Swedish University of Agricultural Sciences
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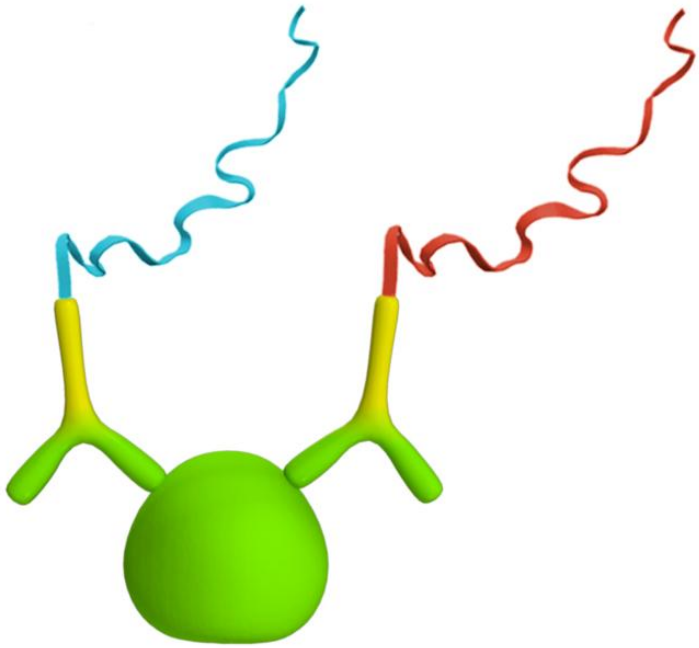

Master´s thesis • 30 hec • Second cycle, A2E
Biotechnology program
Publikation/Sveriges lantbruksuniversitet, Institutionen för livsmedelsvetenskap
No: 364
Uppsala 2012

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Credits: 30 hec
Level: Second cycle, A2E
Course title: Independent project/degree project in Biology
Course code: EX0565
Program/education: Biotechnology

Place of publication: Uppsala
Year of publication: 2012
Title of series: Publikation/Sveriges lantbruksuniversitet, Institutionen för livsmedelsvetenskap
Serie no: 364
Online publication: http://stud.epsilon.slu.se

Key Words: Proximity extension, colorectal cancer, biomarker, real-time PCR

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Abstract

The ability to detect protein-based biomarkers, which are linked to different diseases like colorectal cancer, is very important as a diagnostic tool. Usually complex biological samples like blood are studied which will contribute to different technical issues when performing an assay. The aim with the project is to optimize and develop the high throughput protein detection technique Proximity Extension Assay, PEA, into a 96-plex panel, in hopes of discovering an expression profile for colorectal cancer. PEA was developed by Olink Bioscience and allows specific proteins in a sample to be quantitatively transformed into nucleic acid sequences that are subsequently detected and quantified with real-time PCR. Two proximity probes containing oligonucleotide sequences bind pairwise to target protein and when brought in proximity, a DNA polymerase will extend a hybridization arm from one probe over to the second forming a double-stranded DNA sequence that can serve as a template in real-time PCR. The results showed that there is no significant difference in sensitivity, specificity, recovery or efficiency between assays performed in single plex, lower or higher degree of multiplex. Higher sensitivity of the assay was achieved by optimization of factors such as, concentrations of proximity probes and hybridization oligo arm. The results also show that the recovery will not be affected by higher concentration of plasma or by using other assay formats. Work proceeds to develop a 96-plex panel with just as high sensitivity and recovery, which would make PEA the most, multiplexed immunoassay with high sensitivity so far.

Sammanfattning

Möjligheten att upptäcka proteinbaserade biomarkörer, som är kopplade till olika sjukdomar som kolorektal cancer, är mycket viktig som ett diagnostiskt verktyg. Vid studier av komplexa biologiska prover som blod bidrar matrisen vanligtvis till olika tekniska problem. Syftet med projektet är att optimera och utveckla proteindetektionsmetoden Proximity Extension Assay, PEA, till en 96-plex panel, med förhoppning om att upptäcka en uttrycksprofil för kolorektal cancer. PEA har utvecklats av Olink Bioscience och tillåter specifika proteiner i ett prov att omvandlas till nukleotidsekvenser som senare upptäcks och kvantifieras med realtids-PCR. Två prober, så kallade *proximity probes*, består av oligonukleotidsekvenser som binder parvis till målproteinet. När de kommer i närhet, extenderar ett DNA-polymeras en hybridiseringsarm från en prob över till den andra och bildar en dubbelsträngad DNA-sekvens som kan fungera som en templat i realtids-PCR . Resultaten visade att det inte finns någon signifikant skillnad i känslighet, specificitet, återhämtning eller effektivitet mellan de assays som genomfördes i single-plex, lägre eller högre grad av multiplex. Högre känslighet uppnåddes genom att optimera faktorer som koncentrationen av prob respektive hybridiseringsarm. Resultaten visar också att återhämtningen inte påverkas av högre koncentration av plasma eller genom att använda andra analysformat. Arbete fortsätter med att utveckla en 96-plex panel med lika hög känslighet och återhämtning vilket skulle göra PEA till den hittills mest multiplexade immunoassay med hög känslighet.

Content

Aim

The aim of my master thesis was to learn more about how to optimize and develop the high throughput protein detection technique Proximity Extension Assay, PEA, in hopes of discovering an expression profile for colorectal cancer. The overall aim with the project is to develop a 96-plex panel where 96 different potential biomarkers for colorectal cancer are detected and analysed in one single plasma sample. This would make PEA the most multiplexed immunoassay with high sensitivity so far. Hopefully it leads to progresses in in fields of understanding of the molecular mechanisms of the disease and the development of a diagnostic tool that may discover the disease already at an early stage.

Introduction

PROACTIVE

PROACTIVE is a project with the vision to develop a validated high throughput technique for detecting and analysing a large set of putative cancer protein biomarkers in human blood samples. In other terms, the aim is to discover a way to separate healthy from cancer diseased individuals, by either detecting new biomarkers or by observing combination of several specific biomarkers to obtain an expression profile for colorectal cancer. The PROACTIVE project is funded by the European Community and started 2008 and will proceed for 3 years. The main participant is Olink Bioscience (Uppsala, Sweden) but the project is collaboration between several partners, both universities and companies. Integromics (Madrid, Spain) developed a web-based laboratory information management system (LIMS) in order to have a convenient way of sharing data within the project. The proximity probes were prepared by Innova Biosciences (Cambridge, UK) and Olink Bioscience is responsible for the biomarker profiling and panel evaluation. The other partners in the project are Fujirebio AB (Gothenburg, Sweden) source of antibodies and antigens, Uppsala University (Uppsala, Sweden) performing the statistical analysis, Department of Surgical Gastroenterology (Copenhagen, Denmark), and Copenhagen University (Copenhagen, Denmark) are providing the clinical samples and run comparative ELISA analysis.

Biomarkers in cancer diagnostics

Colorectal cancer, also known as bowel cancer, is one of the most common types of cancer. Around 5% of the population in the western world has some form of colorectal cancer. Colorectal cancer is localized in the large intestine and rectum and has an epithelial origin. The prognoses for a patient with colorectal cancer depends on the differentiation of cancer cells, tumour stage and if/how tumour cells are spread to other organs and form so called metastatic, secondary, tumours. If metastasis is discovered in a patient undergone surgery, after 5 years the survival rate is 10% compared to 90% for when no metastasis has occurred. Therefore it is crucial to increase the knowledge about the molecular mechanisms behind the development of colorectal cancer, for example by finding biomarkers that can identify the disease already at an early stage (Belov *et al,*. 2010).

Some of the antigens used in the assay are known protein biomarkers for colorectal cancer and some are new, and have not been studied before, but are believed to play a role in development of the disease. One of the proteins that are studied in majority of the experiments is Interleukin 8, IL-8, is chemokine produced by macrophages and play an important role in inflammatory response. Previous investigations suggest that IL-8 is produced under development of colorectal cancer. (Malicki *et al*,. 2009)

Cancer cells have similar features as normal cells and therefore it can be difficult to separate cancer cells from their normal surrounding. The one thing that will distinguish cancer cells from healthy are that they have uncontrolled growth meaning that they will need more nutrients than healthy cells with controlled cell growth. Vascular endothelial growth factor, VEGF, is needed for high proliferation and metabolism of cancer cells. VEGF is a signal protein which is well studied as a stimulating growth factor for vasculogenesis, i.e. the formation of embryonic circulatory system, and angiogenesis. Angiogenesis is the formation of new blood vessels and usually does not occur in adults unless tumours are involved. Angiogenesis is thus a crucial step in the transition from inactive to malign state of a tumour (van Oosten *et al*,. 2011).

Another protein studied in the experiments is Carcinoembryonic antigen-related cell adhesion molecule 5, CEACAM5. CEACAM5 is a known colorectal cancer biomarker. It is important for intracellular signalling and is a cell adhesion molecule that is expressed on the surface of most of the solid tumours. It is also highly studied in cancer therapy diagnostics since there is high concentration of CEACAM5 in colorectal cancer tissue, shown by gene array studies in the Swedish Human Proteome Resource project (Govindan *et al*,. 2009).

Antibodies

Antibodies, i.e. immunoglobulins, are naturally occurring glycoproteins in our body produced by a white blood cell type called *B cells* or *plasma cells*. The antibodies exist either in a secreted soluble form or is membrane bound to a B-cell referred to as B-cell receptors. The immune system uses antibodies to attack and neutralize bacteria and viruses. The part of the antibody that recognizes the specific part of the antigen is called paraepitope or antigen-binding site and the specific part of the antigen is called epitope.

Monoclonal antibodies are clones obtained from hybridomas and they are mono-specific, meaning that they have affinity for a single epitope on a specific antigen. Examples of monoclonal antibodies in the experiments are CA242 and CEACAM5. In contrast, polyclonal antibodies originate from different B cells and will recognize different epitopes on one specific antigen. Polyclonal antibodies are produced from polyclonal antisera taken from an animal immunized with the target antigen. Blood cells are usually suspended in blood plasma and when blood cells and clotting factors are removed it is called blood serum. Polyclonal antibodies are affinity purified from antisera with affinity chromatography. Polyclonal proximity probes are produced by dividing antisera into two portions and coupling oligonucleotides with free 5´ends to each portion.

Affinity-purified polyclonal antibodies were raised against the whole native recombinant protein. While polyclonal antibodies recognize more than one

epitope on the protein, monoclonal antibodies are said to be more target specific since they only bind one single epitope (Gullberg M *et al*,. 2004).

Polymerase chain reaction

Polymerase chain reaction, PCR, is commonly used in microbiology work to amplify specific DNA sequences. Each PCR cycle is divided in several steps and the first step is called *initial denaturation step* where the reaction is heated to around 95ºC to eliminate non-specific amplification. The following step is the *denaturation step* where the reaction is kept at around 95ºC. This high temperature will separate the hydrogen bonds between the complementary nucleobases in the double stranded DNA forming two single stranded DNA strands, referred to as template. Now the temperature is decreased to around 60ºC, depending on the primers design. This step is called *annealing step* since the primers anneal or bind to the DNA strand. The DNA polymerase will recognize the attached primers and bind to the template before it starts its DNA synthesis. The following step is called *elongation step* and the temperature is changed to 72ºC where DNA polymerase has its optimal activity. In the PCR reaction mix, there are deoxyribonucleotide triphosphates, dNTP, which is a gathering name for the four deoxyribonucleotides; dATP, dCTP, dGTP and dTTP. Each deoxyribonucleotide consist of a nucleobase, a deoxyribose sugar and phosphate groups. The DNA polymerase adds dNTP's that is complementary to the template. When the PCR reaction is most efficient, it will double the DNA amount for each cycle. Usually the PCR reaction runs for 30-40 cycles. After the last cycle a step called *final extension* can be added that will hold the reaction at around 70ºC to assure that the all single stranded DNA has been extended (Saiki RK *et al*,. 1988)

Real-time polymerase chain reaction

Real-time polymerase chain reaction or quantitative polymerase chain reaction, qPCR, is a commonly used technique in molecular biology. The technique is based on, and follows the principle of polymerase chain reaction, PCR, that uses temperature changes and a heat stable DNA polymerase to amplify DNA sequences. The advance with qPCR is that an amplified DNA sequence can be detected in real time (Fredriksson S *et al*,. *2002a* and Sano T *et al*,. 1992). The most common detection methods are use of fluorescent dyes that bind non-specifically to double stranded DNA or by using DNA probes, that are coupled to fluorescent reporters that hybridize to specific DNA sequences. So by using DNA probes, only the DNA sequence that are complementary to the DNA probe gets detected which increases the specificity. Since the probes are target specific, they can be used in mutliplex assays and reduce the risk of primer dimers being detected even though the primer dimers might exist and cause an inhibitory effect.

The DNA probe, called molecular beacon, consists of oligonucleotides and has at one end a fluorescent reporter and at the opposite end a quencher of fluorescence. When the reporter and quencher are in close proximity, it will prevent the reporter from emitting fluorescence. Under extension, the Taq polymerase will work along the DNA strand and eventually break the proximity between the reporter and quencher by its 5´ to 3´ exonuclease activity. This will release the reporter and allow it to emit fluorescence that is detected after excitation with a laser in the qPCR machine. For each PCR cycle, the product will increase exponentially and the increase of product

detected by the probe will be proportional to the increase of fluorescence emitted by the released reporter (Heid CA *et al,*. 1996).

A DNA polymerase is an enzyme that has an essential role in DNA replication since it catalyzes the polymerization of deoxyribonucleotides into a DNA strand. There are different types of polymerases existing and DNA polymerase I consists of a large subunit called Klenow fragment and a smaller called Exonuclease I. When Klenow fragment is cleaved from DNA polymerase I, it will possess 5´ to 3´ polymerase activity and 3´ to 5´ exonuclease activity, which mediates proofreading but loose its 5´ to 3´ exonuclease activity. In the experiment, a mutated variant of Klenow fragment is used that does not have any exonuclease activity in order to study the polymerase activity. Exonuclease I, Exo I, possesses 3´ to 5´ exonuclease activity and works only on single stranded DNA. Exo I works by cleaving nucleotides from the 3´ end of the polypeptide chain by causing a hydrolyzation event that will break up the phosphodiester bonds in the polynucleotide chain (Xie P *et al,*. 2011).

Multiplexed proximity extension assay for biomarker profiling

The ability to detect protein-based biomarkers, which are linked to different diseases like cancer, is very important as a diagnostic tool. Usually complex biological samples like blood are studied which will contribute to different technical issues when performing an assay. The specificity, recovery and linearity can be negatively affected (Fredriksson *et al,*. 2007).

Proximity extension assay is a relatively new *in vitro* protein detection technique that allows specific proteins in a sample to be quantitatively transformed into nucleic acid sequences that are subsequently detected with qPCR. PEA is developed from and is similar to the protein detection technique Proximity ligation assay, PLA. In PLA, enzymatic ligation is used to connect the proximity probes when bound to the target protein, which is illustrated in *Figure 1*. The accuracy of the assay can be validated by calculating the recovery which will reveal how well the response of data generated agrees with the former obtained reference values from either the same or another method. To see how the detection is affected by factors, such as high concentration of plasma or multiplex, samples are treated in different ways and results are compared. If no reference sample is available, a blank sample can be used that is spiked with a known concentration of the desired factor. When doing recovery experiments or other experiments that measure the effectiveness of the assay, it's important to be careful and mimic the sample preparation and experiment set up to exclude variation or differences that can affect the assay and give unreliable results. When the assay is not inhibited by for example components in blood plasma or by running multiplex, the recovery is high, meaning a robust assay. The negative aspect of using a ligase is that the recovery loss will be higher when studying proteins in complex biological fluids like blood plasma (Fredriksson, 2002b and Gustafsdottir *et al,*. 2006).

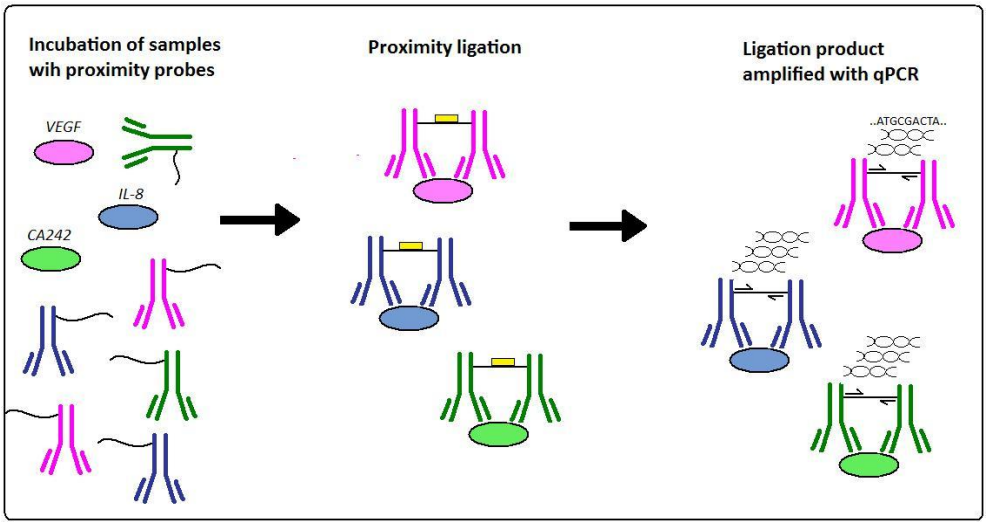


Figure 1. Schematic illustration of the performance of homogenous-phase proximity ligation assay (PLA). The sample containing proteins are incubated with proximity probes. A ligation and amplification mix is added to allow proximity ligation where the free 3´and 5´ends of the probes hybridize to the connector oligonucleotide, when they bind to the target protein. The ligated product is amplified and detected with qPCR.

PEA uses a pair of proximity probes that are illustrated in *Figure 2* and called probe A and B. These probes are antibodies, either mono- or polyclonal, and each probe is conjugated to a DNA oligo. To probe A, a 56-mer DNA oligo is hybridized. This hybridization oligo consist of 40 nucleotides complementary to the DNA oligo on probe A, a spacer of 7 nucleotides and the last 9 nucleotides are complementary to the DNA oligo on probe B. The probes bind the protein they have affinity for and when the proximity probes are near each other, in so called close proximity, they will hybridize to each other. In the next step, DNA polymerase is added that will extend the hybridization oligo arm over the second probe leading to a double stranded DNA sequence. This double stranded DNA will serve as the template and be detected and quantified by qPCR. Since the probes have to be in close proximity and only this dual recognition of the antigen will initiate an amplified detection signal, this results in a very target specific method (Fredriksson *et al*,. 2002a and Lundberg *et al*,. 2011a).

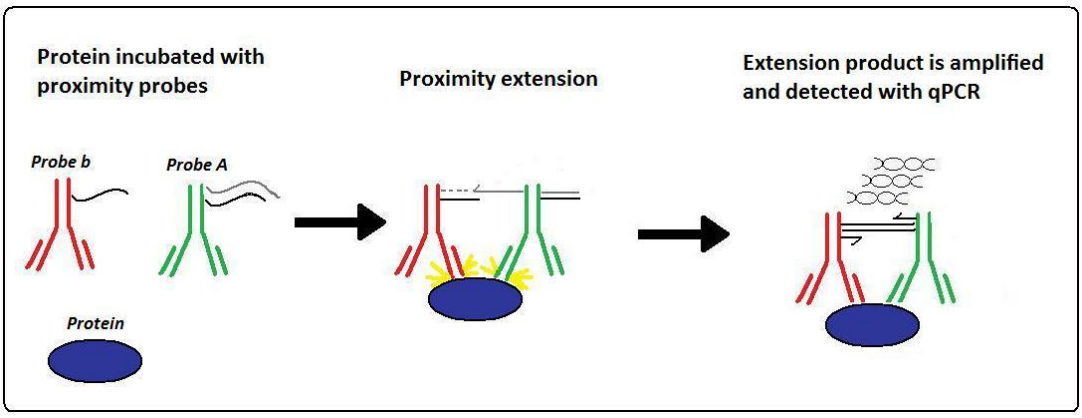


Figure 2. Illustration of the performance of PEA. Protein sample is incubated with proximity probes whereas probe a is connected with a hybridization oligo. When dual recognition of target protein occurs, the polymerase will extend the hybridization arm over to the second probe which is called proximity extension and results in a double stranded DNA strand. This extension product will be amplified and detected with qPCR.

Before qPCR, a pre amplification step is done to have enough amplicon that will give more reliable result since low number of amplicons can lead to a less robust amplification. If different primer pairs are used, it can contribute to unevenness in amplification since they can have for example different extension temperatures and effectiveness. So to improve unevenness in pre amplification, one pair of primers called universal primers are used for all samples which is illustrated in *Figure 3*. To enable this, universal sequences has been added to outer probe regions. Uracil-DNA glycosylase, UNG, is a DNA repair enzyme, which removes uracil bases from DNA due to miscorporation in place of thymidine which otherwise can lead to mutations. UNG excise uracil by cleaving (hydrolyze) the N-glycosylic bond between the deoxyribose sugar and uracil and start the base-excision repair (BER) pathway (Lindahl T *et al,*. 1977 and Longo *et al,*. 1990). To avoid that the universal primers not would interfere with the qPCR reaction by continuing to amplify the sequences produced in pre-amplification, they are digested by UNG. Since UNG will cleave off uracil bases from a DNA strand, the universal primers and the outer regions of the probes, where the universal primers bind, are designed to contain uracil residues. UNG is in a mix with Endonuclease IV. UNG is responsible for the cleaving of uracil bases from the DNA strand resulting in an abasic, inactive, site. Endonuclease IV will then cleave the phosphodiester bond at his inactive site generating several digestion fragments. When these universal sites are cleaved off, only the specific sites are left that are amplified with specific primers and a detection site for detection with DNA probe in qPCR (Lundberg *et al,*. 2011b).

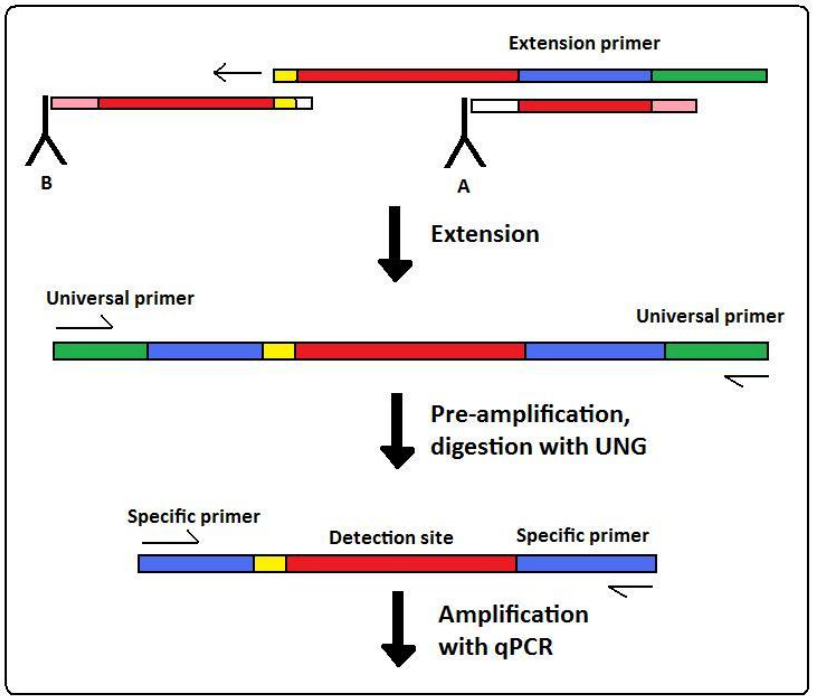


Figure 3. Schematic illustration of the probe design in PEA.

The procedure used in the beginning of the project was Proximity ligation assay, PLA, but due to low recovery signals and that normalization was necessary, PLA was later replaced with Proximity extension assay, PEA, which proved to have the same sensitive with better recovery and better quantification of proteins. For example, PLA has an average recovery value of 33% whereas PEA has an average of 77%. Previously, in the second part of the project, the number of proteins detected and unique probes in one sample where 24, called 24-plex, which where done for 4 panels. Now in the third part of the project, the aim is to have one 96-plex panel and also run a

4 times 24-plex panel, the ones used in the second part, which would make it the most multiplexed immunoassay with high sensitivity so far. In the second part, plasma samples from 75 healthy and 75 colorectal diseased individuals where analysed. In third part of the project, the same number of healthy and diseased will be studied but also plasma samples from 75 individuals with adenoma and 75 with other glandular diseases will be analysed (Ekström B,. 2011).

A new probe design was developed to reduce unspecific background and in silico analysis was made to avoid formation of hair-pin structures. Only the probe pair that match will hybridize to each other. Several designs of extension primers (hybridization oligos) where evaluated in an effort to make the assay even more sensitive. Different designs where tested that had varying length of nucleotides, from 6-11, complementary to the probe. Other modifications where also made like changing some of the complementary bases to inosine to improve the strength of hybridization. The salt concentration was optimized just like the incubation temperature. The extension primers with highest signal in combination with maximum sensitivity were selected, with no modified bases and 9 nucleotides complementary to the probe (Lundberg *et al*,. 2011a).

Concepts and calculations in experiment results

The presented experiment results are based on several calculations that are explained and described in the coming section.

A low detection signal is correlated with a high Ct (cycle threshold) value. This is because a low copy number of DNA, in other words a small amount of starting material in qPCR, will take longer time for reaching the threshold level. A high detection signal is correlated with a low Ct value since an already high number of DNA will not need as many cycles to reach the threshold level. That is why the diagrams presented in the results have values in reversed order on the y-axel.

Threshold Ct

The exponential phase of amplification in qPCR is when all the components needed for the reaction are still in excess resulting in an increase of product at a constant rate. The threshold base line should be set at the beginning of the exponential phase to reduce background found in early cycles of amplification. At the end of the qPCR run, the reaction will eventually run out of the essential component, such as primers, template and polymerase. The reaction then reaches a plateau where the DNA is amplified at a much lower rate or not at all. After the plateau the graph can sometimes begin to decline, even at lower cycles when the primer concentration is high. This is called the *hook-effect*. Stable binding of the molecular beacon is important for the production of a good signal since the flourescent signal is proportional to the target quantity. When there is a surplus of primers, they will outrival the molecular beacon, thereby reducing the hybridization signal. The amplified strands will reanneal before the probes can bind and emitt fluorescence, a phenomena known as the *hook-effect*.

Standard deviation

Standard deviation is a measurement of the distribution of the resulting values, usually replicates, how they vary or diverge from a mean value.

Specificity and sensitivity

An assay with both high specificity and sensitivity of detection is usually the desired outcome when performing qPCR. Specificity refers to the ability of the assay to distinguish between target molecules in detection. High specificity is correlated with a higher accuracy and a more reliable detection method. Sensitivity is correlated to the assay's range of detection. The sensitivity is high when the assay has high signal to background in samples with both low and high concentration of target protein.

Delta Ct

A cycle is the constant temperature changes that are made in the qPCR in order for the DNA to be amplified. The Ct, cycle threshold, is the cycle when the signal from a sample crosses the threshold. Delta Ct, abbreviated as dCt**,** is a term used in qPCR analysis to describe the efficiency. The dCt can be defined as the difference between the detected Ct value of the sample (cross-over threshold) and the Ct value for a reference gene (or a negative control). An increase of material will give a positive dCt value and a decrease of material will have a negative value. dCt is defined as:

$$\mathbf{dCt = Ct_{Buffer} - Ct_{sample}}$$

Efficiency

Efficiency is a measurement of how much DNA that will be amplified, which reveals how well the qPCR run went. The efficiency is usually given in percent, whereas an efficiency of 100% means a doubling of the amount DNA at each cycle. The lower the percentage, the less DNA is amplified resulting in a lower number of DNA copies. When the efficiency is 100% the slope of the curve is -3,32. Efficiency in percent is defined as:

$$\mathbf{Efficiency\ \% = 10^{(-1/slope)} - 1}$$

The amount of DNA after *n* cycles is described as $\mathbf{[}\boldsymbol{efficiency}\mathbf{]^n}$

Normally when evaluating the performance of a primer set, a serie dilution is made of the target molecule. R2 value is a measurement of the correlation between variations in y-values compared to variations in x-values. When R2 = 1, this means that there is no variation.

Recovery

The recovery value measures the accuracy of the assay when influenced by different factors.

When the assay is not inhibited or influenced by factors the recovery is high, meaning a robust assay. To perform a recovery test, a sample with and without the factor is compared to see how the detection signal varieties. Recovery values are usually given in percentage whereas a high value correlates with a good recovery, meaning that there is no difference between the samples with and without the factor. When calculating recovery, the Ct values have to be first converted to amplicons. Amplicon is the amount DNA produced by the qPCR run and is defined as:

$$\boldsymbol{Amplicon = 2^{(30-Ct)}\ or\ 2^{(38-Ct)}}$$

Recovery, in percentage, is defined as:

$$\boldsymbol{Recovery\ \% = (Amplicon_{Sample+Ag} - Amplicon_{sample-Ag}) / (Amplicon_{Buffer+Ag} - Amplicon_{Buffer-Ag}) * 100}$$

Normalisation

Normalisation is a way to improve the variation in sample efficiency in qPCR and errors in sample quantification. Normalisation is done by introduce a reference gene in the assay and divide the sample values with the normalizer. Worth remembering is that the normalizer itself decides how good the normalisation quality will be.

Coefficient of variation, CV (%)
The coefficient of variation, CV, is a normalized measure of the dispersion of data points in a series around the mean value. It can also be described as the ratio between the standard deviation to mean value. A high value means a high variability and a low CV means a low variability between a sample set. CV allows comparison in the degree of variation between different series even though the means are totally different in each series. The CV in percentage is defined as:
$$\%CV = (\text{Standard deviation}_{\text{serie mean}}) / (\text{Mean}_{\text{serie mean}}) * 100$$

Comparison of PEA with other protein detection techniques

ELISA

Enzyme-Linked ImmunoSorbent Assay, ELISA, is a solid phase protein detection technique. The crucial difference between ELISA and PEA is that 100µl of sample is needed for performing ELISA when only 1µl is needed in PEA. Such low sample consumption is desirable in clinical diagnostics and medical treatments when performing hematologic tests of human or lab animals. Several washing steps are also required in ELISA which is not needed in PEA. Material such as target protein can sometimes detach from the solid surface leading to a less efficient assay. PEA has a lower assay time than ELISA and also higher recovery. The disadvantage with PEA is that it requires access of a heating block and adding pre-extension and extension mix to the plate while it is in the block which can lead to more pipetting errors.

The positive aspect of ELISA is that it is a cheap and easy method with a lot of antigen specific kits available with very high sensitivity. The disadvantage is that with ELISA, there is no probability to quantitate the DNA in real time. In PEA, there is a reverse translation of proteins into nucleic acids encoded signals that can be amplified by qPCR or analysed with techniques used for DNA.

Western blot

Western blot is one of many examples of a protein detection technique. The disadvantage that unites them is that there is no reverse translation into DNA so the target can not be quantified or amplified.

Immuno-PCR

Immuno-PCR is a relatively new protein detection technique that is said to have higher sensitivity than ELISA. Immuno-PCR works just like ELISA but a reporter gene is connected to the antibody, usually via a streptavidin-biotin link. Through this reporter gene, qPCR can be applied for this method. When performing PEA; there is a dual recognition of target protein leading to a higher specificity compared to single recognition.

Material and Methods

Plasma samples and antigens

EDTA blood plasma samples were provided by Nils Brunner, member of the Proactive consortium at Copenhagen University and were collected from healthy and colorectal cancer diseased individuals. The blood samples were centrifuged 10 min at 2500 x g at 4ºC. The plasma was aspirated and stored at -80ºC. The antigens, recombinant human proteins, were from RnD Systems (Minneapolis, USA). Antigens were kept in PBS + 0.1% BSA at -80ºC.

Antibodies and proximity probes

All polyclonal antibodies were purchased from RnD Systems (Delhi, India). To make the proximity probes, a single batch of affinity-purified polyclonal antibodies split into two or matched monoclonal antibody pairs were covalently linked to two different 5´-phosphate free 40-mer oligonucleotide sequences. The conjugation of oligonucleotide to antibody was generated by Innova Biosciences (Cambridge, UK) by using their conjugation technique Lightning-Link^{TM}. SDS-PAGE was used to check the quality of conjugation. The 40-mer 5´oligonucleotide sequence used for conjugation consisted of a 20 bp universal sequence that functions as hybridization site and also as detection site for molecular beacon. The last 20 bp sequence is unique and will function as target for qPCR detection. The hybridization oligo, previously called extension primer, was hybridized to the 5´-free oligonucleotide of the proximity probe conjugate. The conjugation was at a 2:1 oligo-to-antibody ratio.

Proximity Extension Assay

Only 1 µl sample is required for performing proximity extension assay, PEA, which could be human EDTA plasma or PBS + 0.1% BSA buffer +/- antigen spike when running controls. The plasma sample was mixed with 3µl of probe mix (100 pM of each PEA conjugate, 25mM Tris-HCl, 4mM EDTA, 0,016mg/ml single stranded salmon sperm DNA (Sigma Aldrich) and 0,02% sodium azide). This 4µl reaction mix was incubated in 200 µl tubes, either 2 h at room temperature or 1 h at 37ºC.

After probe incubation the samples were put in a thermal cycler and kept at 37ºC. 76µl dilution mix (40µM of each dNTP's, 70mM Tris-HCl, 17.7mM ammonium sulphate and 1.05mM dithiothreitol) were added to each sample. The mixture was incubated at 37ºC for 5 min. Then, to start the extension, 20µl extension mix was added at 37ºC for 20 min. The extension mix contains 66.8mM Tris-HCl, 16.8mM ammonium sulphate, 33mM magnesium chloride, 1mM dithiothreitol, 62.5 U/ml T4 DNA Polymerase (or 62.5 U/ml Klenow fragment exo(-), 125 U/ml Klenow fragment and 125 U/ml DNA Polymerase I from Fermentas (Maryland, USA)). In some experiments 250 U/ml Exonuclease I from Biolabs (New England) was added to the extension mix to study its optimal concentration. The extension reaction was then heat inactivated at 80ºC for 10 min.

Pre –amplification with PCR

The pre-amplification was done with PCR in a total volume of 20µl, by adding 10µl extension product to 10µl PCR mix (PCR mix contains 1X PCR Buffer from Invitrogen (Stockholm, Sweden), 1mM dNTP (Invitrogen), 15mM $MgCl_2$ (Invitrogen), 0.2µM of each forward and reverse

PRE-AMP primer and 7.5U Platinum Taq polymerase (Invitrogen). The PCR reaction was run for 17 cycles with an initial incubation for 10min at 95ºC, followed by 2 cycles for 15 s at 95ºC, 10min at 46ºC (for single and 8-plex assay), 2min at 60ºC and 15 cycles for 15 s at 95ºC, 2 min at 54ºC and 2min at 60ºC.

UNG treatment

To digest PRE-AMP primers used in pre-amplification step, the samples have to be treated with UNG, Uracil-DNA-Glycosylase. 15µl of pre-amplification product was mixed with 15µl UNG mix (final mix contains UNG diluted 200x and 3x PRE-AMP buffer). The mix was incubated for 20min at 37ºC and heat inactivated for 10min at 70ºC.

Real-time PCR analysis

The proteins detected in PEA were analysed with real-time PCR, qPCR, on an ABI HT Fast (Applied Biosystem) 7900. The proteins were analysed in separate qPCR reactions with individual primer pairs on a 96- or 384-well microtiter plate. In each well, 4µl of extension product was mixed with 6µl qPCR mix (the extension mix contained 7.5mM magnesium chloride, 25mM Tris-HCl, 50mM potassium chloride, 8.3mM Trehalose from Acros Organics (New Jersey, USA), 8.3mM ammonium sulphate, 333µM of each dNTP, 1.67mM dithiothreitol, 833nM of each primer, 417nM Molecular Beacon from Biomers (Stuttgart, Germany), 41.7 U/ml recombinant Taq polymerase (Fermentas) and 1.33 µM ROX reference (Biomers). The qPCR was run as a two-step method, with initial denaturation at 95ºC for 5min, followed by 45 cycles of 15s denaturation at 95ºC and 1min annealing/extension at 60ºC.

Results

Standard curve for a known biomarker of colorectal cancer

A standard curve is often used in biological experiments as a tool to quantify the concentration of proteins. Various samples with known concentrations of the protein of interest, in this case IL-8, were plotted and the obtained detection signal was then used to compare values of samples with unknown concentration. In this experiment, the standard curve of three different assays, with different probe sets were compared.

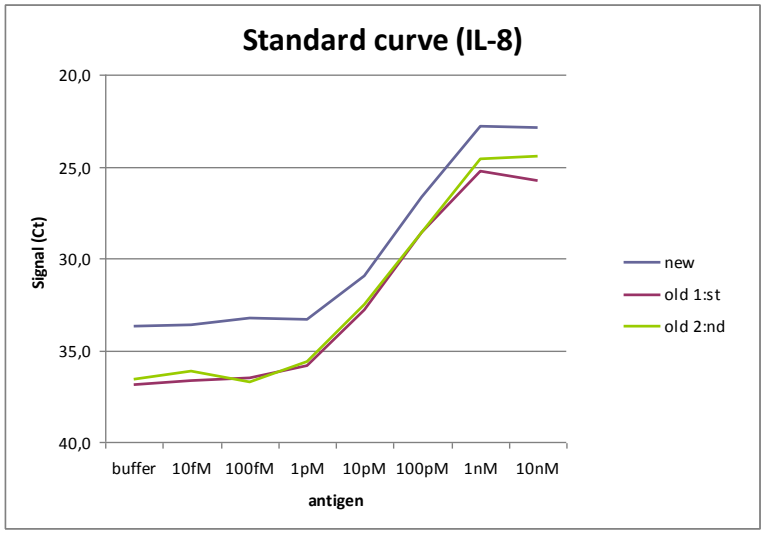


Figure 4. Illustration of a typical 8-points standard curve of IL-8. Two sets of old probe designs were tested against a new probe design.

By looking at *figure 4*, it can be stated that the new probe design have higher detection signal and the two older probe designs have almost identical standard curves. The two older probe sets have the best sensitivity since they have a more extended exponential phase, allowing measurement of the lowest concentrations of the antigen at 1 pM. A low concentration of the antigen is correlated with a high Ct value which can be seen by looking at the Y-axel. The Y-axel has reversed order of the values in order to get an easier understanding of the reaction events. One event occurring is the hook-effect which happens at around the same concentration, for all probes. When the concentration of antigen reaches 1nM, the exponential phase changes to a plateau that begins to decline. Now the DNA is amplified at a lower rate the reaction running out of necessary components. The primers will limit the molecular beacon's binding capacity to the template and when the molecular beacon do not bind any target it will not emitt fluorescence, seen in the graph as a declining signal.

Sensitivity tests to reduce background by studying combination of polymerase and exonuclease activity in single plex assay

In order to test how the sensitivity of the assay is affected by polymerase and exonuclease activity, an enzyme test is made, with 16 concentration combinations of Klenow fragment and Exo I. Probes directed against VEGF are used at 50 pM. Klenow fragment has 5´ to 3´ polymerase activity but lacks 3´ to 5´ exonuclease activity. Exo I has 3´ to 5´ exonuclease activity. The outcome should be to find the ultimate mix between the two enzymes in order to reduce background and sustain signal for better sensitivity.

Table 1. dCt values for sample spiked with 100 pM antigen with 16 concentration combinations of Klenow fragment and Exo I. The highest concentration is 1 and lowest 4. Klenow fragment is the first number and Exo I is the second number.

1:1	**1:2**	**1:3**	**1:4**	**2:1**	**2:2**	**2:3**	**2:4**	**3:1**	**3:2**	**3:3**	**3:4**	**4:1**	**4:2**	**4:3**	**4:4**
7.3	6.7	4.9	3.9	7.7	7.0	5.6	4.6	6.1	6.0	6.3	5.9	7.9	8.0	7.5	7.5

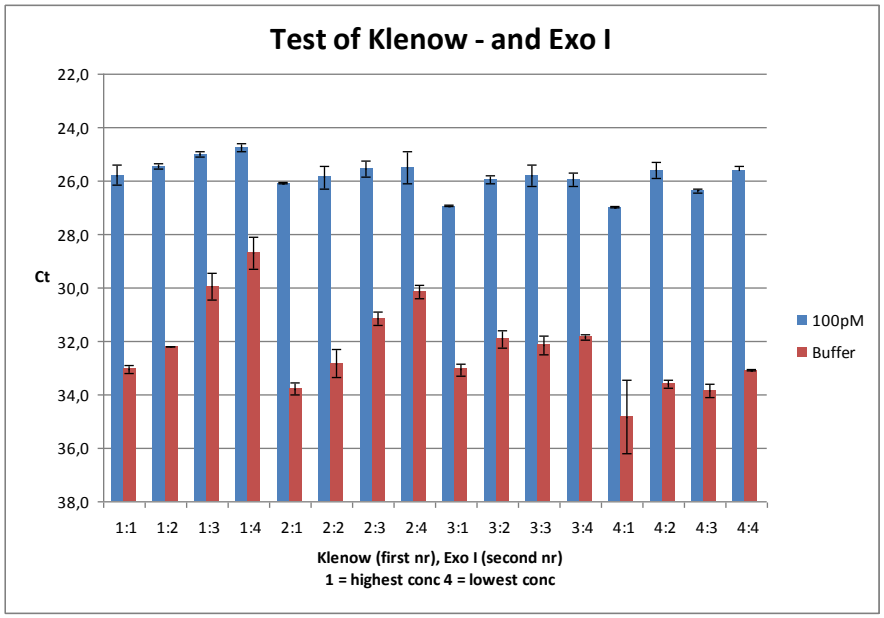


Figure 5. Enzyme test of Klenow fragment, with polymerase activity and Exo I, with 3´ to 5´ exonuclease activity. VEGF probes are used. The more Klenow fragment used the more Exo I is needed to get high signal to noise.

Results from *figure 5* shows that when the concentration of Klenow fragment is high, the more Exo I is needed to give highest signal to background. This can be seen as a high dCt value, in *table 1*. A low concentration of Exo I results in high background and low dCt value. A low concentration of Klenow fragment will be unaffected by the Exo I.

Another sensitivity test was made in single plex regarding the polymerase and exonuclease activity. This experiment is a variant of the previous sensitivity test but now two different probe concentrations are tested to see if background signal increases with increased probe concentration. 8 concentration combinations are used of T4 and Exo I. T4 polymerase is used having the same polymerase and exonuclease activity as Klenow fragment. Probes directed against VEGF are used with either the normally used probe concentration 50 pM or with a five times higher concentration at 250 pM. Enzyme T4 is used at concentrations of 1 and 0.2 units. Exo I is used at concentrations 0.4, 0.08, 0.016 and 0 units.

Table 2. Shows the dCt values of samples with concentration combinations of T4 and Exo I. Samples that have lower probe concentration give a higher dCt value. Number 1 illustrate the highest concetration and 4 the lowest concentration.

[T4] : [Exo I]	**Probe [50pM]+ VEGF**	**Probe [250pM]+ VEGF**
1:1	8.5	6.4

1:2	8.7	6.2
1:3	8.3	6.2
1:4	8.7	6.6
2:1	8.6	5.6
2:2	8.3	6.9
2:3	8.8	6.2
2:4	7.5	6.2

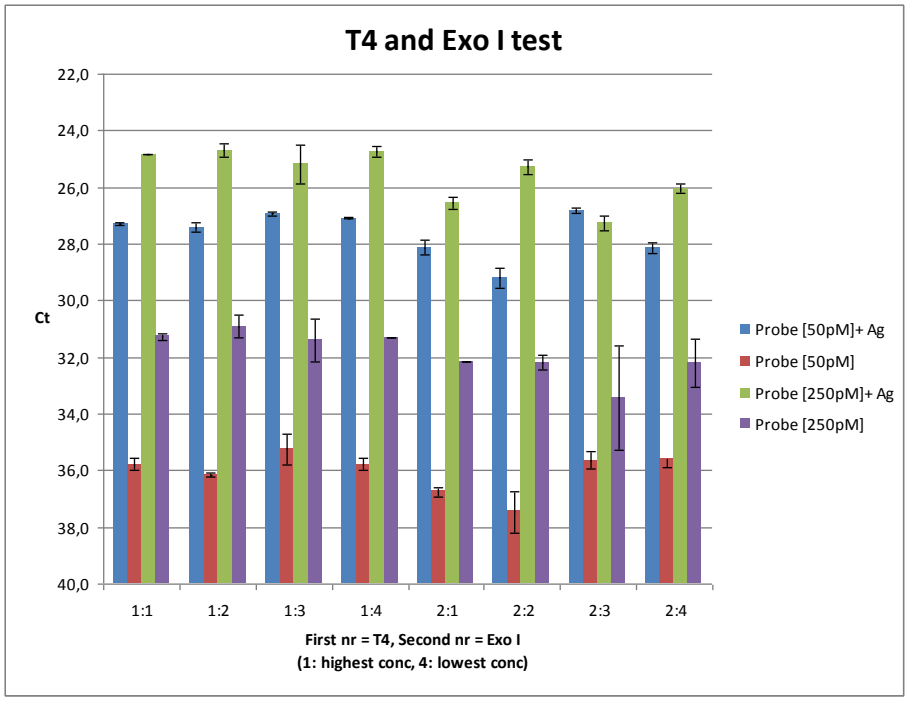


Figure 6. Samples with different concentration combinations of T4 and Exo I. Probes specific for VEGF are used at two different concentrations, 50 and 250 pM. Higher probe concentration leads to higher background as well.

Figure 6 shows that high probe concentration increases the signal but also the background level. The dCt for the samples can be seen in *table 2.* dCt is lower for higher probe concentration since background also increases more. There is no great difference in signal in samples with T4 polymerase when Exo I is added or not.

Optimization of sensitivity in multiplex by testing different proximity probe concentrations

To evaluate how the sensitivity in a multiplex assay is affected by changing the probe concentration, a test is made in 8-plex with the use of 4 different probe concentrations; 25, 50, 250 and 500 pM. This is done to see how the signal for background is correlated with an increased probe concentration.

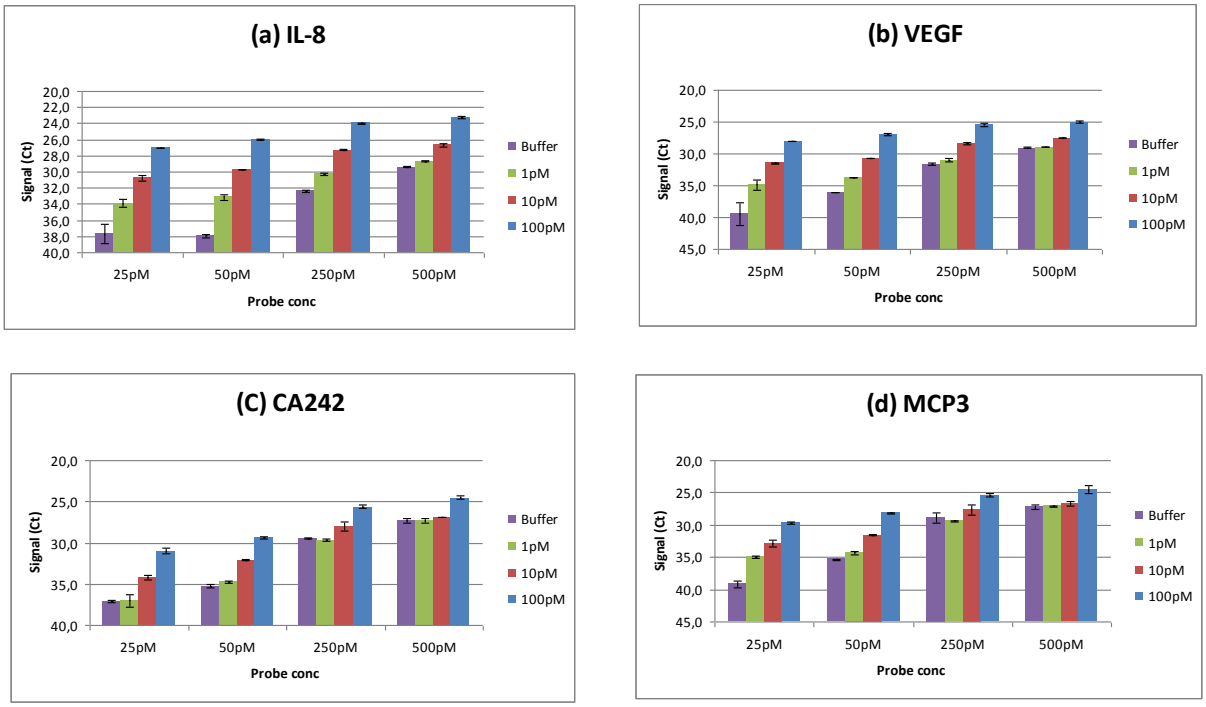


Figure 7. Shows 8-plex assay with 4 different probe concentrations where the two lowest (25 and 50 pM gave highest signal to background. Only detection with 4 primers are shown. Detection with primers for IL-8 can be seen in (a), VEGF in (b), CA242 in (c) and MCP3 in (d).

Detection with qPCR was only made with primers for IL-8, MCP3, VEGF and CA242. Results from *figure 4a-d* show that probe concentration 25 and 50 pM worked well for all assays whereas concentrations 250 and 500 pM gave very low signal to background.

Optimization of sensitivity in multiplex by testing different hybridization oligo concentrations

It is not known how many oligos that are actually attached to each antibody. Previous studies have shown that an increased amount of oligo can increase the signal but also the background. Optimization of hybridization oligo concentration was done, in order to get a higher sensitivity without an increased background signal. 4 concentrations of hybridization oligo were analysed against 2 different probe concentrations. Normally, 2x hybridization oligo is hybridized to the probe. This means that when the oligo is conjugated to the antibody, there is twice, 2x, the amount of oligo compared to antibody. Now 2x, 4x, 6x and 8x hybridization oligo is tested in an 8-plex assay. The probe concentrations chosen were the ones that gave the best result in previous experiments; 25 and 50 pM.

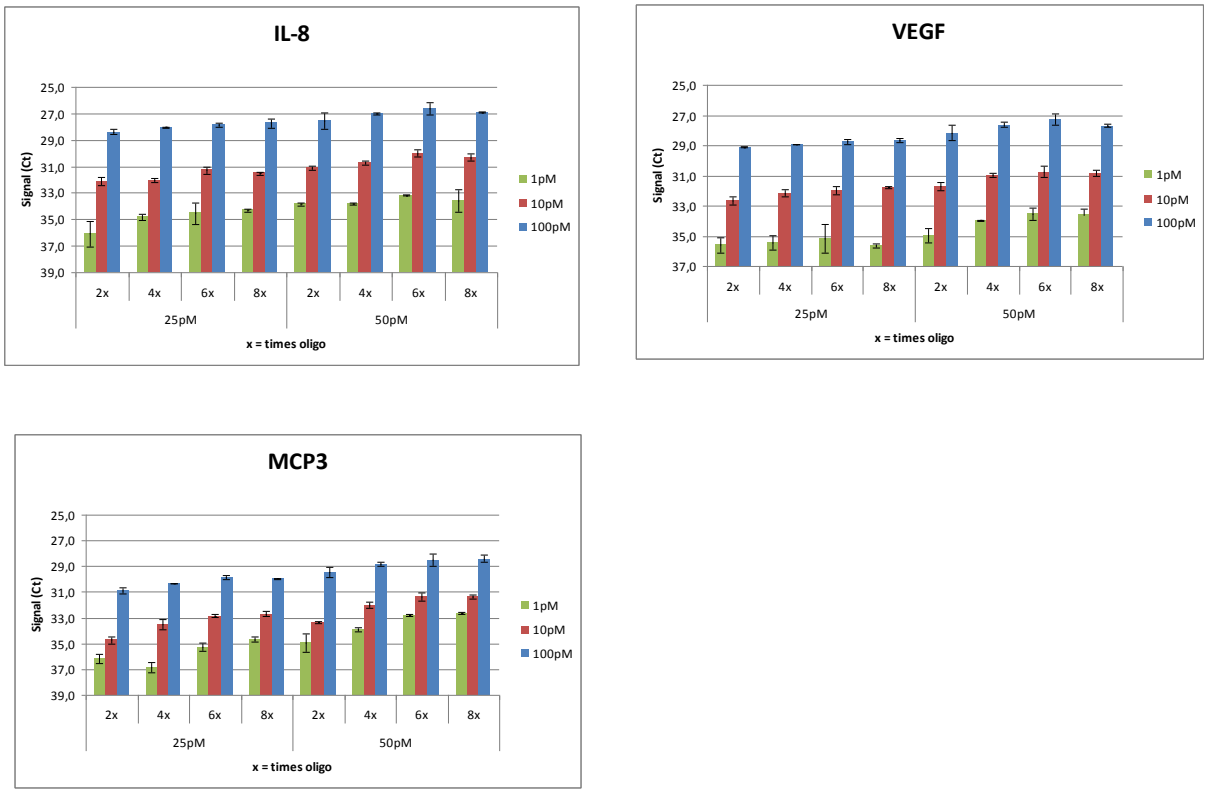


Figure 8. Test of 4 different concentrations of hybridization oligo -2, 4, 6 and 8x. The highest signal to noise was achieved with concentration 6x at 50 pM probe concentration for all assays. Detection can be seen for primers against IL8, VEGF and MCP3. Background values are eliminated due to too high standard deviations.

Detection with qPCR was made with primers for IL-8, MCP3 and VEGF. The highest signal for all assays was observed when using 4-6x hybridization oligo concentration and 50 pM probe concentration, which can be seen in *figure 8*. When hybridization oligo concentration increased to 8x, the signal to background decreased. The variation between buffer samples makes the results unreliable and they are therefore excluded from the figures.

Validation of qPCR primer efficiency in multiplex

Depending on how the qPCR primer has been designed, there will always be a difference in for example annealing temperature, how well they hybridize to the template or other primers resulting in primer-dimer formation and these factors correlates with qPCR amplification efficiency and sensitivity. For this experiment, pre-amplified material was used (which was run 15 cycles) and a 2-fold dilution is made in order to measure the efficiency in an 8-plex assay.

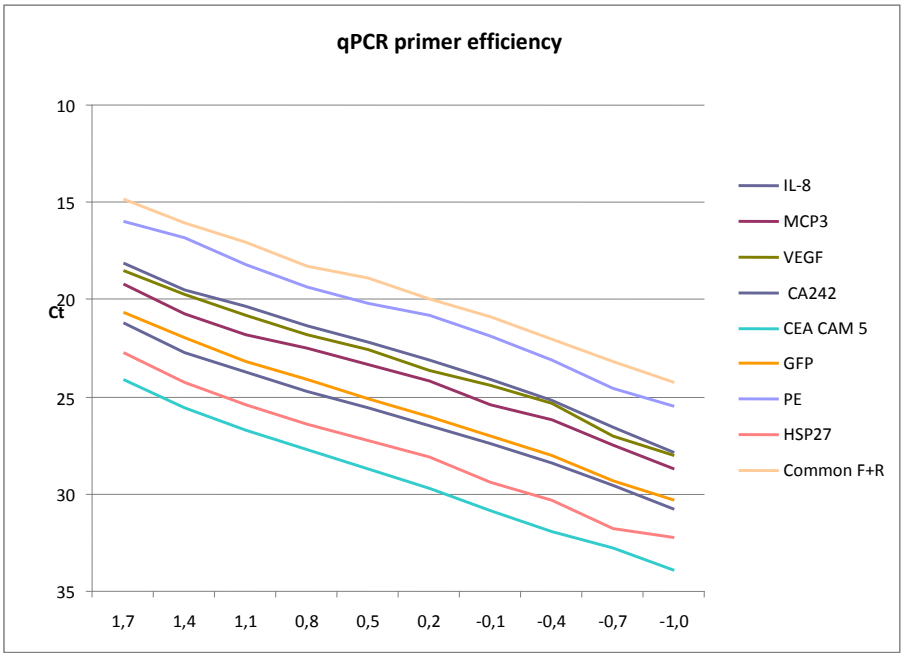


Figure 6. Shows a test of qPCR primer efficiency.Primers concentration is shown at the X-axle. More diluted samples give lower signals which can be seen in the figure as higher Ct values. Primers for CEACAM5 gave lowest signals.

The highest and lowest values of the dilution series gave unstable values and were excluded. The results from *figure 9* showed that a more diluted sample gives lower signal, a higher dCt value. The qPCR efficiency lies around 100%, which can be seen in *figure 10*. The assay that gave the highest qPCR efficiency was MCP3 and lowest was observed for HSP27.

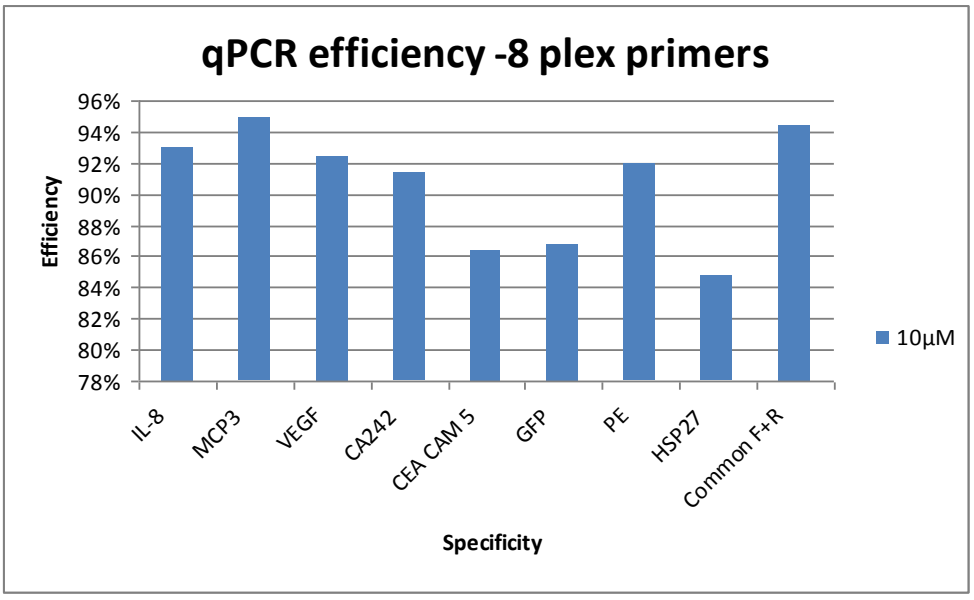
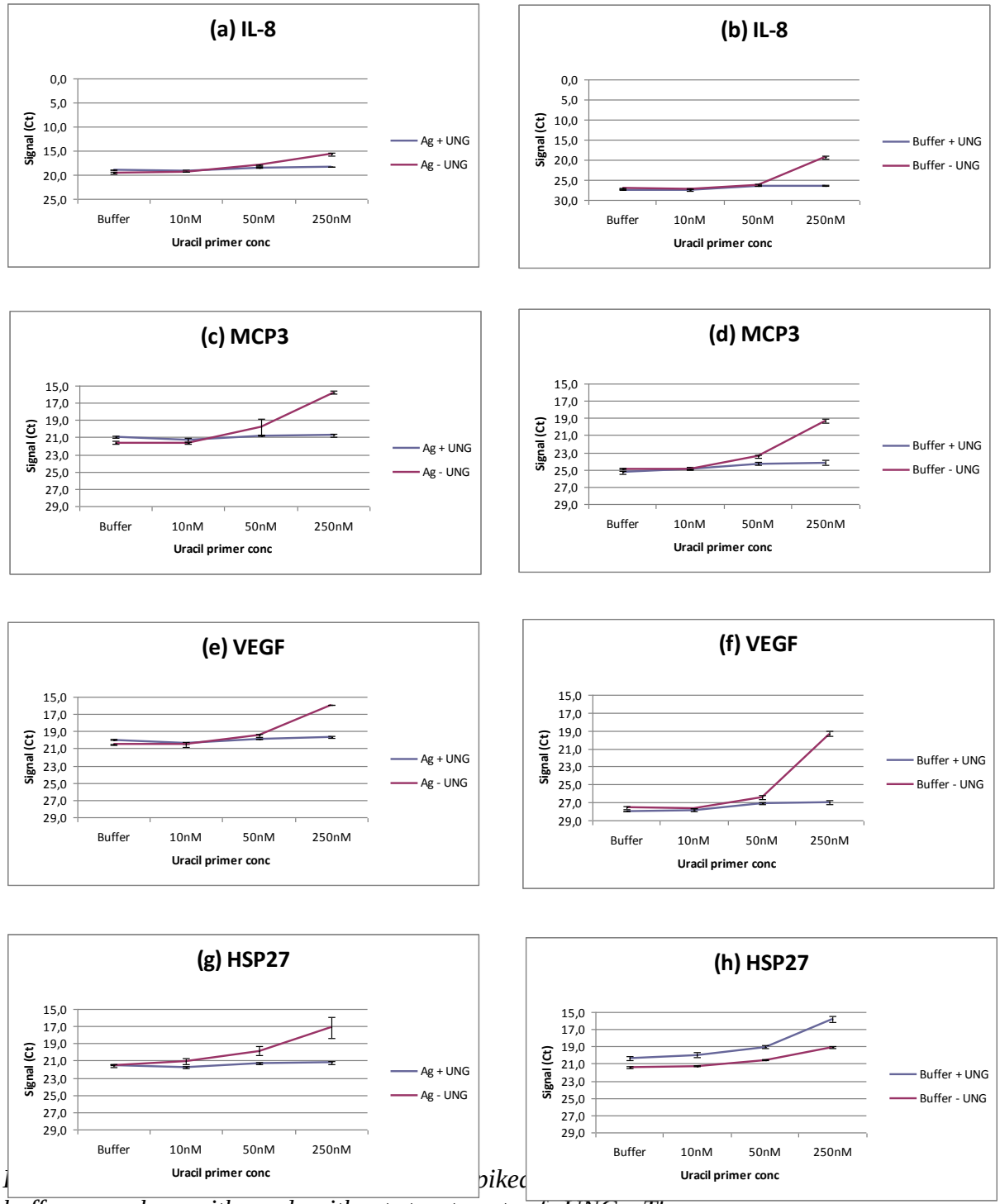


Figure 7. Evaluation of qPCR primer efficiency.

Specificity test by the use of UNG treatment in order to study the interference in qPCR at an increased pre-amplification primer concentration, performed in multiplex

UNG digestion is an essential step after pre-amplification so the pre-amplification primers will not interfere with the qPCR reaction by amplification of other analytes. Pre-amplification primers were incubated

with and without UNG. Different concentrations of pre-amplification primers were used; 0, 10, 50 and 250nM to validate the effect of unspecific amplification in qPCR. Pre-amplified samples in 8-plex were used with and without 100 pM spiked antigen.



buffer samples, with and without treatment of UNG. The assay was performed in multiplex and analysis of IL8 in spiked samples can be seen in (a) and in buffer samples in (b). Analysis of MCP3 can be seen in (c) and (d), VEGF in (e) and (f) and HSP27 in (g) and (h). Signal does not increase at higher primer-spike in concentration when treated with UNG.

Detection was made with primers for IL-8, VEGF, MCP3 and HSP27. Results show that when UNG is added to the samples, regardless of spiked antigen, the signal does not increase much compared to samples without UNG which can be seen in *figures 11a-h*. Even if the primer concentration increased, the signal will not get higher in presence of UNG. The opposite

happens in absence of UNG, when primer concentration increases also the signal gets higher. UNG seems to be working fine at 250 pM of primer concentration. The buffer samples will give around 5 Ct lower signal. The *figures 8g and h*, for HSP27, should look the same since there is no difference between them, none of the reactions contain HSP27 antigen. Probably the buffer samples have been mixed or contaminated with UNG.

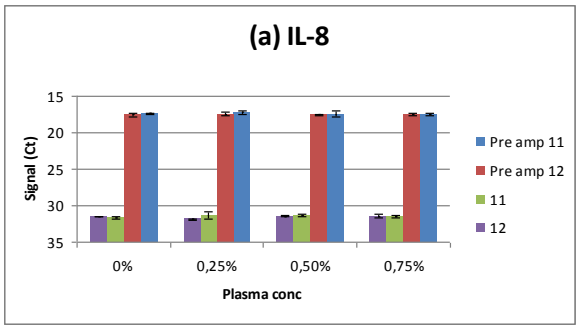
Recovery test of pre amplification by the use of different plasma concentrations, performed in multiplex.

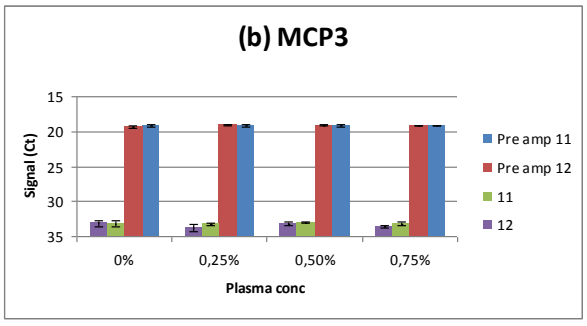
Recovery test of plasma samples, comparison between before and after pre amplification. When using a 100% pure plasma sample, the resulting plasma concentration is 0.5% in pre-amplification mix. In this experiment, the plasma samples were spiked with four concentrations of plasma; 0, 0.25, 0.5 and 0.75%, to study the inhibition of amplification. These concentrations where chosen in order to see how a lower or higher concentration of plasma would influence the assay performance and if a more diluted sample would result in higher sensitivity.

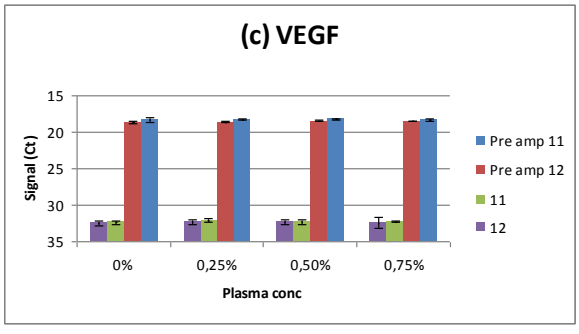
Table 3. dCt values before and after pre amplification for IL8, MCP3, VEGF and HSP27. Regardless of plasma concentration added, all reactions showed a dCt of 14.

	IL8		MCP3		VEGF		HSP27	
	11	**12**	**11**	**12**	**11**	**12**	**11**	**12**
0.75%	14.0	13.9	14.0	14.4	13.9	14.0	13.7	13.9
0.50%	13.9	13.9	13.8	14.1	14.1	13.9	16.1	15.4
0.25%	14.1	14.4	14.0	14.7	13.8	13.8	13.6	15.6
0%	14.3	13.9	14.0	13.8	14.1	13.8	15.7	14.5

Detection was made with primers for IL-8, VEGF, MCP3 and HSP27. The results from *figure 12*, show a 14 Ct difference between samples with and without pre-amplification (also see *table 3)*. No clear difference between samples spiked with high or low concentration of plasma was oberved.







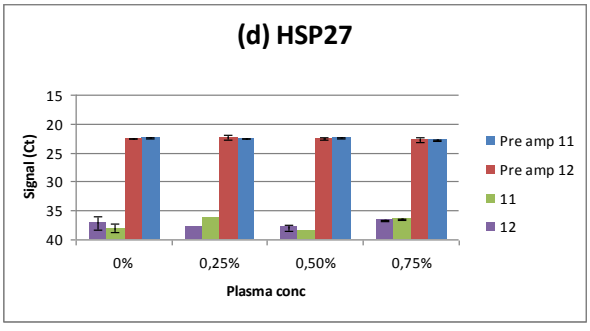


Figure 12. Recovery test of IL-8, VEGF, MCP3 and HSP27 before and after pre-amplification in the presence of different concentration of plasma. No inhibition of samples by plasma occurs in any assay.

Comparison between experiment setups in the 96 vs 384 format

When performing PEA, both a 96 and 384 well microtiter plate can be used. The positive aspect of using 384 formats is that 4 times as many samples can be analyzed compared to 96 formats. The concentrations of all components needed for PEA will be the same in both formats but since a 384 well microtiter plate has smaller wells the volumes have to be reduced in order to fit in the wells. Additional pipetting step in the extension is therefore needed to get the same dilution (1/100) of the reaction as in 96 format.

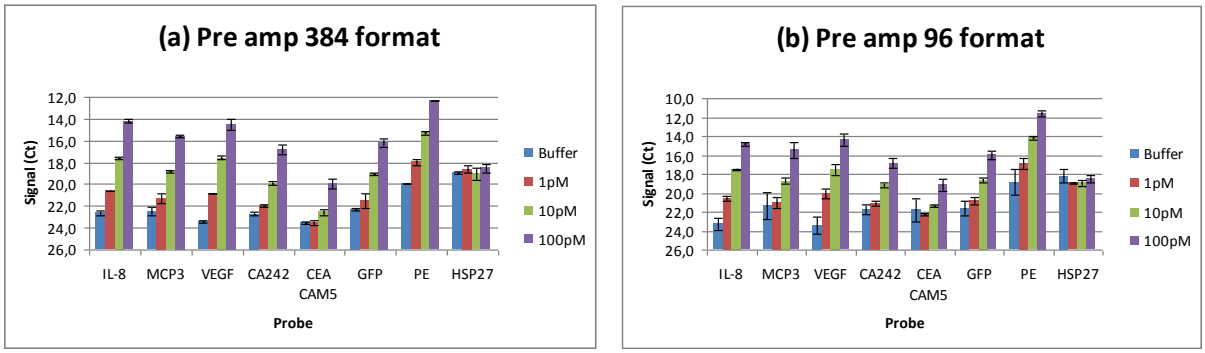


Figure 13. qPCR result for 8-plex in (a) 384 format and (b) 96 format.

Detection was made with primers for IL-8, VEGF, MCP3, CEACAM5, CA242, HSP27, PE and GFP. *Figures 13a and b* show that 384 format just as sensitive as 96 format, both figures show the same trend in signal for all eight detections.
Regarding the pre-amplification experiment; samples are diluted 1 time before pre amp (10+10) and two times after pre amp (15+15 and 30+30) which should result in a theoretical signal decrease of 3Ct. The Ct difference between samples with and without pre-amplification of 15 cycles is 14 Ct. After the dilution steps the expected Ct difference will be: 14-3= 11Ct.

Recovery test of plasma samples with known low recovery values, performed in multiplex

A recovery test was made to compare buffer and plasma samples that are known to have low recovery values. The samples were spiked with 0, 10 or 50 pM antigen and the assay was performed in 16-plex.

Table 4. Recovery test for 16-plex assay with recovery values in %. Results show strange values due to already high protein concentration in the samples.

Plasma sample	KNG1	GDF-15	CD55	METAP1	XIAP
A	18.1	51.0	76.3	104.1	20.1
B	-34.5	46.5	9.5	107.9	41.5

The detection was made with primers for KNG1, GDF-15, CD55, CCK4, CAT, MRC2, METAP1, RACK1, Nbs1 and XIAP, however without

antigen available for CCK4, CAT, MRC2, RACK1 and Nbs1. The recovery was good for METAP1, almost 100% which means that there is no recovery loss (see also *table 4).*

Optimization of amplification efficiency in qPCR by the use of different magnesium concentration, performed in multiplex

This test was made to optimize the amplification efficiency in qPCR by using different magnesium concentrations performed in 16-plex assay. Magnesium is needed as a cofactor for the thermostable polymerase to be active and to synthesise successfully. With a low magnesium concentration in qPCR, the polymerase will be less active and the reaction will be slow. High magnesium concentration leads to a more efficient reaction and also contributes to make the double stranded DNA more stable. On the other hand, a too high concentration will make the polymerase work very fast, which may result in a rapid reaction, but also incorporation of more errors in amplification leading to unspecific strands being amplified. Therefore it's important to find an optimal magnesium concentration to get the highest reaction efficiency.
Pre-amplified buffer samples spiked with 50 pM antigen were used and an 8-fold dilution series were made in order to calculate the efficiency. The magnesium concentrations in the final qPCR reaction were 3, 5 and 7mM (normal) to evaluate which concentration that would give optimal efficiency. The primer concentration was changed from 0.5µM to 0.9µM.

Table 5. Shows a test of amplification efficiency when using different magnesium concentrations in qPCR in a less detailed table. Values of efficiency are shown in %. Highest efficiency was achieved with lowest magnesium concentration, 3mM.

7mM MgCl								5mM MgCl		3mM MgCl	
KNG1	**GDF-15**	**CD55**	**CCK4**	**CAT**	**MRC2**	**METAP**	**XIAP**	**KNG1**	**METAP**	**KNG1**	**METAP**
78%	84%	86%	91%	84%	91%	92%	91%	96%	92%	103%	95%

The detection was made with primers for KNG1, GDF-15, CD55, CCK4, CAT, MRC2, METAP1 and XIAP. By comparing the efficiency at the different magnesium concentrations, the best primer efficiency showed to be at 3mM $MgCl_2$ where the average efficiency was almost 100%, which can be seen in *table 5*. The other magnesium concentrations also worked well for all detections. It was no clear difference between the different magnesium concentrations when detecting METAP1.

The optimization experiments of the effect of different magnesium concentrations were continued but now additional assays where run in qPCR to study detection of additional antigens at different magnesium concentrations. In the previous experiment only two assays where analyzed in qPCR at magnesium concentrations 3 and 5mM. Additional assays were performed to get more information and to see if previous results were repeatable.

Table 6. Amplification efficiency with magnesium concentration 7mM in qPCR. Average efficiency was 87%.

Arbitrary units	**Original log**	**KNG1**	**GDF-15**	**CD55**	**CCK4**	**CAT**	**MRC2**	**METAP**	**RACK1**	**Nbs1**	**XIAP**

25	1,4	20,3	17,3	18,9	23,9	20,4	17,2	22,6	21,7	18,3	22,6	
12,5	1,1	21,9	18,8	20,2	25,6	21,7	18,5	24,1	22,7	19,6	24,1	
6,25	0,8	23,1	19,3	21,4	27	22,8	19,8	24,8	24,1	21,1	25,3	
3,125	0,5	24,3	20,8	22,9	27,3	24,1	20,8	26,1	25,4	22,2	26,3	
1,5625	0,2	25,4	22,1	23,9	29	25	21,9	26,7	26,3	23,3	27,5	
0,78125	-0,1	26,3	23,3	24,9	29,9	26,3	23,2	27,7	27,3	24,4	28,4	
0,39	-0,4	27,8	24,3	26	31,6	27,4	24	28,9	28,8	25,2	29,4	
0,195	-0,7	28,6	25,1	26,5	32,6	28,4	24,6	29,5	28,9	26,4	30,4	average
	slope	-3,87	-3,76	-3,7	-4,01	-3,76	-3,59	-3,24	-3,6	-3,78	-3,62	-3,69
	exponential amplification	1,81	1,84	1,86	1,78	1,84	1,9	2,03	1,9	1,84	1,89	1,87
	Efficiency	81%	84%	86%	78%	84%	90%	103%	90%	84%	89%	87%
	R2	1	0,99	0,99	0,99	1	0,99	0,99	0,99	0,99	0,99	0,99

Table 7. Amplification efficiency with magnesium concentration 5mM in qPCR. Average efficiency was 91%.

Arbitrary units	**Original log**	**KNG1**	**GDF-15**	**CD55**	**CCK4**	**CAT**	**MRC2**	**METAP**	**RACK1**	**Nbs1**	**XIAP**	
25	1,4	21,9	18,7	19,9	25,7	21,7	18,2	23,8	22,3	19,1	23,8	
12,5	1,1	23,2	19,7	21,2	26,7	22,7	19,5	24,9	23,6	20,7	24,7	
6,25	0,8	24,3	20,9	22,4	27,9	24	20,6	25,9	24,7	21,8	25,9	
3,125	0,5	25,6	22,2	23,5	28,9	24,8	21,8	27,1	26	23	27,1	
1,5625	0,2	26,4	23,2	24,5	29,8	26	22,7	27,9	27,2	24	28,4	
0,78125	-0,1	27,6	24,1	25,5	30,9	27,2	24	28,6	28	25,1	29	
0,39	-0,4	29	25,3	26,4	32	28,3	24,8	29,7	29,1	26,1	30,6	
0,195	-0,7	29,6	25,9	27,8	33,4	29	26	30,4	29,9	26,9	31,8	average
	slope	-3,69	-3,55	-3,63	-3,58	-3,54	-3,64	-3,12	-3,59	-3,64	-3,81	-3,58
	exponential amplification	1,87	1,91	1,89	1,9	1,92	1,88	2,09	1,9	1,88	1,83	1,91
	Efficiency	87%	91%	89%	90%	92%	88%	109%	90%	88%	83%	91%
	R2	1	1	1	1	1	1	1	0,99	0,99	0,99	1

Table 8. Amplification efficiency with a magnesium concentration of 3mM in qPCR. All values were flagged/marked in the qPCR machine meaning the Ct values are unreliable because the curves are too flat or show unstable detection measurement, therefore only the values of amplification efficiency are shown.

KNG1	**GDF-15**	**CD55**	**CCK4**	**CAT**	**MRC2**	**METAP**	**RACK1**	**Nbs1**	**XIAP**	**min**	**max**	**average**
77%	89%	89%	83%	91%	99%	83%	91%	96%	94%	77%	99%	89%

The results showed that all the Ct values for samples with magnesium concentration 3mM were flagged in the qPCR, meaning that they are not reliable and the figures were also flat, meaning low signal to background, compared to the figures for 5mM and 7mM magnesium. The efficiency values can be seen in *table* 8. The average efficiency for 7mM was both in the previous experiment, and now 87%, which can be seen in *table 7*. The average efficiency for 5mM was in previous experiment 94% and now 91%, which are both good values, which can be seen in *table 7*.

Validation of normalization in multiplex and comparison between qPCR mixes

Normalization is made to minimize the variation and quantification errors in samples. First experiment was made in an 8-plex assay were normalization was done with GFP, PE and oligo139. GFP, Green Flourescent Protein, and PE are no human proteins and will not occur naturally in plasma. Oligo139 is a fully extended product and function as a positive extension control. Since oligo139 is already extended it does not have to be incubated with probes and therefore it will be less variation for number of amplicons in these samples. The normalization is done by dividing the number of amplicon for target protein with the value for protein that is used for normalization. GFP was choosen to be normalized against since that is a protein that is not naturally occuring in the human body.

Table 9. Shows the %CV values for the number of amplicons for Oligo139, GFP and PE without normalization.

Oligo139	GFP	PE
50	77	85

Table 10. Shows %CV values for the number of amplicons after normalization with Oligo139, GFP and PE. All values are lower than compared with values before normalization.

Oligo139-normalized		GFP-normalized		PE-normalized	
GFP	PE	Oligo 139	PE	Oligo 139	GFP
24,1	31,2	22,8	8,9	27,1	9,2

The results from *table 9 and 10* shows that after the samples have been normalized they have lower %CV value. In other words, the variation in the samples within a series has decreased.

A test of the effect of normalization was performed in a 32-plex assay with 7 plasma samples. The final concentration of GFP was 25 pM. Also, commercial qPCR mix "FUMM" was compared to qPCR mix with Platinum taq polymerase with a magnesium concentration of 5mM. Platinum taq polymerase was used because it is cheaper and usually used in qPCR since it extends DNA well.

Table 11. Variation of number of amplicons before and after normalization in 32-plex assay. %CV is higher after GFP-normalization.

%CV before normalization				
GFP	MCP3	METAP1	XIAP	IL-1β
13.4	10.5	24.4	14.2	25.8
%CV after GFP normalization				
MCP3	METAP1	XIAP	IL-1β	
13.9	21.2	15.1	40.5	

Table 12. Recovery (%) before normalization in 32-plex assay, values of recovery shown in %. Recovery is higher before GFP-normalization.

Sample	GFP	MCP3	METAP1	XIAP	IL-1β	Average
1	123	133	167	66	117	121
2	105	111	248	68	160	138

3	145	142	288	82	111	154
4	105	122	164	73	101	113
5	120	112	184	67	99	116
6	157	121	260	103	59	140
7	80	104	199	64	97	109

Table 13. Recovery (%) after normalization in 32-plex assay. Recovery is lower after GFP-normalization.

Sample	MCP3	METAP1	XIAP	IL-1β	Average
1	111	106	20	69	77
2	110	56	14	137	79
3	97	29	-5	57	44
4	93	117	-5	63	67
5	90	56	-4	64	52
6	83	93	2	13	48
7	80	12	-7	68	38

Normalization of plasma sample with GFP increased the variation in %CV compared to the value before normalization which can be seen in *table 11*.

Table 14. Comparison in number of amplicons between qPCR run with either FUMM mix or with Platinum Taq (normally used in the assay). Results indicate that the use of Platinum Taq gives a has lower %CV.

qPCR mix	Row 2	Row 4	Row 6	Row 8	Row 10	Row 12	Row 14	Row 16	Row 18	Row 20	Row 22	Row 24	Average
Platinum Taq	8	12	2	15	8	11	8	9	9	4	10	3	8
FUMM	14	9	12	14	22	15	16	8	9	14	18	8	13

Results show that samples with Platinum Taq had lower %CV than FUMM (*table 14)*. There is no clear trend over the qPCR plate why some values are higher than others.

Specificity test, comparison between single and multiplex

An evaluation of the specificity of IL-8, MCP3, KNG1, GDF-15, CD55 and VEGF, comparison in single plex (Proseek), 8-plex and 32-plex. This specificity test was performed to investigate if the specificity changed when performing multiplex and if the number of probes would have an effect on the specificity of the assay specificity.

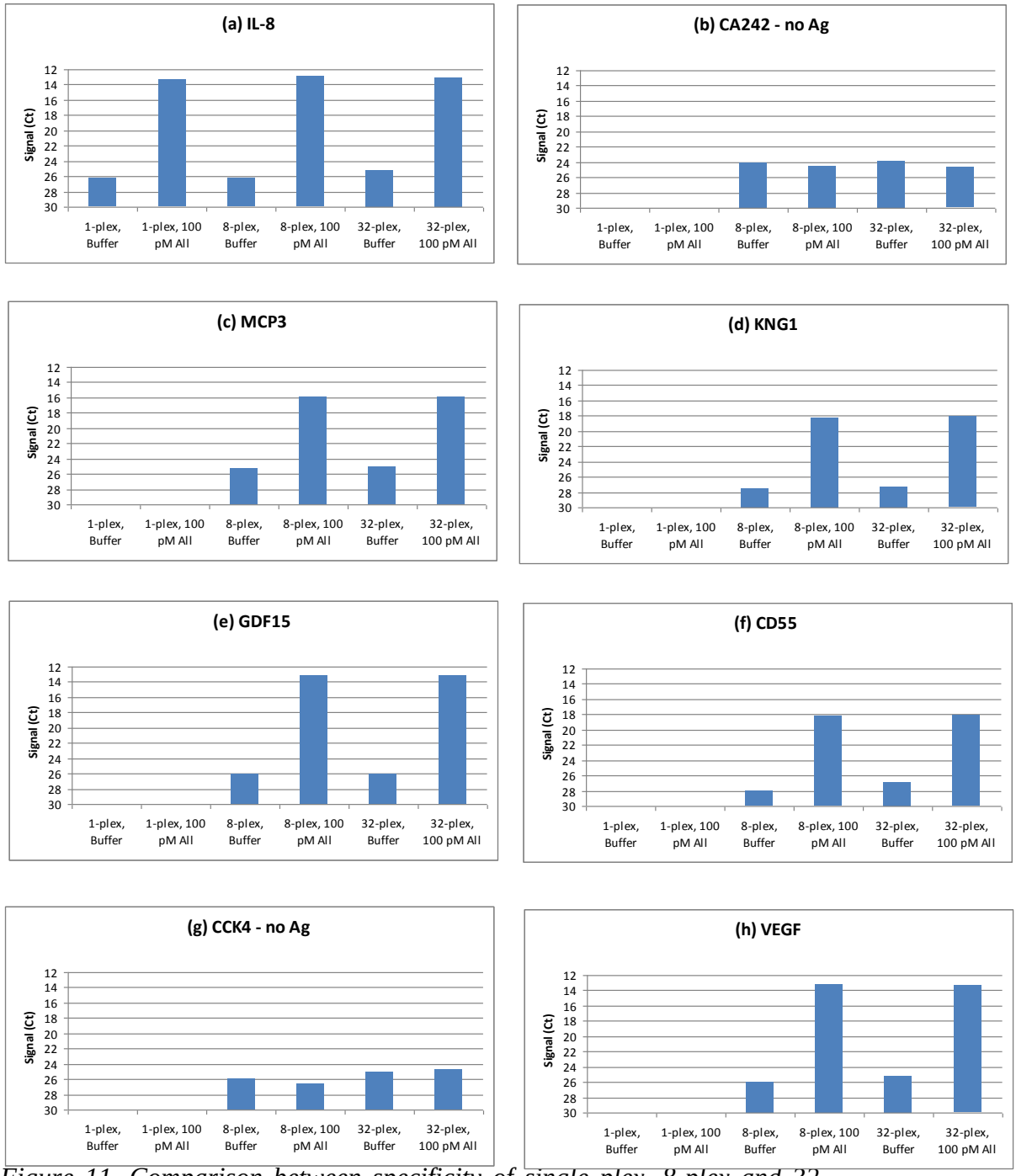


Figure 11. Comparison between specificity of single plex, 8-plex and 32-plex of probes detecting (a) IL-8, (b) CA242, (c) MCP3, (d) KNG1, (e) GDF15, (f) CD55, (g) CCK4 and (h) VEGF). Results show no significant difference in specificity between single plex and multiplex, with the exception of IL-8, where there was significant differences in signal between single and multiplex.

Comparison between single and multiplex assay showed that there was no significant difference in specificity between 8-plex and 32-plex in any assay seen in *figure 11*. All values of signal to noise, with 100 pM spiked antigen, were around 13Ct showing a high detection signal, wtih the exception of CA242 and CCK4. The only assay performed in single plex was IL-8, *11a*, which showed a significant difference between single plex, 8-plex and 32-plex (*figure 11a*).

Discussion

Sensitivity tests in single and multiplex

Increasing sensitivity is an important issue in method development. In this work, the assay optimization was done to maximise the signal to background *i.e* increasing the signal without increasing the background. Achieving a balance between polymerization and exonuclease activity is important for that reason. In high concentration, Klenow fragment exhibits polymerase activity which will lead to a high degree of extensions, both signal and background. Exo I, which specifically digest single stranded DNA, will on the other hand reduce background which otherwise will be amplified due to high polymerase activity of Klenow fragment. The experiment was done to find a balans between polymerization and exonuclease activity. The exonuclease activity should not be too high, since this will lead to a lower signal, which can be seen in *figure 5*. When there are high concentrations of both enzymes, *i.e.* Exo I and Klenow fragment, Exo I will digest oligonucleotides bound to antibodies regardless of antigen binding. This is due to "on and off"-hybridization between the hybridization oligo and oligonucleotide coupled to the b probe. Even if some proximity probes are attached to antigens, the oligonucleotide part of the probe will be digested if it is not in the moment hybridized to a hybridization oligo and will therefore be degraded of Exo I as ssDNA. The results show that when there is a low concentration of Klenow fragment, the reaction is not affected much by the concentration of Exo I. This is probably because the polymerase activity is low leading to a "slow" reaction and the exonuclease activity has time to reach the same speed. Although too high concentration of Exo I leads to lower signal in general.

Results from *figure 6* show that high probe concentration increases both signal and background. Although, the reaction becomes more robust if the background signal increases from 40 Ct which shows a low detection signal it will be low number of template randomly transferred from PEA to detection in qPCR. There is no clear effect from between adding Exo I to samples with T4 polymerase and this is probably because T4 polymerase has a strong exonuclease activity itself. However, if there is an excess of Exo I, it will compete with T4 polymerase for the DNA strands and due to "on and off"-hybridization, it will cleave the strands that are not hybridized at the moment and also probes that are not bound to an antigen. This will result in a lower signal, even for the background.

Optimization of sensitivity in multiplex

The more components you add to a reaction usually the more complicated it gets because there is a larger number of interactions and events that can go wrong. In order to test the sensitivity of PEA in multiplex, different probe concentrations were tested in 8-plex assay. The 8-plex assay was done before proceeding to 96-plex. The probe concentrations that gave highest sensitivity, in other terms the highest signal to background were 25 and 50 pM. The same was found for all assays tested i.e. IL-8, MCP3, VEGF and CA242, *figures 7a-d*. When probe concentrations were higher, the signal but also the background increased. Possibly the background for IL-8 (*figure 7a),* should have been higher at 50 pM because of the increasing signal trend that can be seen in background signal at probe concentration 25 to 250 pM. There is a 5 folds concentration difference between 50 pM and 250 pM and it is possible that a concentration between them could have worked well,

since the signal and background would have been higher and resulted in a more assertive reading.

Optimization of an assay includes changing and testing a large number of parameters to get the desired outcome. By changing the concentration of the hybridization oligo, a higher signal can be achieved. The results from *figures 8a-c* all show that highest signal is achieved when having 6x hybridization oligo with 50 pM probe. The different assays showed differences in dCt for the highest signal. The variation between buffer replicates representing background was too high, the signal was also quite low therefore dCt values are not reliable. Probably a high, but not too high, hybridization oligo concentration leads to more oligo arms getting hybridized, more active probes that can detect more proteins which results in higher detection signal. In contrast, a too high concentration of hybridization oligo will increase the background. There are plenty of free hybridization oligo diffusing in the sample which coincidently could bind to probe b. This would lead to extension even without hybridization to probe a, and an increasing in background. When a protein sample is incubated with proximity probes, there will also be a lot of free probes that can interact. At a higher probe concentration, there will be an increased chance of interaction between free probes. Even if the probes are free and do not bind any antigen they will still be detected. Basically, background can be described as the extension events that are not proximity dependent, and not antigen dependent.

The efficiency of qPCR is an important factor for good assay performance since it determines how many copies of DNA that will be produced and also how much variability it creates between analytes. The average qPCR efficiency for 8-plex primers was good, between 86-95% meaning that almost all DNA in the reaction, for some primers, gets doubled in each cycle (*figure 10).* A more diluted sample gave a lower signal (*figure 9)* and this is quite obvious because it contains a lower concentration of DNA and will reach the threshold later in the qPCR reaction, which will result in a high Ct value. One reason why the assay for HSP27 gave such low efficiency has been discovered later in test. HSP27 has very “sticky” probes so in the incubation step free probes will be more prone to bind to each other creating a high background.

Pre-amplification primers are digested with Uracil-DNA glycosylase, UNG, in order to not interfere with the qPCR reaction. An experiment was done to study the effect of UNG treatment. When UNG was added to samples, the detection signal did not increase with higher primer concentration (*figure 11a-h).* This means that the enzyme is efficient in digesting primers and template that contains uracil bases, that otherwise will interfere with the qPCR reaction. When UNG is not added, there will be a higher detection signal at a high primer concentration. This is because the pre-amplification primers continue to amplify in the qPCR and compete with the qPCR primers of the energy in reaction. The pre-amplification primers are not assay specific and will amplify all analytes present in the particular sample and then be detected in the qPCR. In the qPCR, this will lead to one detection signal created by pre-amplification primers, that are not analyte specific and qPCR primers that are analyte specific. The result is seen as a too high / “false” signal of the analyte, (*figure 11a-h).*
The *figure 11g and h,* for HSP27, should look the same since both reactions are in absence of HSP27 protein. One reason that they don’t, could be that

one of the results was contaminated by samples that contained UNG since the two graphs are almost parallel in, *figure 8h*.

When samples are pre-amplified 15 cycles, it will give a 14 Ct lower signal which can be seen in *figure 12a-d*. Why it does not lead to a 15Ct increase is because one cycle will not be detected in the qPCR since the molecular beacon binds to the complementary strand and will not give any signal until one cycle has run and the complementary strand is amplified. There is no great difference between samples spiked with high and low concentration of plasma so one conclusion from the result is that the pre amplification reaction is not inhibited by plasma.

Another way to optimize the efficiency of the assay performance is to use a plate with more wells. This allows analyses of more samples than with a regular 96-microtiter plate increases the throughput and reduce the variation between samples when analyzed in the same plate. An experiment was performed that compared the same assay with two different plates; 96 vs 384-microtiter plate.
The pre amplification reaction is not dependent on which type of plate setup is used, 384 format will give just as high sensitivity as the 96 format for 8-plex assay, which can be seen in *figure 13a and b*. The figure for HSP27 shows a background that is just as high as the signal. Since there is no HSP27 protein in the reaction, this means that the probe doesn't bind unspecific to their antigens. The IL-8 samples that had not been pre-amplified showed very high Ct values. This is because the signal was too low and therefore undetectable.

Recovery and normalization
Recovery values can sometimes vary a lot between analytes (and different plasma samples) in recovery tests, which is illustrated in *table 4* and often it depends on the fact that the sample already contains a high concentration of the proteins of interest. This can be noticed by comparing Ct values for buffer and plasma samples. For example, for CD55 the Ct value for buffer is around 32 while in plasma sample 11, with no spiked antigen, the Ct value is 24.8 (results not shown). This means that there is already high levels of CD55 protein present in plasma. It shows that much higher levels of CD55 can be detected, way more than 50 pM. When the plasma sample, which already contains high levels of the protein of interest, is spiked with 50 pM antigen - no difference is seen in detection signal between spiked and unspiked sample. To make a correct determination of recovery, the plasma sample should not contain more of or the same amount of the antigen of interest, as it is spiked with. There is a high standard deviation for plasma sample 11, spiked with 50 pM antigen. One reason could be that the plasma sample was not homogeneous from the beginning.

In general, when values are flagged in qPCR or when the graphs of detection are flat, this means that there is too low signal to background. As shown in *table 8*, all the samples with the magnesium concentration of 3mM were flagged in the qPCR machine. A conclusion drawn is that the high efficiency values in the previous experiment, *table 5*, are unreliable.
The qPCR reaction can be influenced by a lot of factors. The beacon binds specifically to the template and there will in theory not be any signal if wrong sequence is amplified. However unspecific amplification events could "take energy" from the reaction. Components like ssDNA in the reaction mix can be amplified and if this occurs, this reaction can "steal" the

crucial components needed for the right amplification and then the reaction will not be as efficient as it can be. When too high magnesium concentration is added to the qPCR, unspecific primer binding can increase and lead to amplification of wrong sequences.

Normalization is done in order to identify samples showing too high or low detection signals. By normalizing samples, a more correct and easier quantification can be obtained. Experiments have been done with normalization events to see how variation will be affected. Normalization was done with GFP, PE and Oligo139. Since these proteins are not naturally occurring in plasma, the signal for these analytes should in theory not vary between plasma samples. In other words, a known average detection signal can be set. When you have samples with many different analytes, these known detection values can be used to correct the signal for other analytes if they are for example too low due to inhibition. When studying analytes of plasma from patients, normalization can be used in data handling in order to separate healthy from cancer sick patients. The results from *table 9 and 10* showed that the variation amongst different samples decreased upon normalization.
Normalization can sometimes increase the variation, which can be seen in *table 12 and 13* as higher %CV values. In this experiment the samples were normalized against GFP. Since GFP is not human, it will not be found in plasma that otherwise could contribute to a greater variation. When the values for different analytes were normalized against GFP this results in a greater spread of the values at each point meaning higher %CV. If replicates are used in qPCR, this pipetting variation will not be corrected by normalization.

As mentioned before, when adding more components to a reaction, more events, both specific and random, can occur. An experiment was performed to see how the number of probes in multiplex will affect the sensitivity of the assay. The results showed no significant difference in sensitivity between single plex, 8-plex and 32-plex (*figure 14a-h)*. Our conclusion is that the binding capacity of these probes is not inhibited or influenced by the number of probes added to the reaction. If it would have been a big variation in signal to noise values between single and multiplex, then the assay would have been influenced by the number of probes. The variation can depend on cross reactivity of probes, primers and also of antibody binding to wrong antigen (tests have been done in multiplex that prove that it would be unlikely to occur).

Conclusions

The purpose of this project was to investigate the possibility to optimize PEA into a high multiplex assay to find a way to separate healthy from colorectal cancer sick individuals already at an early stage. So far, we did not observe any problems by doing PEA in multiplex with new biomarkers. Even if there was only time to evaluate 32-plex assays the results showed no significant difference in sensitivity when comparing single plex with lower degree of multiplex. It is hard to forsee how the 96-plex panel will turn out and only further investigations can answer that question. Hopefully the continuation of adding probes will not lead to a less sensitive or specific signal. If problems in sensitivity would arise, the panel can be divided into several panels. If the problem concerns biomarkers that do not show any clear connection to the disease, they can be excluded and replaced with new biomarkers that hopefully will contribute tohigh sensitivity in multiplex if no more optimization can be done.

Cancer research is known to be complicated and when the molecular mechanisms behind the why or how cancer develops is not well known, it makes it even more difficult to find a technique that can discover cancer at an early stage. If it would be possible to create a 96-plex panel with the same sensitivity as single plex, it would be a step in the right direction. Even if it not will be used as a diagnostic tool, the technique and knowledge will be spread and increase the possibility to discover new biomarkers for cancer. By looking at such large numbers of analytes at the same time, you get a better general view that facilitates identification of trends in concentrations of specific analytes that can be connected to early progressed cancer.

Optimization is a wide concept, involving a lot of work. By doing a lot of optimization testing, I have learned that all parameters have to be treated as important until the opposite have been proved, to make the reaction, or the assay, more efficient. In most cases all the substances in the reaction affect or depend on each other. Therefore a change of concentration of one substance can have a positive or negative effect on another substance in the reaction leading to a more or less efficient assay. To find a balance between all parameters resulting in a more sensitive and efficient reaction is difficult, even more challenging when the technique is already quite optimized. The experiments done in this project only touched upon the surface of the extent of work to develop a technique to endeavour an expression profile for colorectal cancer I hope the results of the project will come to good use for Olink’s further research.

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