount of target.

1.2 Imaging

The common characteristics for imaging systems are the capability to capture, quantify and digitalize the signal. This can be as simple as taking an image of the sample in black and white and quantify the sample by measuring grey-scales. But other more sophisticated detection systems utilize the photoelectric effect were photons emitted by the reporter are converted to electrons, amplified and quantified by the voltage peak generated [5].

1.3 Western blotting

Since its invention 1981 [6] the Western blotting method has been widely and routinely used. It takes advantage of both a separation of size or/and isoelectric focusing and specific detection of targets with antibodies. Western blot enables at best detection of a specific protein down to a few pg in a complex sample. Once the separation has occurred the proteins are transferred to a membrane and immobilized. The membrane is then probed with primary antibodies against the protein of interest. Once the primary antibodies have bound, a secondary antibody directed to the constant region of the primary antibody is added. The

secondary antibodies are conjugated with a reporter which enables detection of the target (figure 3). There are two types of reporters that are used for detection: indirect and direct reporters. Indirect reporters such as horseradish peroxidase (HRP) and alkaline phosphatase (AP) produces a light emitting product when a suitable substrate is added. The light is collected either by exposure to a photosensitive film or by a CCD based camera. Once the emitted light is collected, the signal is digitalized and quantification can be made. Indirect reporters are stable over a longer time and can be: fluorophores or radioactive labeling. To collect the signal from fluorophores, it must first be excited. This is done by using a laser with a suitable wavelength to excite the fluorophore. The fluoroephore will then release a photon with a different wavelength than the incident light. This signal can then be collected and quantified using image analysis software.

Only a certain number of primary antibodies can bind to the target, and only a ceratin number of secondary antibodies can bind to the primary antibody due to sterical hinders and the affinity for the antigen. Even if it is possible to conjugate quite many reporters to an antibody, affinity decreases due to possible conjugation to antigen binding site and quenching for pluorephores often limits the numbers of reporters/antibody. Having the reporters on a secondary level also boost the signal amplification since for every binding event at primary level, more than one binding event on secondary level can occur. Ready to use reporter conjugated secondary antibodies, often directed to mouse or rabbit primary IgG provides target flexibility and ease of use. There are also kits for labeling primary antibodies with reporters. This is suitable for higher abundant targets, since antigen affinty may be affect by

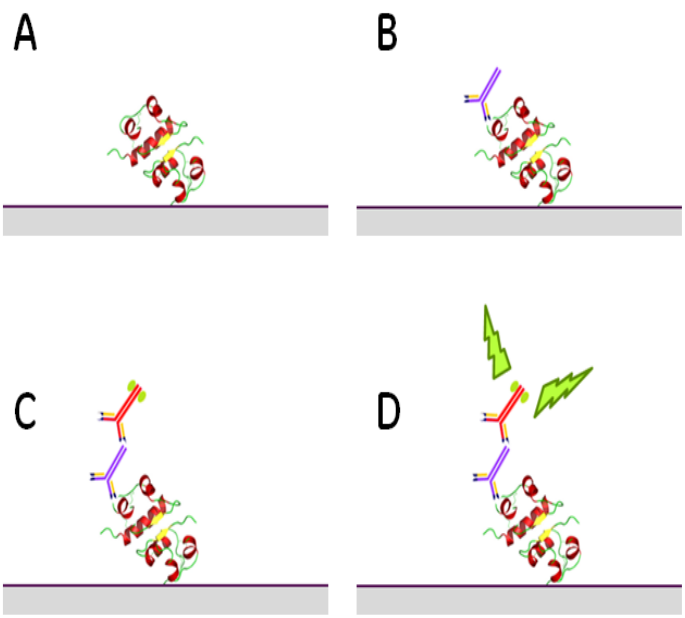

Figure 3. Schematic overview of traditional Western blotting. A) After separation by gel electrophoresis the sample is immobilized on a PVDF membrane. **B)** Antibodies specifically directed towards the protein of interest are added. **C)** Species specific antibodies conjugated with a reporter molecule, often polyclonal anti-IgG from goat, are added. **D)** The reporter is detected with a suitable method and the signal is quantified.

conjugation.

1.4 Proximity ligation assay

The proximity ligation assay (PLA) was first described in 2002 [7]. To give rise to a signal, the PLA requires two independent binding events of antibodies to the target. By the usage of two different oligonucleotides conjugated to these antibodies, targeting different epitopes in close proximity on the target a high specificity is achieved. The conjugated oligonucleotides are designed by the user or bought commercially. After the binding event of the antibodies a splint and a backbone oligo is added to the reaction mix. Upon binding of both antibodies with conjugated oligos in close proximity, the backbone and splint will hybridize to the antibody conjugated oligo and form a circle. Once the circle is formed T4 DNA ligase is added to ligate the splint with the backbone oligonucleotide. Once the nicks in the circular DNA are repaired, phi29 DNA polymerase is added. The phi29 binds to the circle and starts amplification (RCA) of the backbone and splint circle. The product, which consists of around 1000 copies of backbone and splint, will form a sphere of single stranded DNA (ssDNA). Detection reporters such as fluorophores or HRP, complementary to part of the sequence of the RCA product, are added to hybridize to multiple sites of the ssDNA. The fluorescent or the chemiluminiscent signals can be detected using an imager (figure 4) [8].
It is hard to produce specific antibodies since protein usually share epitopes between them and

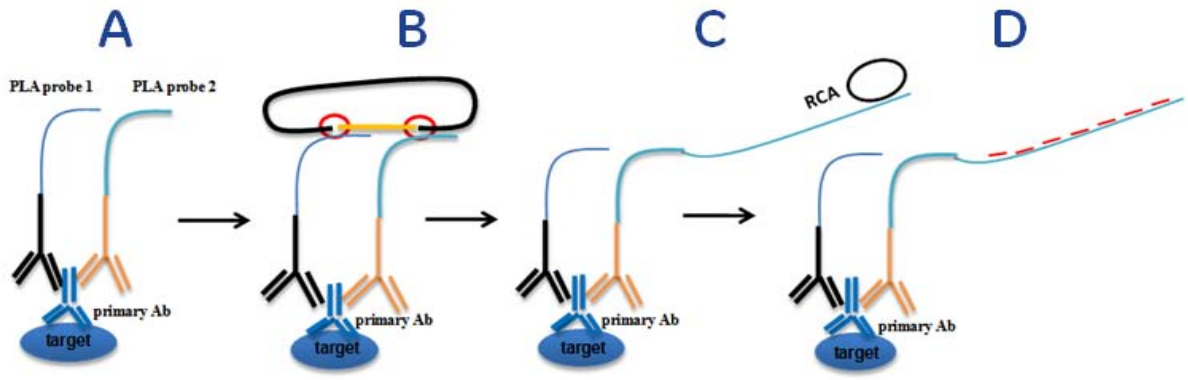

Figure 4. Protocol steps of RCA in PLA. A. Once the added primary antibodies have bound to the target, secondary PLA probes are added which binds to two different epitopes on the primary antibody. **B.** Two oligos (backbone and splint) designed to hybridize to both of the PLA probes are added, and forms circular DNA. T4 DNA ligase is added to repair the nicked DNA (indicated with red circles). **C.** Phi29 DNA polymerase binds to the DNA circle and start RCA, which results in 1000 copies of backbone and splint. **D.** Reporter conjugated oligos complementary to the RCA product hybridizes to the DNA which can be detected with suitable methods.

the preparation used to purify the antibodies does not remove all impurities. Therefore monoclonal and polyclonal antibodies can give rise to unspecific signals. Since PLA requires two independent binding events to give rise to a signal, the specificity is enhanced compared to conventional immunoassays. The possible number of reporters per target is also increased due to the usage of RCA. The drawbacks of PLAs signal amplification compared to methods that uses conjugation of reporters to antibodies are that the PLA protocol requires more steps, takes longer time and the total cost per assay is higher due to the use of expensive phi29 and other reagents needed.

1.5 Purpose

The purpose of the work is to apply the strong specific signal amplification of the PLA assay in a Western blotting environment. This in order to take advantage of Western blotting's molecular weight (Mw) or isoelectric point (pI) separation prior to detection. To maximize the signal amplification, and minimize the background in the readout signal, different parameters for the PLA assay was optimized in a Western environment. With a combination of optimal parameters, the assay's reproducibility and robustness is increased when working in safe parameter intervals with minimal variation. Also, minimal reagent consumption for lowest possible assay cost will be investigated in this report. For ease of use reasons, possibilities for reduction of the time, temperature and number of steps was analyzed.

2. Materials and Methods

2.1 SDS-PAGE electrophoresis and membrane blotting

Six different amounts of Transferrin, Apo-Human Plasma (Calbiochem®) corresponding to 2.5 ng, 1.25 ng, 78 pg, 39 pg, 2.4 pg and 1.2 pg dissolved in 2 x SLB (0.125 M Tris-HCl, pH 6.8, 4 % SDS, 17.4 % Glycerol, 0.2 mg/ml Bromophenol blue, 31 mg/ml DTT) was loaded on to Novex® 12 % Tris-glycine pre-cast gel (Invitrogen) together with ECL Plex rainbow markers. The electrophoresis was run in MiniVE vertical Electrophoresis system (GE Healthcare) at 100 V until the bromophenol blue reached the bottom of the gel. Once the electrophoresis was finished, the gels were equilibrated in transfer buffer (1 x Tris-glycine, 20 % Methanol) for 15 minutes before transfer onto Hybond-P (for ECL readout, GE Healthcare) or a Hybond-LFP (for fluorescence readout, GE Healthcare) PVDF membranes. Transfer was performed using the TE 22 Mini tank transfer unit (GE Healthcare) at 25 V and 4°C for 2.5 h. The blotted membrane was cut in to an approx. 1x5 cm area containing the 72 kDa transferrin protein guided by the Mw marker bands.

2.4 Traditional Western Blotting

The blotted and cut membrane was blocked in 3 % (w/v) BSA (Sigma Aldrich) and PBS buffer with 0.1 % Tween20 (PBST) for 1 h at room temperature (RT) with gentle orbital rotation. The membrane was rinsed twice and washed for 2x5 min in PBST. Next the membrane was incubated with primary polyclonal rabbit anti-human Transferrin (DakoCytomation) over night at 4°C diluted 1:750 in PBST. The next day the membrane was rinsed twice and then washed for 2 x 5 min in PBST. After the washing, ECL Plex goat anti-rabbit IgG, Cy5 (GE Healthcare) at a dilution of 1:2500 in PBST was added to the membrane for fluorescence readout. For ECL readout HRP conjugated donkey anti-rabbit IgG were added at a 1:30000 dilution (GE Healthcare) for 1 h incubation at RT with gentle orbital shaking. After four brief rinses and 4x5 min washing in PBST the membrane was rinsed four times in PBS without Tween20 and finally dried over night at RT protected from light before further analysis.

2.5 Proximity ligation assay based Western Blotting

The blotted and cut membrane was blocked in PLA membrane blocking solution (3 % (w/v) BSA (Sigma Aldrich), 0.1 % Tween20, 100 µg/ml Salmon Sperm DNA (Fermentas) in TBS buffer) at gentle orbital rotation for 1 h at RT. The blocked membrane was incubated with the same primary antibody as in traditional Western blotting above, at a range of tested dilutions (1:750- 1:5000) over night at 4°C. The next day the membrane was first rinsed in TBS with 0.1% Tween20 (TBST) and then washed for 2 x 10 min in TBST. After the wash, the membrane was incubated with Duolink ® II PLA Plus probe (OLINK Bioscience) which consists of anti-rabbit IgG antibody conjugated with an oligonucleotide together with Duolink ® II PLA Plus probe (OLINK Bioscience) which consists of anti-rabbit IgG antibody conjugated with another oligonucleotide for 1 h at RT with gentle rotation. The concentrations of detection probes were: 0.2-1 µg/ml for fluorescence readout and 0.05-0.2 µg/ml for ECL readout in PLA probe diluent buffer (0.5 mg/ml BSA, 5 µg/ml salmon sperm DNA, 5mM EDTA, 0.05 % Tween20 in TBS buffer). The membrane was then briefly rinsed and washed for 2 x 10 min. Next, T4 DNA ligase (Fermentas) diluted to a range of tested concentrations (0.01-0.03 U/µl) in oligo ligation buffer (250 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.5 mM ATP, 0.25 mg/ml BSA, 0.05 % Tween20, pH 7.5) together with a ranged of tested concentrations (1-90 nM) of backbone oligonucleotide (5'-phosphate-CTA TTA GCG TCC AGT GAA TGC GAG TCC GTC TAA GAG AGT AGT ACA GCA GCC GTC AAG AGT GTC TA, Integrated DNA Technologies) and 1-90 nM of splint

oligonucleotide (5'phostphate-GTT CTG TCA TAT TTA AGC GTC TTA A, Integrated DNA Technologies) at gentle orbital rotation for 40 min at 37°C. Next the membrane wash rinsed and washed for 5 min in TBST. After the washing the membrane was incubated with phi29 DNA polymerase (GE Healthcare) diluted to a range of tested concentrations (0.01-0.125 U/µl) in rolling circle amplification (RCA) buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM of dNTPs (dATP, dTTP, dCTP, dGTP), 0.25 mg/ml BSA, 0.05 % Tween20, pH 7.5) for 1 h at 37°C. After two rinses the membrane was incubated with detection probe (5'-AAA AAA AAA CAG TGA ATG CGA GTC CGT CT) conjugated with either HRP enzyme (Biomers) at a 5 nM concentration or Cy5 dye (Integrated DNA Technologies) at a range of tested concentrations (5-45) nM in detection buffer (2xSSC, 0.5 mg/ml BSA, 2 µg/ml salmon sperm DNA, 0-5 % formamide, 0.05 % Tween20) for a range of tested incubation times (10-30 min) at 37°C. As a last step, the membrane was rinsed twice and washed for 3 x 10 min in TBST and finally rinsed in TBS to remove tween20.

Wash buffer B (100 mM NaCl, 35 mM Tris, 165 mM Tris HCl, pH 7.5) was used to wash the membrane after detection probe incubation instead of TBST in the background reduction experiment.

2.6 Detection of reporters

ECL detection – After the final rinse in TBS, the membranes were put on a clean plastic surface and ECL Plus (GE Healthcare) was added until it covered the whole membrane by surface tension. The membrane was incubated in the substrate for 5 min. Then the excessive substrate was removed by tilting and placing the membrane's edge on a paper tissue. The ECL signals on the membrane were detected by ImageQuant LAS 4000 (GE Healthcare) with 10 seconds increment setting up to 5 min.

Fluorescence detection – Fluorescence signals on dried membranes were visualized using the Typhoon FLA 9000 with laser and filters suitable for Cy5 detection. The PMT voltage was set so that the band corresponding to the highest amount of transferrin was just below saturation (100 000 counts/pixel)

2.7 Image analysis

The images generated from the different scanners were imported to the software ImageQuant TL 7.0 (GE Healthcare). To calculate the integrated intensity of a specific band, lane profiles were tightly drawn around the band and background subtraction was performed by using the rolling ball method with a radius of 200 pixels. Background measurements were done by

drawing a square in an area containing only membrane background and average pixel intensity was calculated.

3. Results

3.1 Primary antibody and PLA probe concentration

Fluorescence readout - To investigate the optimal amount of primary antibodies to achieve a strong signal in fluorescence detection (Cy5-detection probe), membranes incubated with primary antibody at 1:750, 1:2500 and 1:5000 dilutions were prepared as described above. From the integrated intensity plotted against the amount of target, one can see that the signal varied slightly between the different dilutions for fluorescence readout (Appendix) but the amount of primary antibody is in the range of the amount needed for traditional Western blotting [9]. Primary antibody in 1:2500 dilution gave the strongest signal but the highest background, and therefore a 1:750 dilution of primary antibody was used, which gave a moderate signal but lower background.

In order to determine the optimum PLA probe concentration, three membranes was prepared as written above. The different PLA probe concentrations used were: 0.2, 0.5 and 1 mg/ml. Here the signal increased with increased PLA probe concentration with 37 % per each additional 0.2 mg/ml increase of PLA probe (Appendix). The background was unaffected by the different concentrations used. Even though a high concentration of PLA probe resulted in a stronger signal 0.2 mg/ml PLA probe concentration was used for further fluorescence studies for cost reasons.

ECL readout - When using the same primary antibody dilutions for ECL readout (HRP probe), the signal: antibody trend is revealed compared to fluorescence readout. Here the 1:750 dilution gave the strongest signal, 1:2500 gave a moderate signal and 1:5000 gave a weak signal compared to the other dilutions that was tested. Usage of 1:5000 primary antibody dilution gave the lowest background, and both the 1:750 and 1:2500 dilutions gave similar, and compared to 1:5000, slightly increased backgrounds (Appendix). Since 1:750 primary antibody dilution gave the highest signal and a background comparable to 1:2500 this concentration was used in further experiment with ECL readout.

Chemiluminescence (ECL Plus) readout compared to fluorescence readout (Cy5) gave a much stronger signal and a lower limit of detection (Appendix). Therefore, further reduced PLA probe concentrations of 0.2, 0.1 and 0.05 mg/ml were used to see if the concentration could be

decreased without sacrificing signal. Here 0.2 and 0.1 mg/ml gave almost the exact same signal, while the use of 0.05 mg/ml PLA probe resulted in a much weaker signal (Appendix A). With the small decrease in background, PLA Probe concentration of 0.01 mg/ml was used for further experiments.

3.2 Background reduction

To gain a deeper understanding of the parameters that affects the background and the signal in the PLA-WB method, nine different PLA-WB membranes with 2.5 and 1.25 ng transferrin was prepared and tested under different conditions. The conditions suspected to affect the background were: no marker during the PLA-WB steps, no primary antibody added, primary antibody incubation together with 1 % BSA, increased salmon sperm DNA concentration in the detection probe buffer (10 µg/ml), 10 min total time for detection probe incubation, detection probe incubation at RT, 0.05 % Tween20 in wash buffer B (Olink) for washing after addition of Cy5 conjugated detection probes and reduced volume of wash buffer. The signal was visualized using the Typhoon FLA 9000 with PMT setting 700 V. As seen in figure 5, the

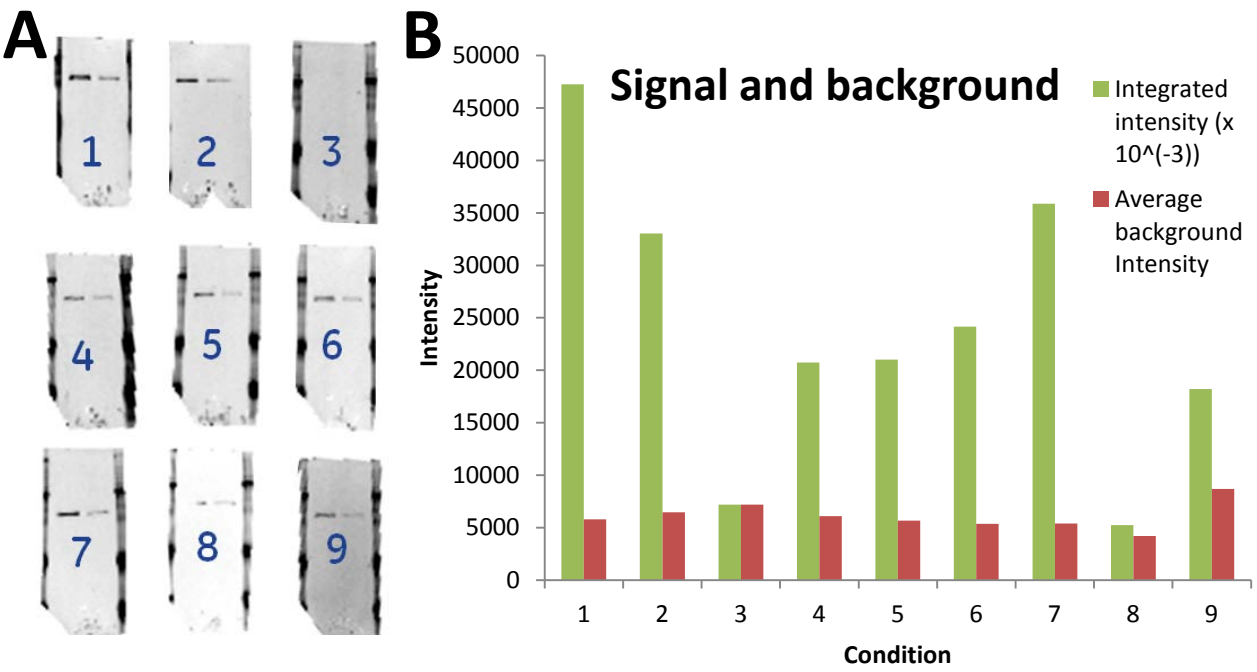

Figure 5. Signal and background intensities under different conditions. A. 2.5 and 1.25 ng transferrin were analyzed under different conditions: **1.** Control, **2.** No marker in the cut membrane, **3.** No primary antibody added, **4.** 1 % BSA added during primary antibody incubation, **5.** 10 µg/ml salmon sperm DNA in detection probe buffer, **6.** 10 min detection probe incubation, **7.** Detection probe incubation at RT, **8.** 0.05 % tween20 in wash buffer B, **9.** No excessive amount of wash buffer. **B.** Signal (integrated intensity) for 2.5 ng transferrin and background intensities under different conditions for condition 1-9.

main parameter that affected the background was the use of reduced volume of washing buffer. A high ion strength washing buffer together with Tween 20 resulted in a lower background but the signal intensity was substantially reduced.

3.3 Backbone and splint oligonucleotide concentration

For the PLA-WB method to give rise to a signal, a complete circle of DNA must be formed. The circular DNA consists of a splint and a backbone oligonucleotide. To determine if different splint and backbone concentration could have an effect on the signal and/or the background, 90, 30 and 10 nM splint and backbone concentrations of were tested using ECL readout. The signal was captured using the ImageQuant LAS 4000 (GE Healthcare) at 10 s exposure time, and the digitalized image was analyzed. Here I found unexpectedly that 10 nM backbone and splint oligonucleotide concentration gave the highest signal. The usage of 90 nM yielded a moderate signal and 30 nM resulted in the lowest integrated intensity. The background was slightly elevated for 10 nM and at similar level for 30 and 90 nM (figure 6). To confirm the trend with lower oligonucletide concentration that resulted in a stronger signal for fluorescence readout, four new membranes were prepared and splint and 90 nM, 10 nM, 5 nM and 1 nM backbone concentrations were tested. As seen in figure 7, a lower

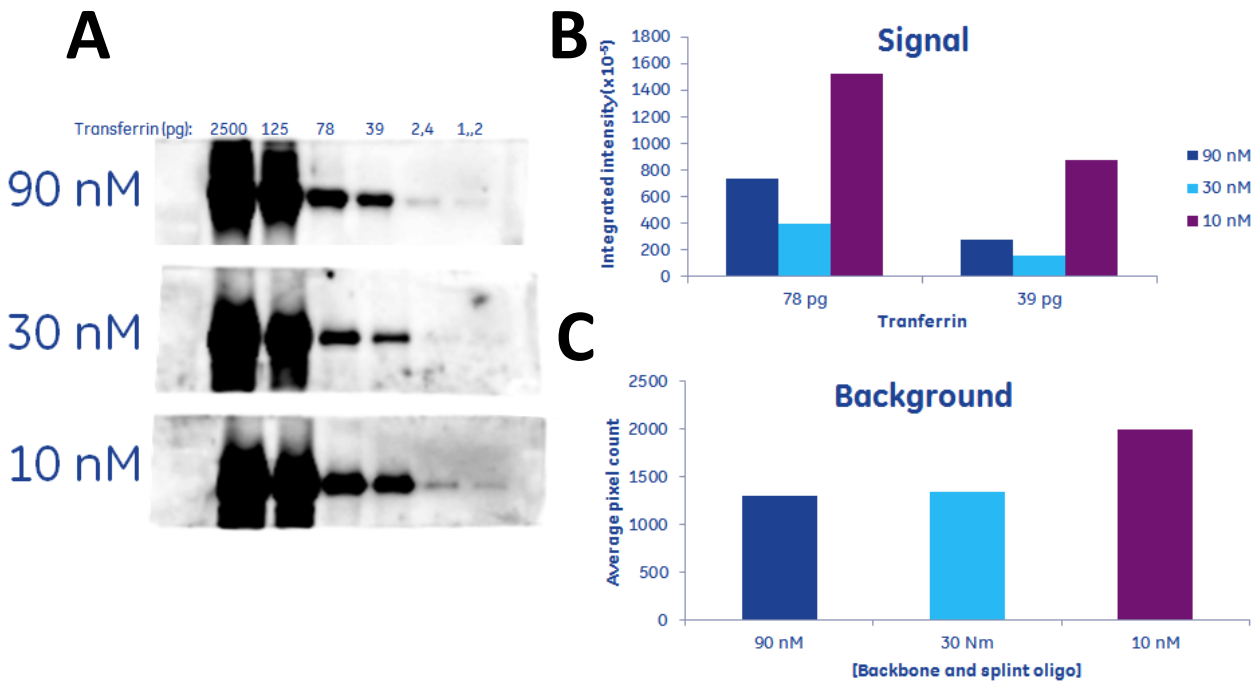

Figure 6. The effect of splint and backbone oligonucleotide concentrations with HRP conjugate detection probe. A. Signal from membranes treated with 90, 30 or 10 nM backbone and splint oligonucleotide captured by ImageQuant LAS 4000 at 10 s exposure time. **B**. Integrated intensities of the membranes plotted against the amount of sample. **C.** Average background for each membrane.

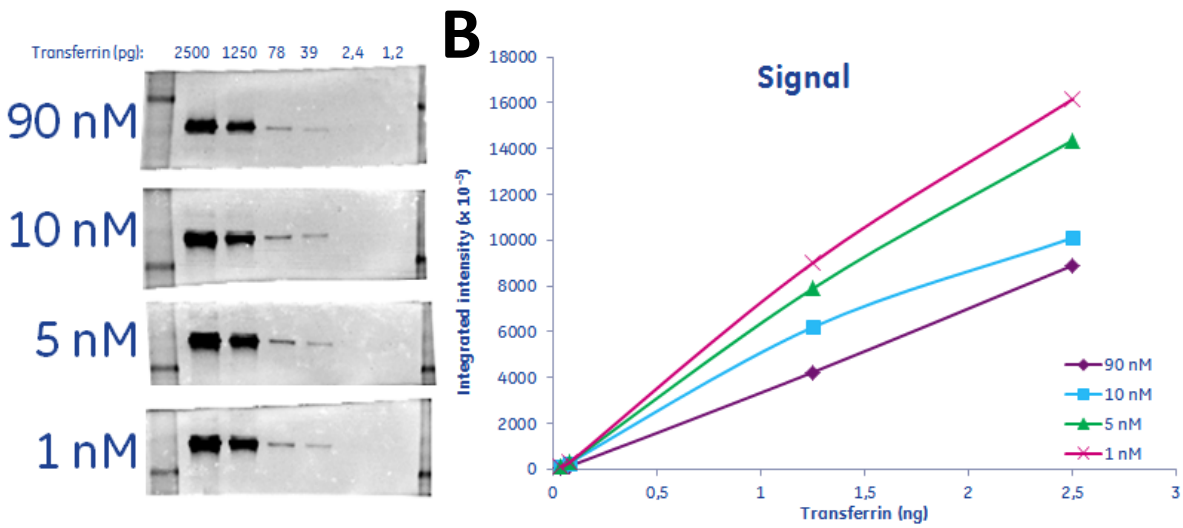

Figure 7. The effect of backbone and splint oligonucleotide concentrations using Cy5 conjugated detection probe A. Membranes treated with 90, 10, 5 or 1 nM splint and backbone oligonucleotide concentration. The image is generated by scanning with the Typhoon FLA 9000 at PMT voltage 650. **B.** Integrated intensities for the different membranes plotted against the amount of protein.

oligonucleotide concentration gave rise to a stronger signal for the abundant transferrin samples but for the lower amount of sample the relative signal increase with lower nucleotide concentration was reduced.

3.4 T4 ligase concentration

To investigate the potential to use a lower concentration of T4 DNA ligase, in order to reduce the cost per assay for PLA-WB, membranes were incubated with 0.01 and 0.03 U/µl were prepared. The usage of a third of the original concentration of T4 DNA ligase does not affect the signal intensity, as seen in figure 8.

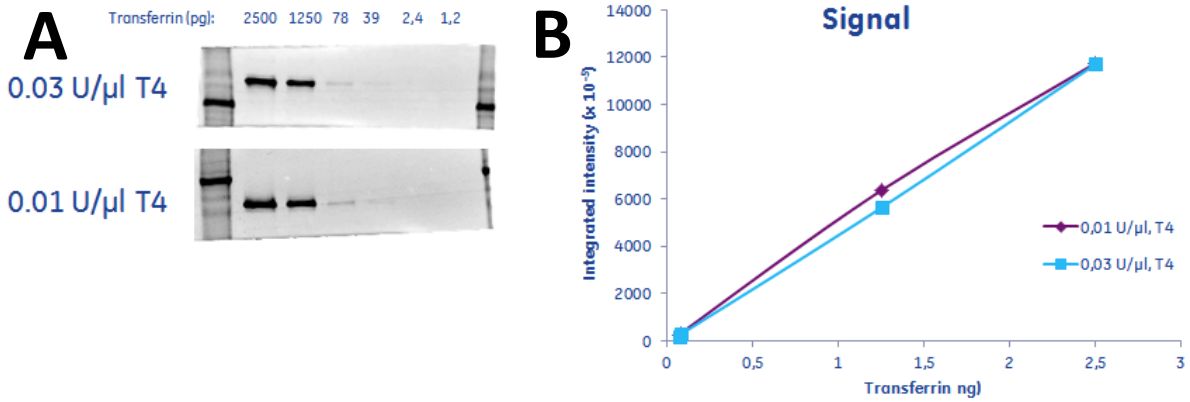

Figure 8. The effect of T4 DNA ligase concentration on integrated intensity. A. Membranes containing 2500, 1250, 78, 39, 2.4, 1.2 pg transferrin incubated with either 0.03 or 0.01 U/µl T4 DNA ligase during the ligation step. **B.** Integrated intensity for the four most abundant transferrin bands plotted against the amount of transferrin.

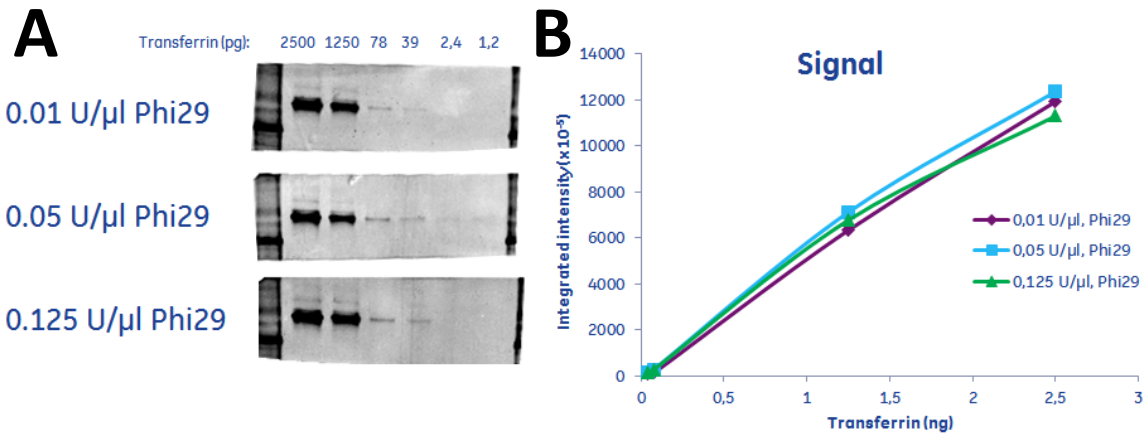

Figure 9. The effect of different ph29 DNA polymerase concentration. A. Membranes obtained using 0.01, 0.05 and 0.125 U/µl phi29 DNA polymerase during the RCA step in PLA-WB. **B.** Integrated intensity for each detectable band plotted against the corresponding mount of transferrin.

3.5 Phi29 polymerase concentration

To find out if it is possible to achieve a stronger signal or if the signal is maintained by using a higher or a lower concentration of phi29 polymerase, three different concentrations of phi29 DNA polymerase were used in PLA-WB. The concentrations evaluated were 0.01 U/µl, 0.05 U/µl and 0.125 U/µl. The membranes were then incubated with Cy5 conjugated detection probes, washed, dried and scanned at PMT voltage 580. The integrated intensities for the different amount of transferrin were plotted for each concentration (figure 9). The three phi29 concentrations resulted in almost the same signal intensity and similar backgrounds (figure 9A).

3.6 Detection probe concentration

In the pursuit of the optimum Cy5 conjugated detection probe concentration, PLA-WB was performed with three different probe concentrations, 45, 15 and 5 nM. The signal intensity and background for each membrane was analyzed. As seen in figure 10 the integrated intensity for the different concentrations are more or less the same for every membrane. However the background is affected significantly. There is a strong correlation between the concentration Cy5 conjugated detection probe used and the average pixel count in the background. The higher the detection probe concentration, the higher background. 5 nM resulted in quite low background, 15 nM moderate background levels and 45 nM had a quite high background. 5 nM was considered optimum.

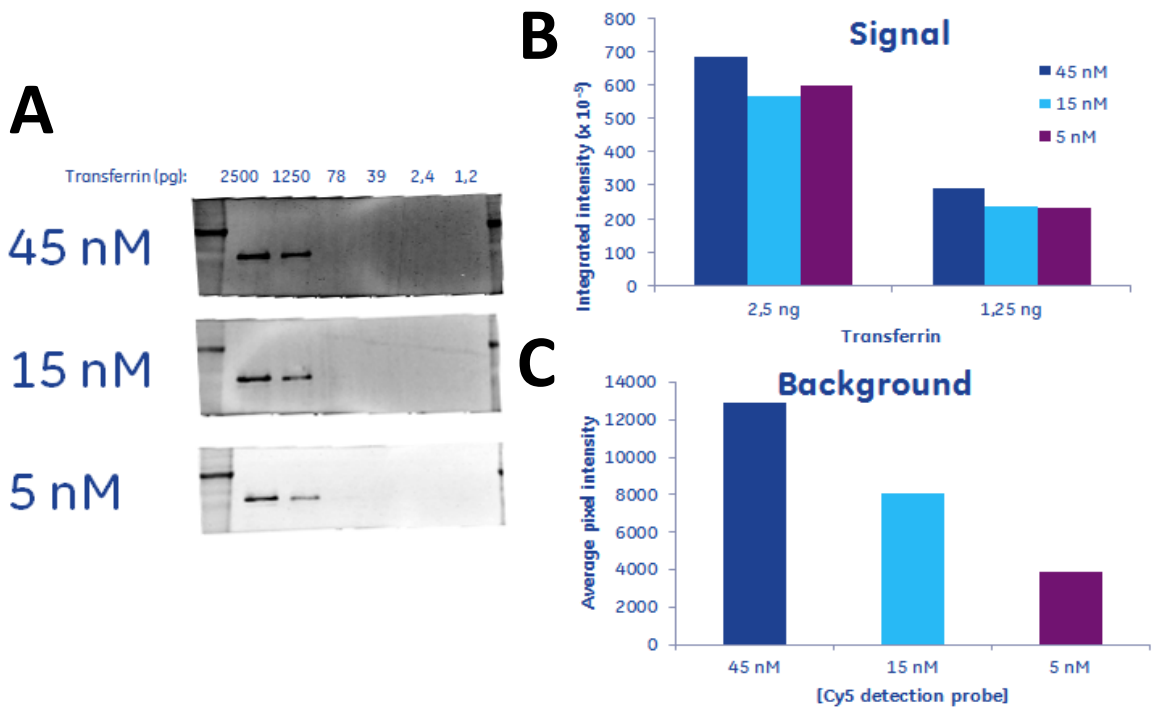

Figure 10. The effect of Cy5 conjugated detection probe concentration. A. Membranes incubated with 45, 15 or 5 nM Cy5 conjugated detection probe during the detection step. **B.** Integrated intensities for bands corresponding to 2.5 ng and 1.25 ng transferrin plotted against the amount of transferrin. **C.** Representative background for each different detection probe concentration.

3.7 Protocol optimization

To increase the versatility of PLA-WB both in terms of throughput and ease of use, some of the washing steps were shortened or merged without losing signal or gaining background (data not shown). To study the effect of PLA-WB when merging the RCA and the detection probe incubation steps, 5 nM HRP conjugated detection probe or 5 nM methylated HRP conjugated detection probe was added during the RCA step. When adding the ordinary detection probe during RCA the both signal and the background was substantially reduced. With the addition of methylated HRP conjugated detection probe, the signal was moderate and the background was quite high compared to a control (figure 11).

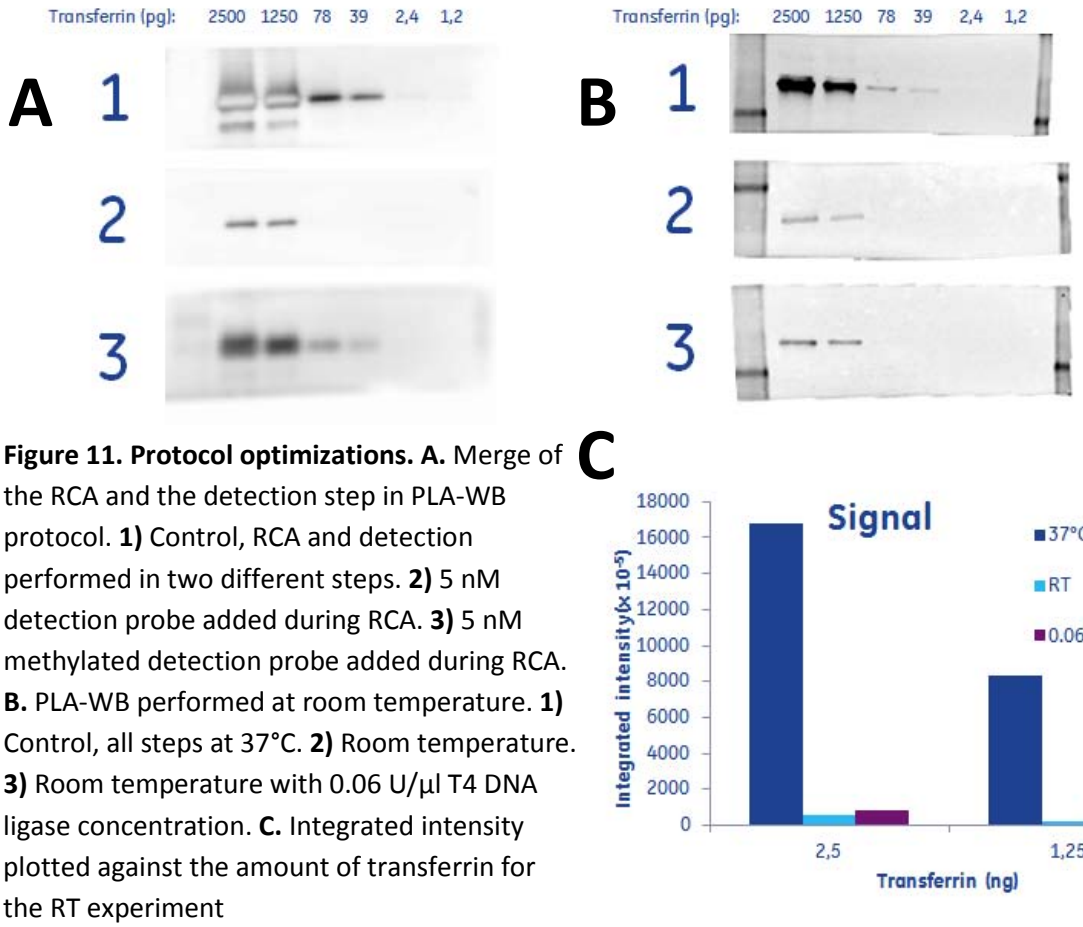

Figure 11. Protocol optimizations. A. Merge of the RCA and the detection step in PLA-WB protocol. **1)** Control, RCA and detection performed in two different steps. **2)** 5 nM detection probe added during RCA. **3)** 5 nM methylated detection probe added during RCA. **B.** PLA-WB performed at room temperature. **1)** Control, all steps at 37°C. **2)** Room temperature. **3)** Room temperature with 0.06 U/µl T4 DNA ligase concentration. **C.** Integrated intensity plotted against the amount of transferrin for the RT experiment

To test PLA-WB compatibility with instruments for automatic Western blotting probing, the whole reaction was performed at room temperature. To compensate for lower enzyme efficiency at RT, the concentration of T4 DNA ligase was increased to 0.06 U/µl and the incubation time for phi29 DNA polymerase was increased from 60 min to 90 min. As can be seen from figure 12 the signal was largely decreased for all membranes compared to the control. The extended RCA incubation did not significantly affect the signal (data not shown). With increased T4 DNA ligase concentration the integrated intensity was slightly increased compared to control at room temperature.

3.8 Scale up

To test if PLA-WB works at larger reaction volumes, the reaction volume was scaled up to 12.5 ml from 2 ml. The optimal concentrations of the reagents were used. Membranes were prepared with a full 2-fold dilution series of transferrin ranging from 5 ng to 0.63 pg. Figure 12 shows that the PLA-WB assays is functional at a larger scale than previously used. The signal intensity for PLA-WB is lowered compared to a small scale control but still stronger

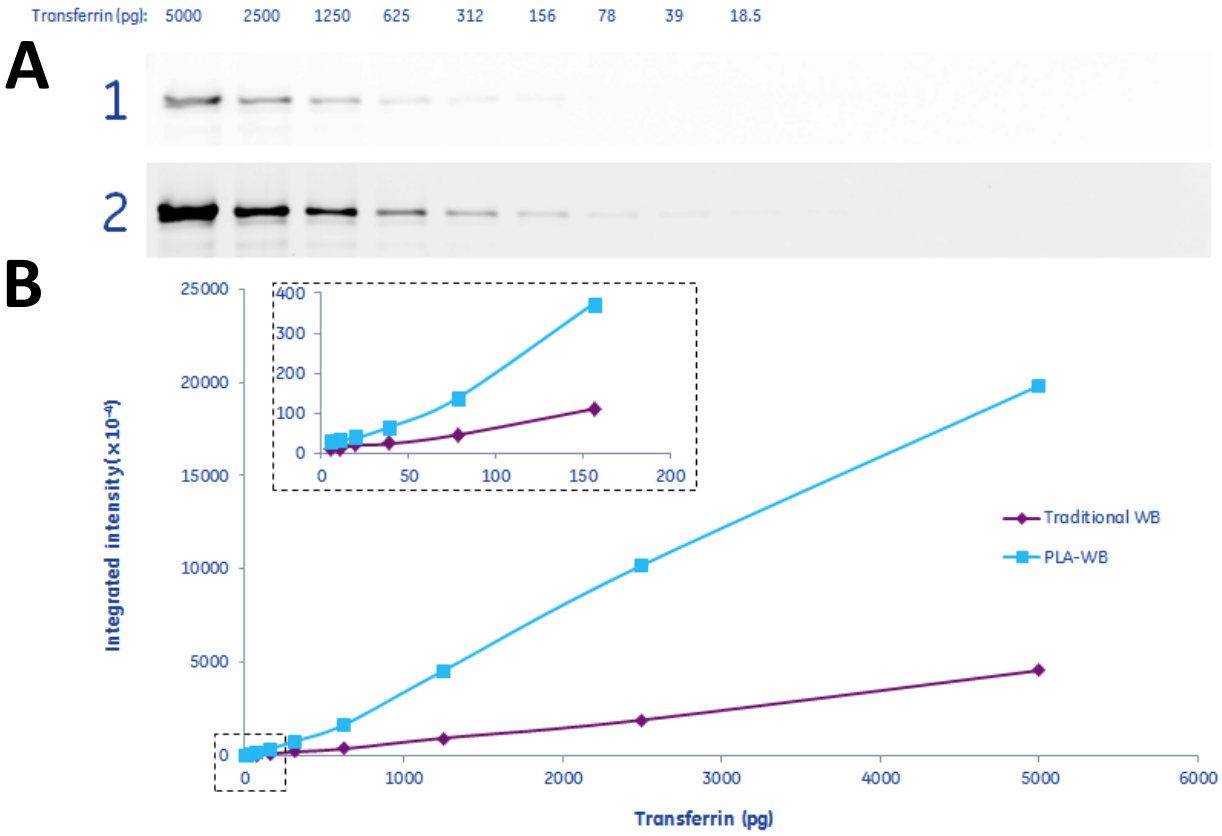

Figure 12. The effect of reaction volume. A. 2-fold dilution series of transferrin ranging from 5000 – 0.63 pg transferred to membranes and scanned. **1.** Detection by traditional WB using Cy5 conjugated secondary antibodies. **2.** Detection by PLA-WB with cy5 conjugated detection probes. **B.** Integration intensities for transferrin bands plotted against the corresponding amount.

than the ECL Plex control. The background is increased in PLA-WB compared to traditional WB.

3.9 Dynamic range and sensitivity of ECL readout for PLA-WB and WB

Once the optimum parameters for PLA-WB had been found, the dynamic range and the sensitivity was evaluated for PLA-WB by preparing a precast gel with a 2-fold dilution series of transferrin ranging from 2.5 ng to 80 fg. In order to detect the whole series simultaneously it was dived into three parts and each part was analyzed on a 1x5 cm membrane. The PLA-WB was performed with HRP conjugated detection probes and as a control traditional WB with ECL Plus was used. The signals were captured using the ImageQuant LAS 4000 with an exposure time of 10 s for both methods to be able to directly compare PLA-WB with WB. Due to substrate depletion and saturated bands only 625- 0.076 pg transferrin were analyzed. Compared to Traditional WB the integrated intensity of PLA-WB was around 80 times stronger, the dynamic range was shifted towards lower amounts of transferrin and the limit of

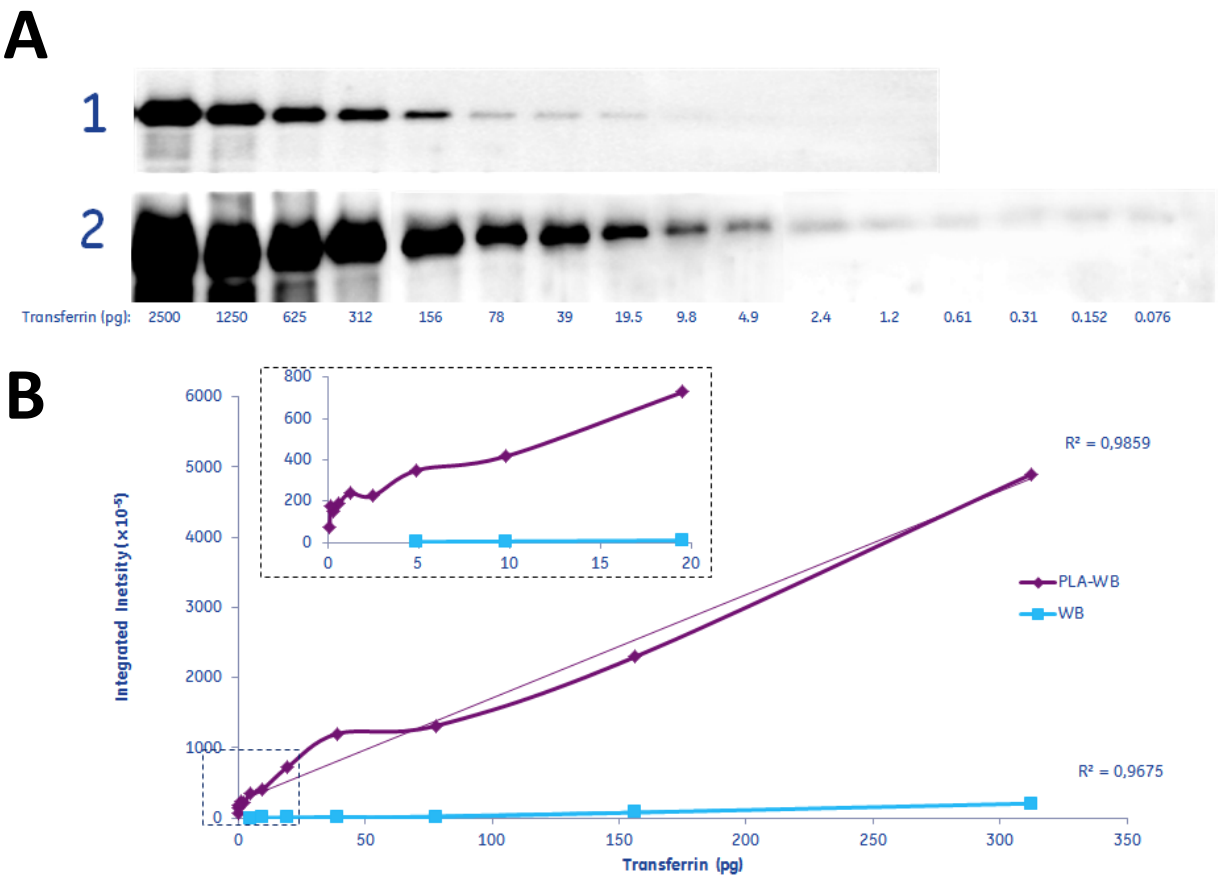

Figure 13. ECL detection of transferrin using traditional WB and PLA-WB. A. 2-fold dilution series of transferrin ranging from 2500 – 0.076 pg transferred to membranes. **1.** Detection using traditional WB and HRP conjugated secondary antibodies. **2.** Detection using PLA-WB and HRP conjugated detection probes. **B.** Integrated intensity for WB and PLA-WB plotted against the corresponding transferrin amount.

detection was better than any other Western detection system. With PLA-WB the LOD was 76 fg and with traditional WB a LOD of 9.8 pg was reached which corresponds to a 128 fold increase in LOD for PLA-WB (figure 13).

3.10 Signal amplification for fluorescence readout

To compare the signal generated by traditional WB and PLA-WB, membranes containing transferrin were prepared. Traditional WB and PLA-WB was performed according to the material and methods section. The membranes were scanned using a PMT based laser scanner at PMT voltage of 580. The digitalized images were analyzed with ImageQuant TL (figure 14). The integrated intensity was calculated and plotted against the amount of transferrin. Compared to traditional WB the signal from PLA-WB was by around 20 times stronger.

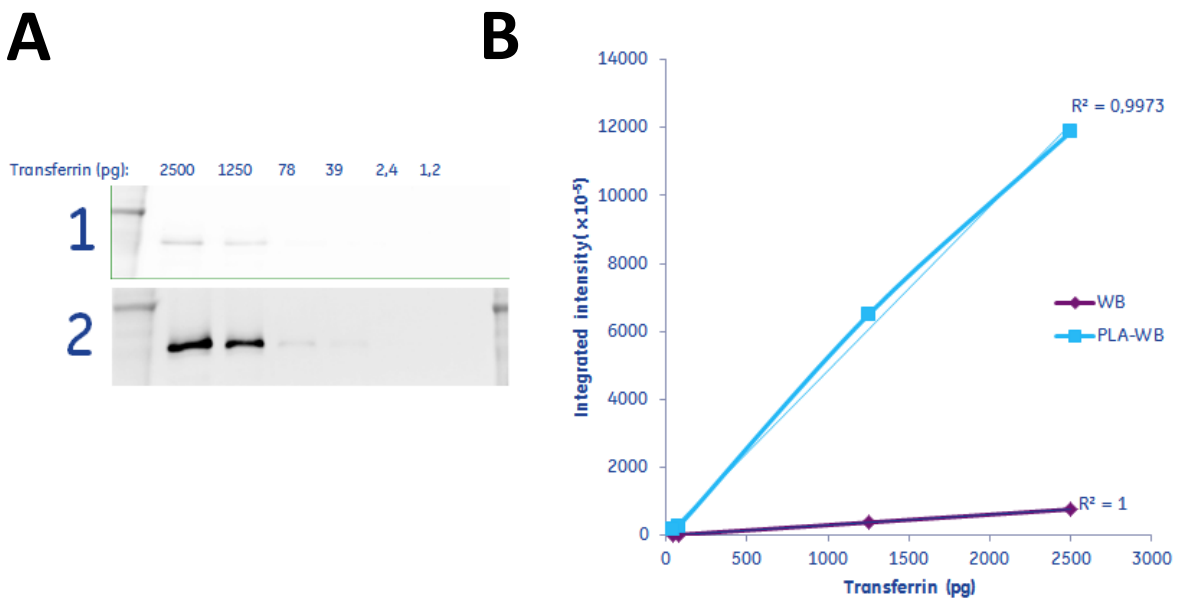

Figure 14. Cy5 detection of transferrin using WB and PLA-WB. A. Membranes containing different amounts of transferrin. **1.** Detection using traditional WB and Cy5 conjugated secondary antibodies. **2.** Detection using PLA-WB with Cy5 conjugated detection probes. **B.** Integrated intensity for WB and PLA-WB plotted against the corresponding transferrin amount.

4. Discussion

One of the biggest advantages with using dual recognition as a demand for signal generation, is not only the increased specificity but it also offers a lowered background by reducing signal from unspecific antibody adsorption to the membrane. By only giving rise to a signal when the plus and the minus probe are in close proximity to each other, the probability of unspecific signal due to PLA probe adsorption to the membrane is minimized. Other methods that take advantage of RCA for signal generation such as immunoRCA lacks this effective suppression of unspecific signal. ImmunoRCA only uses one oligonucleotide conjugated antibody, to which a circular DNA probe is added. The circular DNA is then amplified by RCA. This method will have comparable signal specificity to WB and PLAs signal amplification, therefore the background might be increased [10].

Herein I have showed that the WB-PLA reaction can be optimized in terms of costs and ease of use without sacrificing the linear dynamic range or the limit of detection. By optimizing the signal gained for PLA-WB, the signal: background ratio can be increased and thus less abundant protein bands can more easily be analyzed.

4.1 Reagent concentrations

Compared to already existing western detection systems such as ECL Plex and ECL Plus [9], it has been shown in this report that PLA-WB does not require more primary antibody. The PLA-WB signal is a much stronger signal for fluorescence and ECL readouts (figure 13, 14). By increasing the plus and minus PLA probe concentration further than investigated herein, one can achieve an even stronger signal for PLA-WB with Cy5 conjugated detection probes (appendix A).

The biggest contributor to the background during the PLA-WB protocol is the detection probe incubation (figure 10). Therefore the detection probe concentration needs to be optimized to achieve as low LOD as possible. The RCA product is a DNA sphere, which consists of around 1000 copies of backbone and splint oligonucletides [8]. Thus the number of possible hybridization spots for detection probes are limited. Because of this, the concentration, as shown in figure 10, can be lowered to 5 nM without losing any significant signal. By using 5 nM Cy5 conjugated detection probe, most of the available hybridization spots will be targeted. By increasing the detection probe concentration, a few more will find the corresponding sequence on the RCA product but more will be adsorbed to the membrane and cause an elevated background.

When comparing 90 nM and 1 nM splint and backbone oligonucleotide, the signal surprisingly increases with the lower amount of splint and backbone (figure 6, 7). The oligonucleotides are designed to hybridize and link the plus and the minus probe together, enabling formation of circular DNA. With high concentration of splint and backbone, the probability of two oligonucleotides of the same sort hybridizes to both the plus and the minus PLA probe increases and thus, no functional DNA circle can be formed which later gives rise to a signal. When comparing the signal increase between 90 nM and 1 nM for different amounts of transferrin, the relative signal increase is much higher for the high abundant target in contrast to low abundant. If an increase in signal for the high abundant target does not result in the same increase for the scarce targets, the detection system will be saturated and we will reduce DR. Consequently, it is of great value to further investigate the splint and backbone oligonucleotide concentration, since this will affect the linear dynamic range of the PLA-WB method.

I have also shown that the enzyme concentration can be decreased from 0.03 to 0.01 U/µl for T4 DNA ligase without losing any signal. The T4 ligase first binds to the nicked DNA strand

and links the two strands together, it then releases from the repaired strand to search for a new target. Therefore the end result will be corresponding to a both the incubation time and the concentration of enzyme. In figure 8 there is no signal difference for 0.01 and 0.03 U/µl which means that the incubation time is sufficient to repair most of the nicked DNA for both concentrations. With increased T4 ligase concentration, the incubation time can most likely be shortened due to the fact that more ligation events will occur for a given time. The phi29 DNA polymerase can also be decreased to 0.01 U/µl without affecting the signal. The phi29 binds to the 5' end of the plus PLA probe since the minus probe got a 5' phosphorylation which inhibits polymerase binding. Once the polymerase has bound, rolling circle amplification starts if there is a supply of dNTPs. Here the enzyme is bound to the original backbone and splint oligonucleotide complex for a longer time compared to the T4 ligase, which means that in order to get the optimum signal a crucial concentration has to be reached to saturate the available binding sites. Once this crucial concentration has been reached, the signal will not increase with increased phi29 concentration since all the binding spots are occupied. Figure 9 shows that this crucial phi29 concentration has been reached for 0.01 U/µl.

4.2 Protocol optimizations

One of the drawbacks with PLA-WB is the number of steps and time required in the protocol, which can be seen in the material and methods section. To decrease both of these factors for PLA-WB, the RCA step and the detection step was merged together. By choosing the buffer used in the RCA step, but with addition of 5 nM Cy5 conjugated detection probe, the optimum parameters for RCA is met. Once the elongation starts, detection probes can hybridize to corresponding DNA sequences as the reaction proceeds. When comparing the merged protocol with the standard protocol one can see that the signal and LOD is around 20-fold reduced for the merged protocol. This is mainly due to phi29 proof reading function. Once the detection probe has hybridized to the RCA product, free phi29 will remove it. To inhibit this proof reading function, methylated detection probes at a concentration of 5 nM were added. With the methylated probes some of the signal was rescued but it was still far weaker than the control. The merge of the RCA and the detection step also caused an elevated background. This might be due to unspecific adsorption of detection probes to the membrane since the total incubation time for detection probe is increased from 25 min to 60 min. If this is shown to be true, the background increase can be avoided if the methylated detection probe is added during the last 25 min of the RCA step. The elevated background can also be avoided by adding less detection probes, but the signal might also decrease by this procedure. Even

though the signal was reduced and the background was increased, this shows that it is possible to merge the two steps together and still have a functional method. To make the merged protocol comparable with the standard protocol further optimization is needed.

Another way to make PLA-WB easier to use is to make the protocol compatible with existing system for automatic Western blotting, *e.g.* processor Plus, GE Healthcare. Therefore the reaction volume for PLA-WB was scaled up from 2 ml to 12.5 ml, which is a more common volume for Western blotting. As seen in figure 12 the PLA-WB method works for larger volumes as well as smaller ones. Compared to small scale, the 12.5 ml reaction mix resulted in a decrease in signal and higher background. This can be explained by the increased membrane size, the membrane area increased around 12 times were the volume increased five times. With the increased membrane area per volume unit, the unspecific adsorption of reagents to the membrane is increased. This can have affected the concentrations during the protocol steps.

It has also been shown from the experiments performed in figure 11, that if the PLA-WB method is used at RT, the signal is largely reduced. This is due to lower enzyme efficiency at room temperature. T4 DNA ligase has an optimum efficiency at 37˚C and phi29 DNA polymerase at 30˚C so with temperatures close to 20˚C the enzyme activity is reduced. With increased T4 DNA ligase concentration, some of the signal was rescued. With longer RCA incubation time, or a higher concentration of phi29 the signal stayed at the same levels as standard protocol in room temperature. It has later been shown that PLA-WB can yield similar signal at RT as at 37°C by using a primary antibody dilution of 1:190, 0.06 U/µl T4 DNA ligase and increasing the ligation incubation time to 80 min (Yanling Liu, oral communication, 7th June 2011).

4.3 Signal amplification, dynamic range and LOD

PL-WB offers a significant increase in LOD and signal amplification compared to traditional WB. Figure 13 shows that the LOD of detection when using HRP conjugated detection probes and ECL Plus substrate is increased, from 9.8 pg to 76 fg. This corresponds to a 128-fold increase in limit of detection. The signal amplification is also much greater with the PLA-WB assay. When comparing the integrated intensities for the two methods, it is around 80 times stronger for PLA-WB with ECL readout. The DR of the methods is maintained, but shifted towards lower amounts of target for PLA-WB. For abundant target such as 2.5 ng transferrin

the ECL Plus substrate is depleted because of the strong signal amplification for PLA-WB which causes “white bands” which can not be quantified.

In fluorescence readout the signal from PLA-WB was increased compare to traditional WB with fluorophore conjugated secondary antibodies. In figure 14 the signal amplification was increased around 20 times for all targets for PLA-WB. For fluorescence readout the LOD and DR was similar for PLA-WB and WB.

5. Conclusions

Throughout this report I have shown that the PLA-WB can be optimized for maximal signal intensities and minimal background in many ways. The cost per assay has been reduced by lowering the enzyme concentrations of PLA-WB without affecting the results of method. Simplification of PLA-WB protocol has been increased by shortening a few incubation steps and washes. I have also shown the possibility to merge two steps of the protocol together and thereby reducing the amount of time required per assay. Important factors that affect the background have been investigated, and a decrease in background has been achieved. The signal generated by PLA-WB has been increased which eases the detection of low abundant targets *e.g.* in scarce clinical samples. I have not succeeded with increasing the linear dynamic range, but as stated above it has been shifted towards lower amounts of target. Furthermore I have shown that PLA-WB is capable to detect down to at least 80 fg transferrin, which to my knowledge is a Western blot world record in limit of detection.

6. Acknowledgements

I would like to give my sincere appreciations to all people in the protein analysis unit at GE Healthcare for contributing to a warm and welcoming atmosphere.

My greatest thanks go to my mentor Åsa Hagner-McWhirter. For giving me helpful comments on this report and always having time to answer all my questions. With your guidance I have gained knowledge about experimental design and a deep understanding of protein analysis.

Furthermore I would like to appreciate my gratitude towards Yanling Liu for giving me feedback on experiments and helping me out in the lab.

I would also like to thank my scientific reviewer Anna Edman-Örlefors for providing comments on this report.

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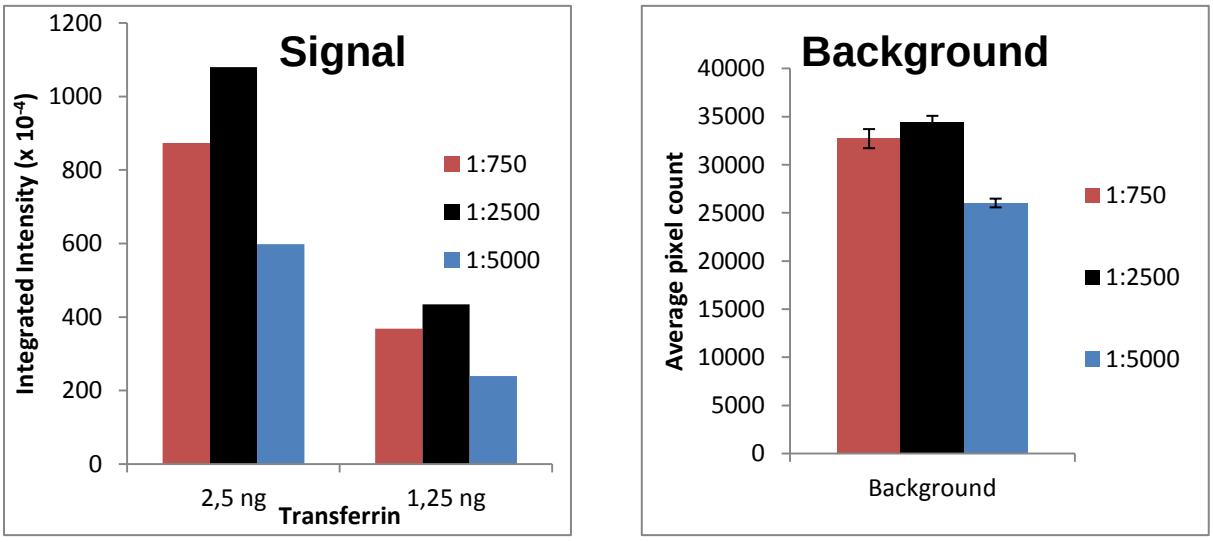
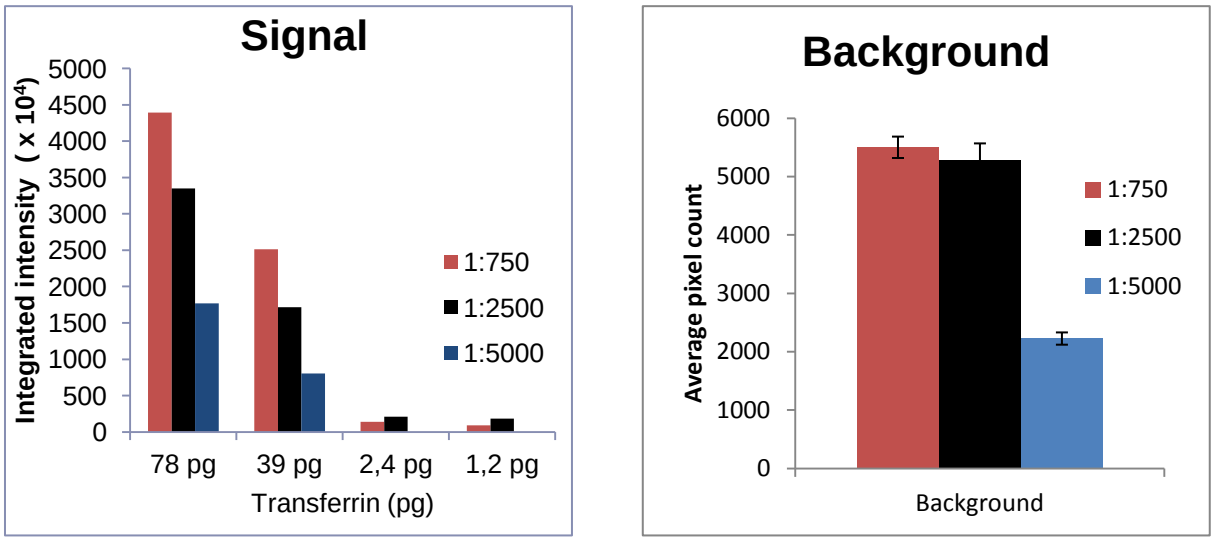
8. APPENDIX



Cy5 readout of integrated intensity and background for membranes incubated with 1:750, 1:2500 or 1:5000 primary antibody dilution



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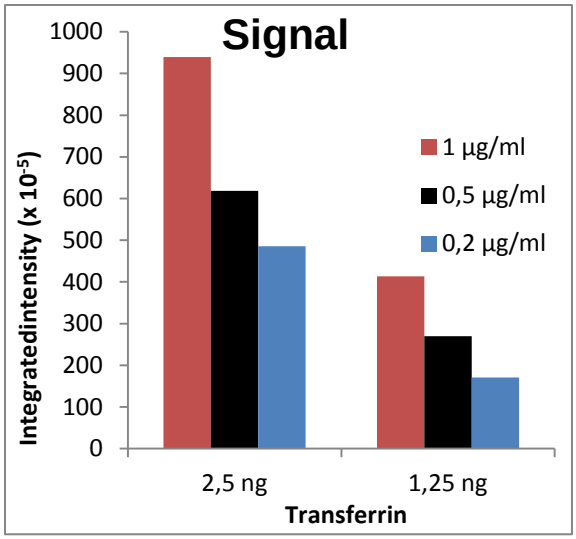
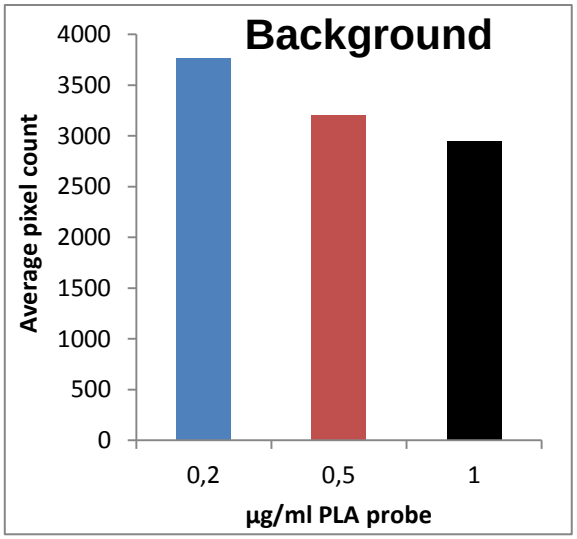
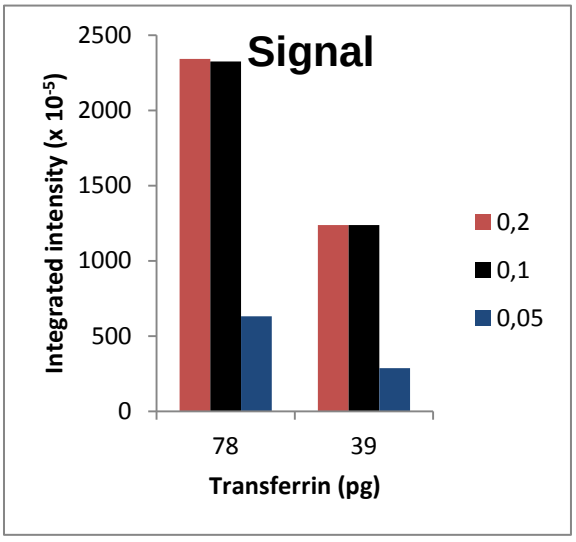
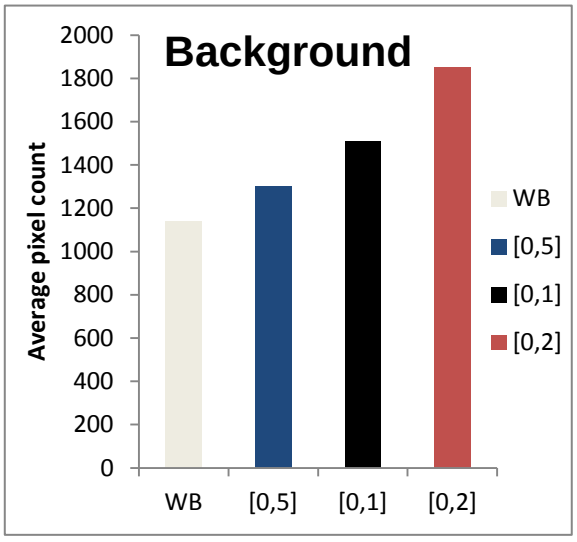




Cy5 readout of integrated intensity and background for membranes incubated with 0.2, 0.5 or 1 µg/ml PLA probes





ECL readout of integrated intensity and background for membranes incubated with 0.05, 0.1 or 0.2 µg/ml PLA probes