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UPTEC X 14 003
Examensarbete 30 hp
Mars 2014

# Development and evaluation of procedures and methods for Proseek Multiplex

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UPPSALA UNIVERSITET

# Molecular Biotechnology Programme

Uppsala University School of Engineering

| **UPTEC X 14 003** | **Date of issue 2014-03** |
|---|---|


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Title (English)

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Title (Swedish)


Abstract

Contemporary proximity extension assays (PEAs) are used for qualitative protein quantifications in serological samples, with possibilities for scaling assays in multiplex. Medical research can however benefit from robust immunoassays functional for assessing protein levels in other types of biospecimens. Formalin-fixed paraffin embedded (FFPE) tissues have long been used for morphological studies. The proteome encapsulated by extensive cross-linking from formalin fixation has however impeded the development of proteomic analysis from the vast biorepositories FFPE-tissues constitute. In this study, I present a proof of concept for assessing FFPE-samples in multiplex format through PEA. Furthermore, a homogenization and protein extraction protocol for assessing fresh-frozen tissue with PEA is presented, together with a novel sample buffer for which remarkable rises in protein detection can be seen in several protein assays. Together, these findings extend the application area of PEA to tissues together with improved quantification characteristics.



Keywords

PEA, immunoassay, FFPE, multiplex



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| Project name | Sponsors |
|---|---|
| Language **English** | Security |
| **ISSN 1401-2138** | Classification |
| Supplementary bibliographical information | Pages **41** |


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# Development and evaluation of procedures and methods for Proseek Multiplex

*Ara-Shant Migoyan*

## Populärvetenskaplig sammanfattning

Proteiner är byggstenarna för allt levande – små molekyler som länge varit svåra att studera och mäta i kroppen hos människor. Numera kan man få relativa indikationer på hur mycket av dessa proteiner som finns i bland annat blodprover, med hjälp av antikroppar som binder till specifika proteiner. Olink Bioscience har utvecklat egna metoder för att hitta och detektera intressanta proteiner i blodprov. En av dessa bygger på proximity extension assay (PEA) teknologi där kända DNA-molekyler är bundna till proteinigenkännande antikroppar. DNA-molekylerna kan sedan kopieras efter att deras tillhörande antikroppar hittat ett protein. Mäter man antalet kopierade DNA-molekyler får man även en uppskattning av antalet proteiner som fanns i provet.

Än så länge används denna teknologi mestadels till blodprover. I detta projekt har applikationsområdet för PEA-teknologin breddats till vävnadsprover som fixerats på objektglas och vävnadsprover som frysts ner efter biopsi. Trots att optimering och kvalitetssäkring kvarstår av resultaten är detta steg mot en generaliserbar proteinmätning från vävnad ytterst intressant ur ett medicinskt perspektiv, då många biologiska prover är i just vävnadsform. Som ett led i utvecklingen av nya applikationsområden har även en alternativ buffertlösning upptäckts – med egenskaper som stärker proteinmätningen och ger högre utslag på proteindetektion vid blandning med prover.

**Examensarbete 30 hp**
**Civilingenjörsprogrammet Molekylär bioteknik**

**Uppsala universitet, januari 2014**

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# Abbreviations

Table 1 – List of reoccurring abbreviations. For full list of acronyms for analysed proteins, see Table 9 in appendix, section 8.1.

| Term | Meaning |
|---|---|
| AR | Antigen Retrieval |
| BCA | Bicinchoninic Acid Assay |
| $C_t$ | Threshold Cycle |
| CV | Coefficient of Variation |
| FFPE | Formalin-Fixed, Paraffin-Embedded |
| HIER | Heat-Induced Epitope Retrieval |
| IHC | Immunohistochemistry |
| LOD | Limit of detection |
| MUX | Multiplex |
| PEA | Proximity Extension Assay |
| PLA | Proximity Ligation Assay |
| qPCR | Quantitative Polymerase Chain Reaction (also known as real-time PCR) |

# 1 Introduction

## 1.1 Background

High-performing detection methods for protein targets are highly required in both research and clinical settings. A great deal of innovative steps are yet to be done in the pursuit for developing novel assays. This is especially true for protein quantification procedures in complex biological samples, where such characteristics as robustness, specificity and sensitivity play a significant role for conducting accurate measurements in volumetrically small samples. The development of such high-performing assays are however difficult – impeding research from further progression[4,11].

Proximity ligation assay (PLA) is a protein detection method using *in vitro* DNA amplification, enabling sensitive and specific macromolecule assays. Early versions of PLAs were based on a proximity ligation event by a simultaneous recognition of target-specific pairs of DNA-aptamers. These aptamers belong to a class of oligonucleotides for which their respective affinity towards target molecules is known beforehand through *in vitro* selection trials. Pairs of DNA-aptamers bind to their target molecule and hybridize their free sequence extensions through the addition of connector oligonucleotides, forming a hybridized proximity probe pair joined together by a DNA ligase. Hybridized proximity probe pairs form a PCR-reporter sequence, and can thus be amplified through PCR, leaving the unhybridized probes unamplified[4]. Qualitative measurements can then reflect the amount of specific proteins in a biological sample. These findings led to later studies where the DNA-aptamers have successfully been substituted by polyclonal or matched pairs of monoclonal antibodies connected to oligonucleotides (called proximity probes) – making the PLA more standardized and generalizable[6].

PLA is however prone to recovery loss when assessing proteins in complex biological samples, such as blood plasma[10], due to the proximity probes being joined with a DNA ligase. In a more recent study, this recovery loss has been overcome by substituting DNA ligase with a DNA polymerase. This technology, referred to as proximity extension assay (PEA) detects proteins through a proximity-dependent polymerization event[11] rather than a ligation event as can be found in PLA. PEA-technology is now commercialized as the Proseek immunoassay, provided by Olink Bioscience.

## 1.2 Aim of study

A broad approach was taken to set up the initial research plan with the principal goal of identifying, defining, evaluating, and performing a specific application to Olink's PEA-technology. A proper methodology for accomplishing the project aim was to include theoretic studies in molecular biology relevant for the chosen application, hands-on immunoassay utilization, qPCR, computer aided data analysis, and even minor features of market analysis to affirm the potential interest of any given application.

This broad approach became considerably narrower when it was decided to examine the compatibility between formalin-fixed paraffin-embedded (FFPE) samples and Olink's PEA-technology. By developing an immunoassay protocol for FFPE-samples, apart from the important scientific value of making use of abundant biospecimens, Olink's existing line of immunoassays would add a field of application to their existing portfolio (serum and plasma samples).

However, during the course of the study, new interesting findings emerged which paved the way for alternative applications and improvements of the contemporary immunoassay. These include extension of the application area to fresh-frozen tissue samples and using alternate buffers in the assay format (Fig. 1).

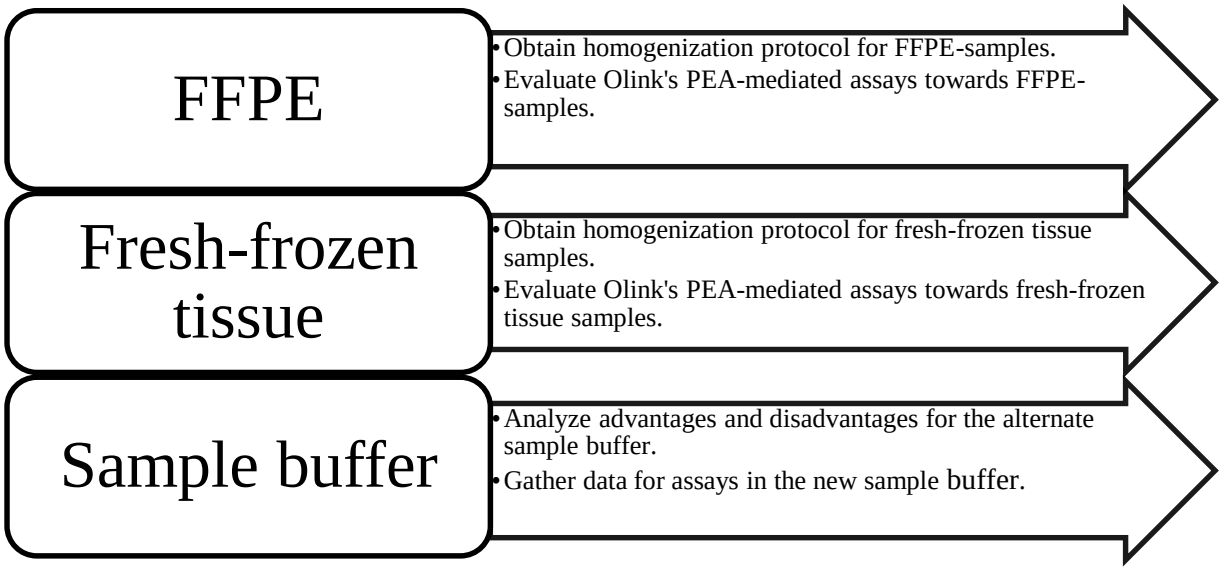


Fig. 1 – General schematics for the FFPE-project and the two subsequent projects.

## 1.3 Tissue samples as biospecimens

### 1.3.1 Formalin fixation and paraffin embedding

Stored tissue samples are a keystone for pathological research by immunohistochemistry (IHC), and vast biorepositories of fixated tissue can be found in clinical and research environments worldwide[17]. Unfortunately, no single standard protocol for tissue fixation has been found to preserve all kinds of epitopes. Therefore, fixating procedures can differ between tissue samples and complicate the handling of fixated tissues when applying them to different analytical procedures[2].

Formaldehyde fixation and paraffin embedding of tissue samples has been, and continues to be, a common method for storing tissue samples. FFPE biopsies are highly stable and can be stored at room temperature for indefinite time periods[7]. Thus, FFPE is a valuable method for preserving histological and morphological features of different tissue types, by which the tissues become suitable for diagnostics by for example IHC[1]. Formaldehyde acts as a reactant

towards certain functional groups of amino acid side chains, resulting in protein-protein and protein-nucleic acid cross-links[16].

The fixation process can vary between desired thicknesses of cut tissues and antigen-antibody pairs. In general, the sliced tissue is fixated in a neutrally buffered formalin solution for at least a couple of hours. The fixated tissue is then dehydrated in a graded series of alcohol solutions followed by a paraffin embedding procedure[1, 2]. However, FFPE reduces the proteomic availability for tissues due to its extensive molecular cross-linking and therefore undermines immunoreactivity. Relatively recent findings have however streamlined antigen retrieval techniques, allowing other protein identification methods than IHC to be compatible with FFPE-samples[1].

### 1.3.2 Antigen retrieval from formalin-fixed, paraffin embedded tissues

A range of different sampling and AR (antigen retrieval) methods for FFPE-samples have been studied and compared to each other. These involve different sample formats, for example cylindrical tissue samples and tissue-sections as well as extraction protocols with different buffers, homogenization steps and incubation procedures[5,7]. HIER (heat-induced epitope retrieval) applied for IHC is one of several classic AR techniques. There is no recognized standard protocol for this heat-induced extraction method, but there are however general hallmarks to be considered when setting up a HIER-protocol for FFPE-samples – 1) deparaffinization and rehydration of FFPE-samples in a graded series of xylene and alcohol, respectively, 2) incubation of the samples near boiling point for different periods of time, 3) using different buffer solutions with ranging pH as boiling medium, with the occasional supplement of stabilizing chemicals such as sodium dodecyl sulphate (SDS)[1,8,17]. Although all anticipated proteins cannot be fully recovered from a single specimen; sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting (WB), protein arrays, enzyme-linked immunosorbent assay (ELISA) and mass spectrometry (MS) have shown that HIER-treatment can extract the majority of proteins encapsulated in both FFPE-samples and fresh-frozen tissue[1]. Intuitively, one would argue that epitopes conserved within a fixated tissue would not withstand such a high-temperature treatment. Research has yet to elucidate the chemical and physical processes enabling HIER to yield recognizable epitopes. In the meantime, one can simply rely on the empirical evidence indicating its adequateness[8].

### 1.3.3 Fresh-frozen tissue

Fresh-frozen tissue, in contrast to FFPE-samples, is less stressful to the conservation of retrieved antigens[7]. Use of fresh-frozen tissue in immunohistochemistry as well as immunoassays can therefore carry conserved antigens that would have been damaged by formalin fixation[2]. However, fresh-frozen tissue requires more rigorous arrangements for storing (at -80°C)[1].

### 1.3.4 Methods for homogenizing and lysing tissue

Contemporary lysis techniques can roughly be categorized into traditional (non-detergent) and chemical (detergent) methods. Traditional methods include mechanical disruption, manual grinding (for example by using pestles) and sonication (high-frequency sound waves). Chemical lysis techniques often include detergents which break lipid-enriched cell structures by solubilizing proteins. The choice of lysis technique should depend on sample type. Due to varying composition of cell membranes and cell walls, the choice of proper detergents is crucial for a successful protein extraction. This also applies to more traditional disruptive and mechanical techniques, due to the rigidness of cells affecting yield and subsequent downstream applications[20].

## 1.4 Proximity Extension Assay (PEA)

### 1.4.1 Principles of PEA

As earlier described, more recent developments based on PLA have focused on improvements of assay performance in biological samples by substituting DNA ligase with DNA polymerase. The enzyme replacement for enabling proximity-dependent DNA polymerization has been proven to reduce the likelihood of enzymatic inhibitions, an intrinsic factor when performing assays in complex biological samples. Thus, PEA is a more robust method for protein quantification in sera, while still inheriting the homogenous assay format from PLA with no washing steps. Antibodies which are to become proximity probes in a PEA are linked with 40-mer oligonucleotides. The oligonucleotides are either linked through their 3'-end or their 5'-end, making so called A-probes and B-probes. Both these probes are required to bind to their target antigen for a proximity-dependent polymerization event to take place (Fig. 2). Furthermore, DNA-polymerase positive for 3'→5' exonuclease activity has been shown to reduce background levels compared to assays run with DNA-ligase[11].

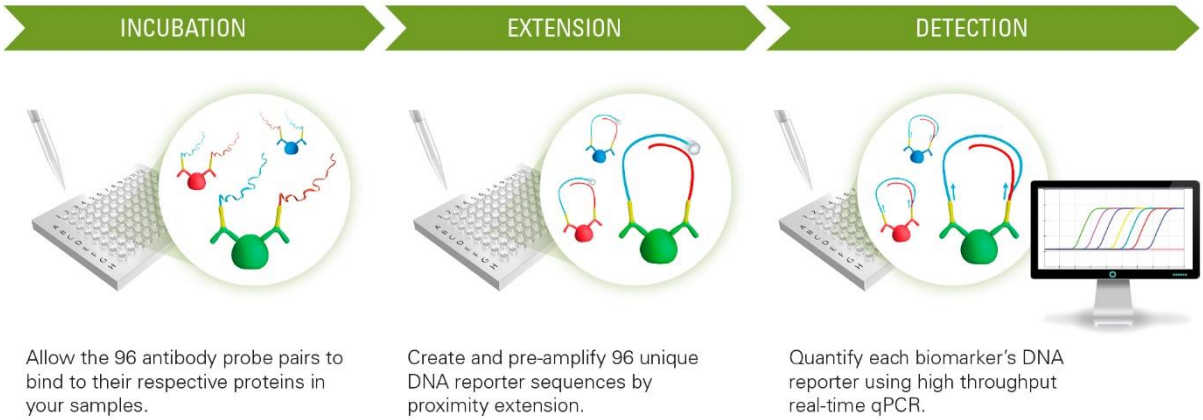


Fig. 2 – Formation of target-specific proximity probes in PEA. The hybridized oligonucleotides are subsequently amplified and detected through addition of DNA-polymerase in qPCR. Copyright © Olink AB 2013. Image courtesy of Olink Bioscience.

### 1.4.2 Olink's Proseek - operating rationale

In a Proseek assay, known oligonucleotide-labelled antibody pairs bind to their correlated antigens in a 1 µl sample. Two adjacent oligonucleotides coupled to their respective antibody pairs form a PCR-reporter sequence by a proximity-dependent DNA polymerization event. The polymerized oligosequences are then detected and quantified by qPCR. Thus far, Proseek's protocol does not differ from the main principles of PEA. However, the assay format is to this date niched and validated for qualitatively indicating certain proteins levels in serum and plasma samples.

### 1.4.3 Multi- versus singleplex

An assay that runs in singleplex can only measure a single analyte at any given time. An assay that runs in multiplex (commonly abbreviated MUX) however, can measure several different analytes simultaneously. Conventional immunoassays, which merely rely on the detection signal from a given antibody bound to a corresponding antigen, are prone to cross-reactivity – causing the assay unreliability when attempting higher grades of multiplexing (Fig. 3).

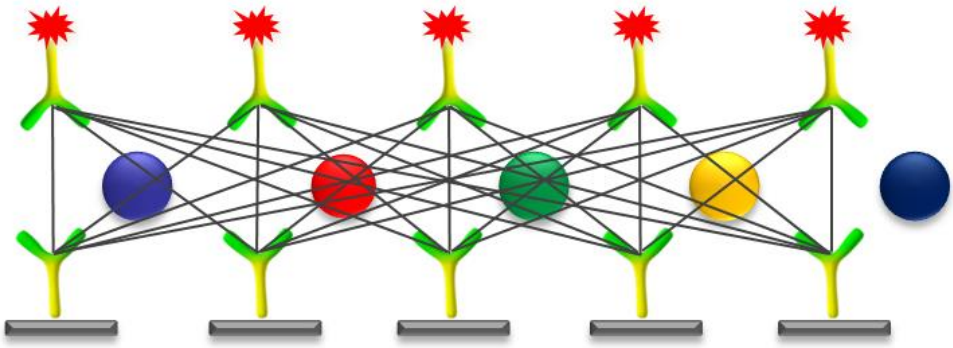

Fig. 3 – Cross-reactivity between antibodies causes unspecific binding and limits the tolerance level of multiplexing in conventional immunoassays. Copyright © Olink AB 2013. Image courtesy of Olink Bioscience.

In the case of Olink's PEA-mediated MUX, detection from cross-reactivity between unwanted antibody-antigen interactions are absent – and thereby eliminating associated false positives (Fig. 4). As of late 2013, Olink offers validated panels in multiplexed assay format for nearly 200 putative biomarkers for cancer and cardiovascular disease.

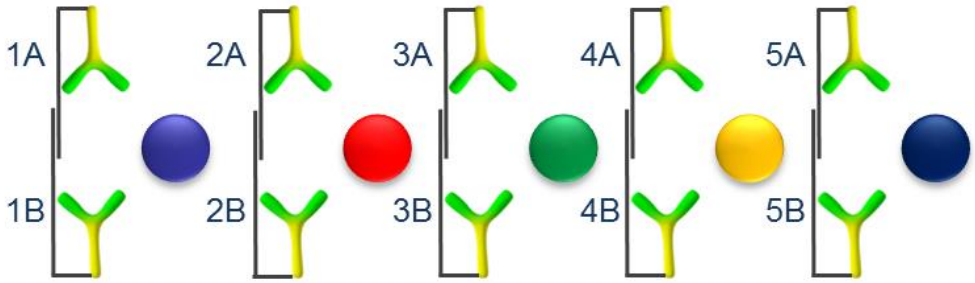


Fig. 4 – Detection from cross-reactivity is absent when coupling unique oligonucleotides to the respective antibody pairs. Copyright © Olink AB 2013. Image courtesy of Olink Bioscience.

The ability to distinguish detection signals in multiplex mode is simply due to measurement of amplicons of antibody-specific oligonucleotides rather than antibody-antigen hybridization events (Fig. 5). Thus, known oligonucleotides can be used to pair fluorescent detection signals with individual proteins. Multiplexing protein detection in PLA or PEA formats therefore provide a more scalable alternative in contrast to heterogeneous methods[3].

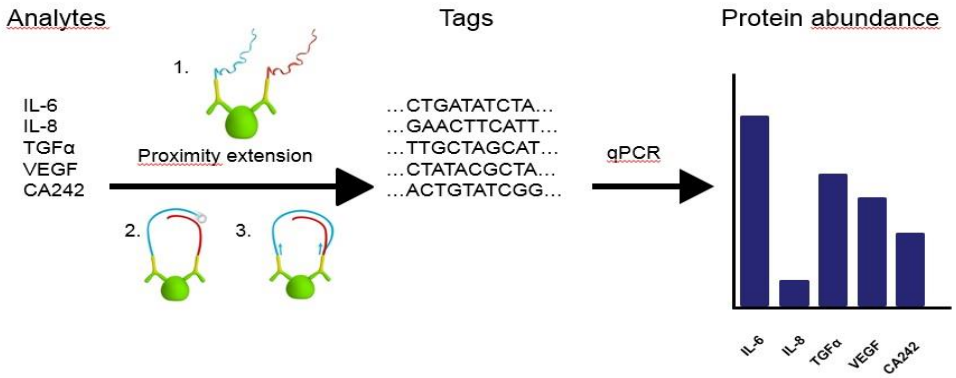


Fig. 5 – In PEA, known oligonucleotide sequences act as bar-codes for protein identification through qPCR. Copyright © Olink AB 2013. Image courtesy of Olink Bioscience.

However, the issue of DNA ligase becoming an inhibiting factor when assessing serological samples remains in multiplex. Optimisation from PLA to PEA in multiplex has been shown to be suitable for detecting plasma biomarkers in samples from colorectal cancer patients[21].

### 1.4.4 Evaluating qPCR

Several aspects of a qPCR must be taken into consideration if one is to properly examine the results. Firstly, a definition of the different parameters involved in the evaluation must be set (Table 2).

Table 2 – Terms and parameters used in qPCR evaluation[9].

| Term/Parameter | Description |
|---|---|
| Dynamic range | Range of linearity of the standard curve. |
| Precision | Usually indicated by the standard deviation between replicates. |
| Standard deviation | Square root of the variance between two data points |
| Efficiency | How efficient a PCR-reaction amplifies a target between cycles. A perfect efficiency (E) doubles the target sequence at each cycle according to $C_n = C_i \times (1+E)^n$, where $C_i$ = initial copy number; $C_n$ = copy number at cycle n; n = number of cycles; E = efficiency of target amplification[9]. |
| Sensitivity | Effectiveness of amplifying and detecting starting template. In PLA, sensitivity is dependent on antibody affinity[6]. |
| $R^2$ | Statistical indication of how well a measured value can predict another when extrapolated. $R^2$=1 indicates a perfect prediction. |
| Rn | Normalized fluorescence signal of reporter dye to reference dye. |
| Baseline | The background fluorescence which is observed in the initial PCR-cycles. |
| ΔRn | Rn normalized to the baseline, ΔRn = Rn - baseline |

| | |
|---|---|
| $C_t$ (threshold cycle) | ΔRn is used to determine $C_t$ by setting it above baseline but within the exponential amplification curve, forming a junction between the threshold line and an amplification curve – a relative measure of target sequence concentration. Lower $C_t$ reflects higher accumulation of fluorescent signal. dCt is the difference between signal of interest and control sample. |
| CV% (coefficient of variation) | Ratio of standard deviation (square root of the variance between two data points) to the mean. In qPCR, the data points are constituted by the number of generated amplicons in individual replicates. |

Secondly, one must take the composition of chosen reagents in assessment when drawing conclusions from deviating parameters. The $C_t$ can be influenced by template-independent factors such as master mix components, reference dye and the PCR efficiency[9]. When measuring protein concentrations, one can minimize potential $C_t$ deviations by mimicking the sample conditions for negative and positive controls, observing the differences in baseline between samples and using a coherent pipetting technique. As $C_t$ only gives a relative indication of antigen concentration, the fluorescent intensity of the reporter dye from samples must be compared to a standard run in the same assay. In the case of assaying protein concentrations, the standard is naturally constituted by a known serial dilution of antigen, forming a standard curve.

# 2 Materials and Methods

## 2.1 Evaluation of FFPE compatibility

### 2.1.1 Rehydration, deparaffinization and HIER

In order to reverse the fixation process, six FFPE-samples of non-cancerous human colon tissue mounted on standard medical slides, 3-4 µm thick (other specifications unknown), where placed in a 75 % xylene 25 % ethylbenzene solution for 10 minutes, followed by a separate 75 % xylene 25 % ethylbenzene solution for 5 minutes, followed by 99.9 % ethanol for 5 minutes, followed by a separate 99.9 % ethanol solution for 3 minutes, followed by 95 % ethanol for 3 minutes, followed by a separate 95 % ethanol solution for 3 minutes and lastly rinsed in deionized water for 1 minute. Care was taken not to let the medical slides dry.

Three slides where then put in two plastic beakers containing 65°C EDTA pH 8 and 1xTarget Retrieval solution pH 6 (Dako), respectively. For HIER-treatment, the respective solutions were heated to 98°C in a pressure cooker with its lid off in an interval of 10 minutes. The lid was then placed on the pressure cocker and the FFPE samples were incubated under overpressure for 18 minutes. After HIER, samples incubated in pH 6 were cooled at room temperature and then placed in 1xTBS at 4°C for storage, whereas slides incubated in pH 8 were stored directly in 1xTBS after HIER.

### 2.1.2 Homogenization of FFPE-samples

After the incubation in 1xTBS at room temperature, tissues were scraped of the slides with a scalpel and placed in 100 µl solution of Buffer B including 1xHalt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), one solution for slides boiled in acidic (pH 6) environment and one for basic (pH 8) environment. Each solution was kept in separate vials. Note that samples for the Proseek assay are conventionally buffered in another buffer, here called Buffer A. The extraction solutions containing the remainder of the deparaffinised tissue samples underwent homogenization through mechanical disruption. Polytron System PT 1200 E (Kinematica) was used for mixing with an attached PT-DA 05/2EC-E85 (Kinematica) as dispersing aggregate, with a dispersing head diameter of 5 mm, suitable for processing volumes of 100 µl minimum. Solutions were mixed in low temperature vials until clear visibility through the vials was reached and then centrifuged at 5 000 rpm for 5 min to separate supernatant from pellet. When not in use, the solutions were stored at -20°C.

No attempt was done to determine the weight of the retrieved tissue, as early experiments had shown that no available scale was suitable for accurately determining such low masses (~0.1 mg) for a standard medical slide, 3-4 µm thick. To determine an accurate weight for small FFPE-samples becomes even more complicated as the tissue must not dry prior to protein extraction, and the wet weight's proportion of the total mass becomes a major source of error.

The protein concentration was measured with BCA (bicinchoninic acid assay), using Pierce BCA protein assay kit with a bovine gamma globulin standard. Measurement samples were diluted 10-fold in 50 mM pH 7.2 Tris-HCl and a total of four replicates were measured for the supernatant and pellet, samples undergone acidic incubation and basic incubation, respectively. The reaction wells were incubated in 120 minutes at 37°C to let the reactions reach equilibrium before spectrophotometric analysis with Tecan Genesis at 562 nm, through accompanying software Magellan v.6.5 (Tecan).

Due to non-detectable protein concentrations in the supernatant for samples incubated in both acidic and basic conditions, a new round of human colon tissue from FFPE-slides underwent HIER-treatment as before. The tissue was added to the existing vials and mixed with the same mixer and dispersing aggregate as before, at full speed for 30 minutes, each 30 minute session divided into 10 minute intervals for both vials, to ensure a higher protein concentration.

### 2.1.3 Protein assay in singleplex

Vascular endothelial growth factor (VEGF-D) was chosen as a candidate biomarker for the FFPE colon samples due to its relative abundance in endothelial cells[19] and also for its existing position as a validated biomarker in Olink's oncological and cardiovascular set of biomarker panels. Thus, polyclonal IgG human VEGF-D antibodies purified from goat (R&D Systems) were labelled with known oligo-nucleotides and assayed against a standard curve of VEGF-D antigen and serum samples as positive control in Proseek singleplex, according to Olink's manual[14].

The 7500 Real-Time PCR System from Applied Biosystems was used for amplifying hybridized oligos in the qPCR step. The accompanying 7500 software Version 2.0.5 was used for compiling the measurement data. The protocol for thermal cycles can be seen in Table 3.

Table 3 – Thermal cycling program for assays run in singleplex

| Step | #Cycles | Time (min) | Temp (°C ) |
|---|---|---|---|
| Pre-extension | 1 | 20 | 50 |
| Activation | 1 | 2 | 95 |
| Amplification | 40 | 0.25 | 95 |
| | 40 | 1 | 60 |
| Melting curve | 1 | ~40 | Ramping |

However, the initial VEGF-D assays for FFPE-samples did not generate a signal distinguishable from LOD, with samples of antigen standard and plasma indicating no malfunctioning of chosen probes. In the light of HIER being an unpredictable method pertaining to conservation of certain antigens, epithelial cell adhesion molecule (EpCAM) proximity probes were prepared through polyclonal IgG human EpCAM antibodies purified from goat (R&D Systems) chosen for a new round of assays due to the protein's abundance in human colon[18]. The assays were conducted with a similar protocol as the ones for VEGF-D and rendered a lower $C_t$ than the baseline for samples taken from the acidic incubation.

### 2.1.4 Protein assay in multiplex

Encouraged by the positive results from the EpCAM assay in singleplex, the project proceeded in multiplex format, simultaneously assaying 92 cardiovascular-related proteins from Olink's CVD I (*cardiovascular disease I*, see section 8.1 for full list of proteins) panel of biomarkers. By this point in the study, efforts and resources were put on developing new sample buffers. As a consequence, only two measurement samples were used for the concluding assay for FFPE-samples, one for each incubation environment (pH 6 and pH 8). The Proseek Multiplex protocol was carried out in accordance with the user manual[15].

## 2.2 Fresh-frozen tissue homogenization, protein extraction and assay in singleplex

0.24 mg of fresh-frozen normal human colon was added to a 480 µl buffer solution of 50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 mM EDTA pH 8, 11 % w/v Triton X-100, 0.1 % w/v sodium deoxycholate, 1 x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific), 1 mM PMSF. The buffer solution was adapted from a protocol kindly provided by Lotta Wik at the Department of Immunology, Genetics and Pathology, Uppsala University.

Polytron System PT 1200 E (Kinematica) tissue with an attached PT-DA 05/2EC-E85 (Kinematica) as dispersing aggregate was used for homogenizing the tissue. The lysate was then centrifuged at 13 000 rpm for 3 min, from which the supernatant was recovered and stored at 4°C.

Protein concentration in the supernatant was measured with BCA, using Pierce BCA protein assay kit with a bovine gamma globulin standard. Measurement samples were diluted 10-fold in 50 mM Tris-HCl pH 7.2 with three replicates for every serial dilution. The reaction wells

were incubated for 60 minutes at 37°C and measured with Tecan Genesis at 562 nm, through accompanying software Magellan v.6.5 (Tecan).

The supernatant of the centrifuged tissue lysate was subsequently assayed in Proseek singleplex according to protocol[14], based on a 10-fold serial dilution of six steps with two replicates, respectively. Plasma samples were used as positive control. All samples were assayed against EpCAM by using the probes conjugated earlier for assessing FFPE-samples (see section 2.1.3). No antigen standard for EpCAM was included in the singleplex assay due to absence of an isolated EpCAM antigen in solution. The chosen protocol is however appropriate for determining whether the homogenization and extraction protocol rendered detectable proteins with the Proseek assay format.

## 2.3 Trials for unconventional buffer

When performing protein assays for evaluation of FFPE-compatibility, an interesting observation and ensuing project emerged. The use of buffers unlike the conventional one, (referred to as buffer A) used in the assay formats, resulted in assays with higher $dC_t$, showing a higher protein concentration in samples that were otherwise equivalent. Furthermore, assays run with separate buffers had a similar baseline – excluding a higher fluorescence background as a factor for falsely high detection signals. The only plausible explanations for the unanticipated results were either that the alternate buffer (referred to as buffer B) inhabits chemical properties suitable for antibody-antigen hybridization in the assay, or that it recovers more antibodies from solution while pipetting or that it is prone to being pipetted in excess volume due to its difference in viscosity and stickiness. The issue of proper pipetting becomes crucial in this case, considering that the Proseek assay is run with 1 µl samples. A volume of 2 µl that might be non-distinguishable during experimentation, doubles the protein concentration of a standard sample. However, repeated experiments all pointed at higher sensitivity for the VEGF-D antigen when using buffer B in comparison to buffer A.

To further examine this so far unexplainable phenomenon, assays for standard curves of all antigens in the before mentioned CVD I panel were run in multiplex, with two replicates for buffer A and buffer B, respectively. The standard curves were diluted 5-fold, ranging from an antigen concentration of 100 ng/ml to 1.28 pg/ml. The sole difference between the antigen samples was the buffer solution in which the antigen pools are solubilized in, all other specifications in the assay format have been identical. Plasma samples with unknown antigen concentrations were also included with two replicates, diluted from stock to 1:2 and 1:8 dilutions. To exclude false positives dependent on pipetting method due to the different viscosities of buffer A and B, a similar setup for an antigen standard was assayed in the same qPCR but with a consciously flawed pipetting method, where samples were diluted independently without any master mix and in a different concentration range (10-fold dilution between 1 000 ng/ml – 1 pg/ml). The multiplex-protocol proceeded in accordance with user manual[15].

Replicates of fresh-frozen colon samples with buffer B as sample buffer were performed in connection with the EpCAM assay on fresh-frozen colon samples in singleplex (see section 2.2), in order to elucidate any difference in assay performance.

## 2.4 Validation of data

This study has focused on development of methods for Olink's contemporary immunoassays. Therefore, efforts have not been strained to statistical analysis of the extensive data collected, as collected data remains at Olink's disposal and can be analysed more extensively when needed. However, CV% of replicates were calculated and will be presented in the results section, due to it being a good indicator of variability between replicate samples and in-run precision. The cut-off value for precise replicates have been set at CV%<30, as this limit is used in Olink's data packages for biomarkers[13].

# 3 Results

## 3.1 Detection of antigen retrieved from FFPE-tissue

Earlier optimization trials have validated a protein concentration threshold of 0.021 mg $ml^{-1}$ (Mats Gullberg, personal communication) as applicable with the Proseek assay format. A supernatant concentration roughly half of the accepted threshold value can therefore be regarded as inadequate (Table 4). Concerning the experienced difficulties of reaching a higher solubility of proteins from the treated FFPE-samples, protein assays in Proseek were conducted despite the low concentrations.

Table 4 – BCA on homogenized and centrifuged FFPE-samples incubated in acidic and basic environments. Measurement samples for BCA categorized as supernatant and pellet, where the protein concentration of the supernatant indicates amount of solubilized protein. Concentration calculated as mean of four replicates.

| **Supernatant** | Protein concentration mg/ml | **Pellet** | Protein concentration mg/ml |
|---|---|---|---|
| pH 6 | 0.010 | pH 6 | 0.079 |
| pH 8 | 0.010 | pH 8 | 0.069 |

$R^2$ for standard curve: 0.9961

Approximately one third of the 92 proteins in CVD I had a $dC_t$ above 1 in comparison to the baseline, out of which several had $dC_t$ above 2 – indicating some functionality between the described homogenization protocol and Olink's multiplexed assay format (Fig. 6). As mentioned in the materials and methods section, no replicates of the FFPE-samples were prioritized and thus, no standard deviation or CV% could be calculated. Four proteins in the CVD I panel (PAR-1, MB, CA-125 and NEMO) did not give away any fluorescent signal in any control sample and are therefore discarded in the results.

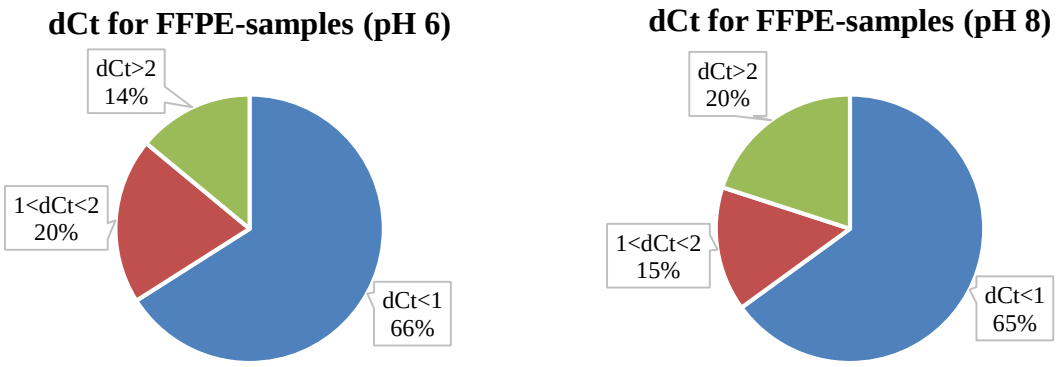


Fig. 6 – Percental distribution of $dC_t$ for individual biomarkers in Proseek MUX CVD I. FFPE-samples undergone HIER in acidic (pH 6) and basic (pH 8) environments, respectively.

21 out of 92 proteins in CVD I had at least one $dC_t$ above 2, either for treatment in pH 6 or pH 8 (Fig. 7). Some proteins showed sensitivity to basic or acidic environments, indicating the importance of acidity for preserving immunoreactive antigen in the HIER-treatment.

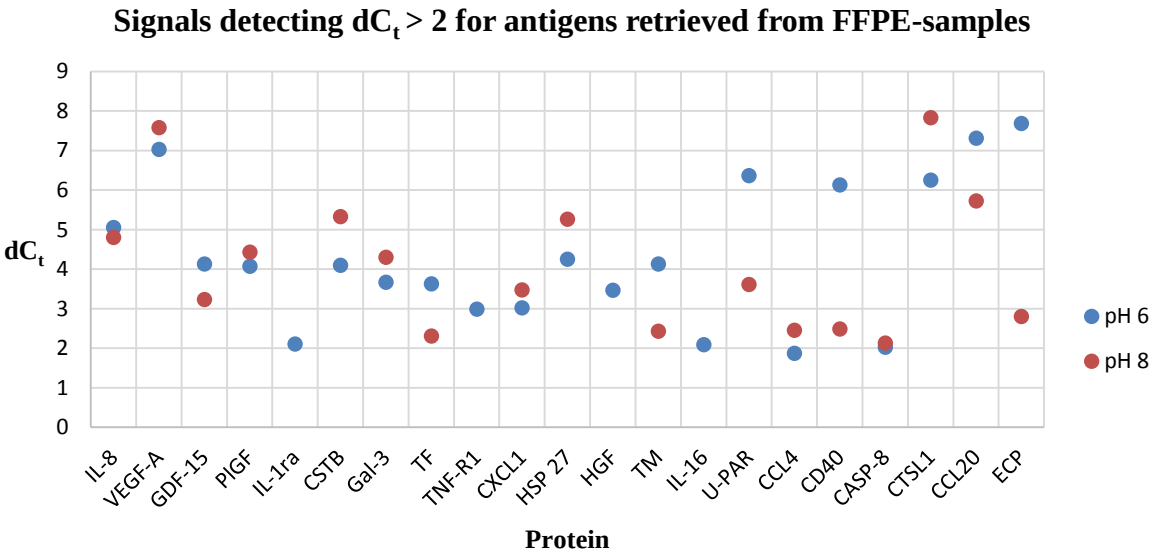


Fig. 7 – $dC_t$ above 2 for single-replicate FFPE-samples assayed with Proseek MUX CVD I. See appendix (8.2) for assays on between-run antigen standards from CVD I.

## 3.2 Homogenization protocol for fresh-frozen tissue

The fresh-frozen tissue lysate contained an adequate amount of protein (Table 5) for a Proseek assay, as a dilution of 1:100 from the original lysate rendered a protein concentration of 0.2 mg/ml.

Table 5 – BCA on homogenized fresh-frozen, human colon samples. Concentration calculated as mean of three replicates

| Dilution of lysate (fresh-frozen human colon) | Protein concentration mg/ml |
|---|---|
| 1:10 | Above LOD |
| 1:100 | 0.206 |
| 1:1 000 | Under LOD |

$R^2$ for standard curve: 0.9574

The subsequent assay on EpCAM concentrations in the fresh-frozen colon sample rendered a fluorescent signal clearly distinguishable from the fluorescence reference (Fig. 8).

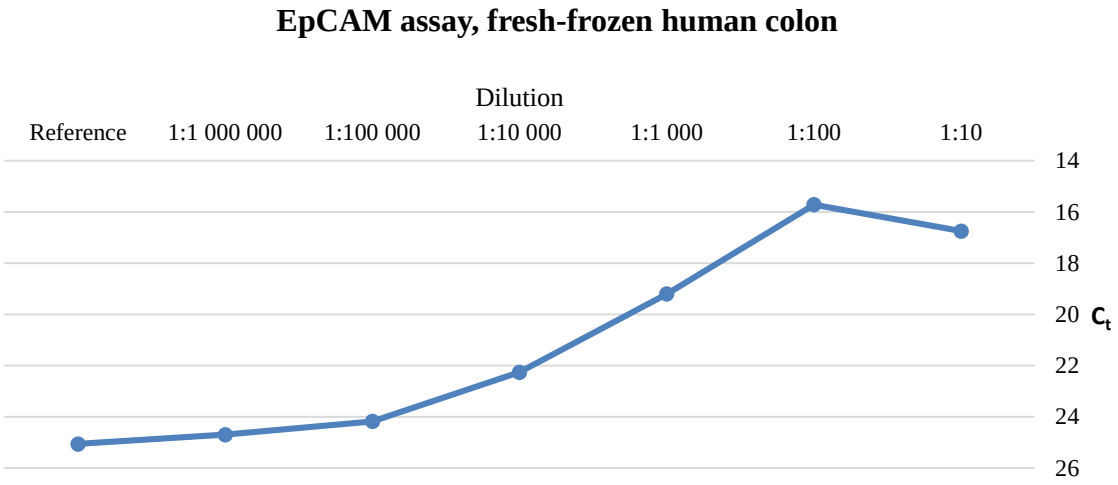


Fig. 8 – $C_t$ for EpCAM assay of fresh-frozen samples (human colon) in Proseek singleplex. All data points had CV%<30 between double replicates.

## 3.3 Lowering $C_t$ in Proseek assays by alternate sample buffer

### 3.3.1 Effects on antigen standard

Practically no protein in panel CVD I showed deteriorated results with buffer B while approximately one third of the biomarkers showed a $C_t$ lowered by 1 in the antigen concentration span 160 pg/ml – 6.4 pg/ml (Table 6), indicating a stronger assay. Furthermore, when examining the distribution of antigen concentrations showing lower $C_t$ in buffer B (Fig. 9), certain antigens are distinguished by their lower $C_t$ for a wider range of antigen concentration. Thus, lower $C_t$ does not appear to be randomly distributed among the 92 biomarkers in CVD I as some antibody-antigen pairs appear to have a stronger inclination for hybridizing in buffer B. In contrast, where $C_t$ has been lower for buffer B compared to buffer A, the drop in $C_t$ has almost exclusively been lower than 1 and had a character of random distribution. The protein assays which indicate a weaker assay with buffer B are SRC and PAPPA (Fig. 9). Individual assays of all 92 biomarkers are graphically represented and compared in appendix section 9.2, buffer A to buffer B. Three proteins in the CVD I panel (PAR-1, MB and CA-125) did not give away any fluorescent signal in any control sample and are therefore discarded in the results.

Table 6 – Differences in $dC_t$ for buffer B compared to buffer A in Proseek MUX CVD I. Diluted antigen samples.

| | **Percent of biomarkers in CVD I meeting condition for $\Delta dC_t = dC_t$ Buffer B - $dC_t$ Buffer A** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Buffer B rendering higher $dC_t$ ($\Delta dC_t > 0$) | | | | Buffer B rendering lower $dC_t$ ($\Delta dC_t < 0$) | | | |
| **Antigen concentration** | $\Delta dC_t > 0{,}5$ | $\Delta dC_t > 1$ | $\Delta dC_t > 2$ | $\Delta dC_t > 3$ | $\Delta dC_t < 0{,}5$ | $\Delta dC_t < 1$ | $\Delta dC_t < 2$ | $\Delta dC_t < 3$ |
| 100 ng/ml | 22% | 4% | 0% | 0% | 6% | 2% | 1% | 1% |
| 20 ng/ml | 30% | 7% | 2% | 1% | 6% | 2% | 1% | 0% |
| 4 ng/ml | 46% | 16% | 3% | 1% | 1% | 1% | 1% | 0% |
| 800 pg/ml | 64% | 18% | 6% | 1% | 1% | 1% | 0% | 0% |
| 160 pg/ml | 65% | 35% | 7% | 0% | 1% | 0% | 0% | 0% |
| 32 pg/ml | 65% | 36% | 2% | 0% | 1% | 0% | 0% | 0% |
| 6.4 pg/ml | 52% | 27% | 2% | 0% | 2% | 0% | 0% | 0% |
| 1.28 pg/ml | 40% | 18% | 1% | 0% | 1% | 0% | 0% | 0% |

Out of 1 728 data points gathered from two replicates, 1 639 (94.8 %) had a CV%<30 (Fig. 10) signifying within-run assays with high precision. The experiment indicated that certain proteins were prone to higher CV% and are over-represented in the share of unreliable values (such as CHI3L1). However, the proteins with high CV% between replicates show sign of being assayed for concentrations beyond their dynamic range when compared to earlier validation studies[12].

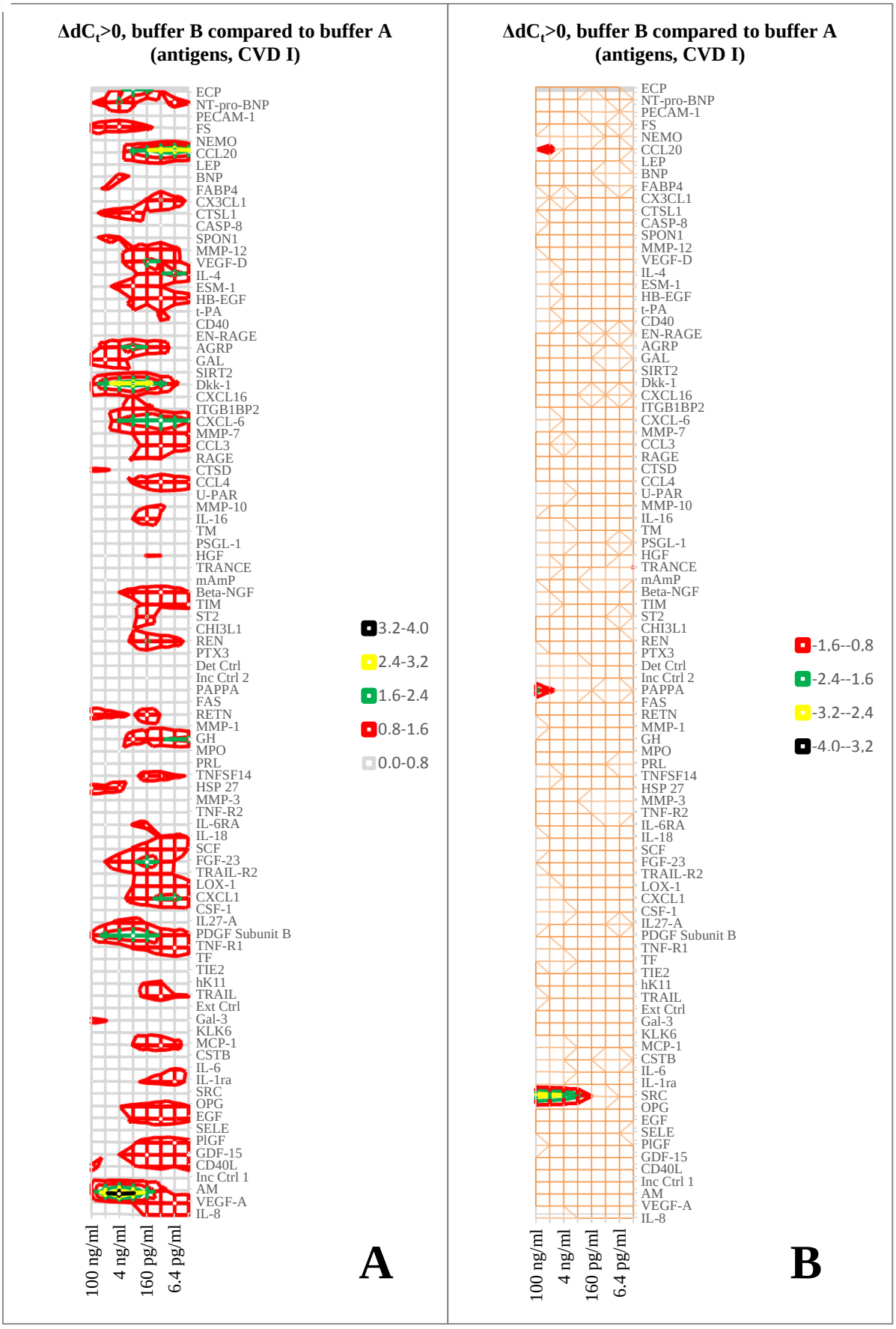


Fig. 9 – $\Delta dC_t$ for antigen standard (1.28 pg/ml to 100 ng/ml, 5-fold dilution) for CVD I antigen standard in Proseek MUX, buffer B to buffer A. **A**: Buffer B rendering higher $dC_t$; **B**: Buffer B rendering lower $dC_t$.


Fig. 10 – CV% of $C_t$-measurements for CVD I antigen standard (100 ng/ml to 1.28 pg/ml, 5-fold dilution, two replicates) in Proseek MUX, buffer A and buffer B. Values indicate within-run precision.

When examining the lower antigen concentration levels, which were affected the most between samples diluted through a rigorous method, and those in a flawed method, a correlation could be seen towards lower $C_t$ between both pipetting techniques for buffer B (Fig. 11). To which extent different pipetting procedures affect results is cumbersome to evaluate from this single experiment with arbitrary concentrations. However, there is a clear tendency towards higher sensitivity for different concentrations with both pipetting techniques.

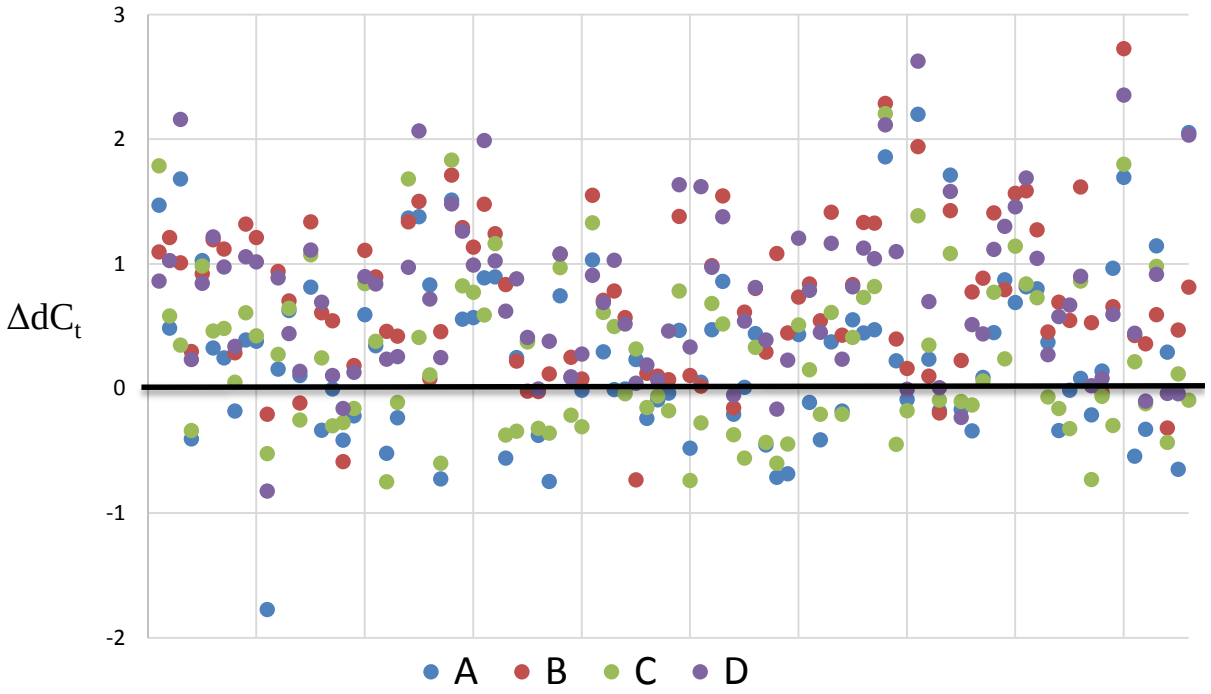


Fig. 11 – Compared $\Delta dC_t$ antigen standard, buffer B to buffer A performed in Proseek MUX CVD I. **A**: 100 pg/ml flawed pipetting; **B**: 32 pg/ml appropriate pipetting; **C**: 10 pg/ml flawed pipetting; **D**: 160 pg/ml appropriate pipetting.

### 3.3.2 Effects on biological samples

No effect on $C_t$ can be found in plasma samples when comparing assays run with buffer B to buffer A (Table 7). For instance, a great contrast appears in the two buffers' effects on $C_t$ when comparing the curves for adrenomedullin (AM) as antigen standard and detection from plasma samples (Fig. 12).

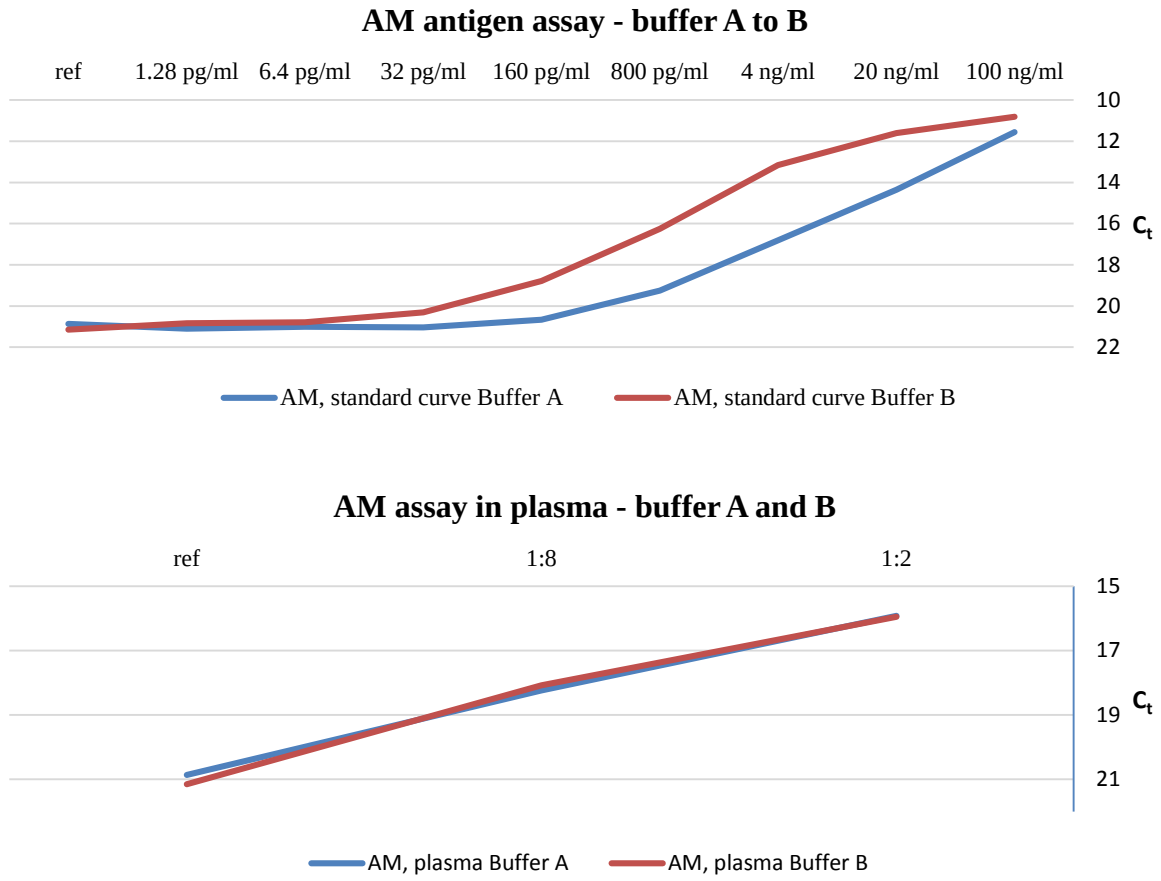


Fig. 12 – $C_t$ for adrenomedullin assay in Proseek CVD I MUX, antigen standard and plasma. Performed with assay buffer A and B, respectively.

Table 7 – Differences in $dC_t$ for buffer B compared to buffer A in Proseek MUX CVD I. Diluted plasma samples.

| | **Percent of CVD I assays on plasma meeting $\Delta dC_t$ condition, buffer B to A** | | | |
|---|---|---|---|---|
| Plasma dilution | $\Delta dC_t>0,5$ | $\Delta dC_t>1$ | $\Delta dC_t<0,5$ | $\Delta dC_t<1$ |
| 1:2 | 2 % | 0 % | 4 % | 2 % |
| 1:8 | 2 % | 0 % | 8 % | 2 % |

CV% for plasma samples were mostly under the threshold of accepted precision of CV%<30 (Table 8), including essentially all CV% plasma samples in buffer B and the 1:2 dilution of plasma in buffer. However, a major disparity arises in the 1:8 dilution of plasma for buffer A, where roughly a fifth of all data points have CV%<30.

Table 8 – CV% of $C_t$-measurements for CVD I assay on plasma samples (diluted 1:2 and 1:8, two replicates) in Proseek MUX, buffer A and buffer B. Values indicate within-run precision.

| | **Percent of CVD I assays on plasma meeting CV% condition** | |
|---|---|---|
| Plasma dilution | Buffer A – CV%<30 | Buffer B – CV%<30 |
| 1:2 | 98 | 99 |
| 1:8 | 17 | 98 |
| Reference | 83 | 89 |

Comparing within-run singleplex assays for EpCAM with fresh-frozen human colon further validates the positive effects of substituting buffer A against B (Fig. 13). $C_t$ levels for plasma were unaffected by buffer B, further validating the results from previous multiplex assays.

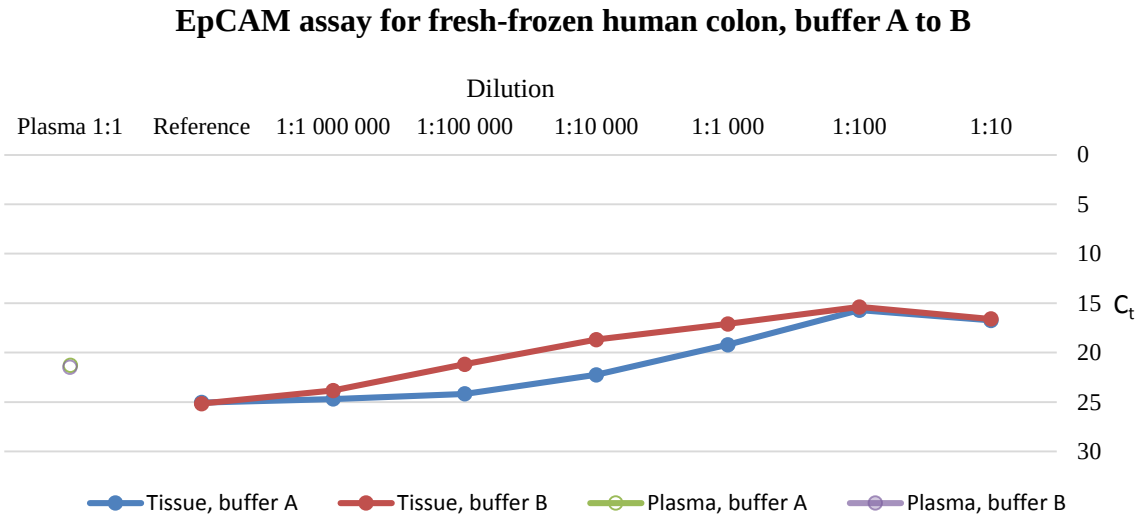


Fig. 13 – $C_t$ for epithelial cell adhesion molecule assay for fresh-frozen human colon samples in Proseek singleplex, run with sample buffer A and B, respectively. All data points had CV%<30 between double replicates. Plasma samples used as positive control, run with sample buffer A and B, respectively.

# 4. Discussion

## 4.1 Tissue samples and the Proseek immunoassay

As demonstrated, the described methodology for extracting proteins from FFPE-samples is suitable for retrieval of antigens with immunoreactive epitopes, functional in a Proseek assay. However, the generalizability of the protocol leaves much to be desired as few proteins gave satisfying levels of detection, and pH during HIER affects the antigen recovery. Yet, the set of proteins detectable in CVD I may be useful in comparative studies with lower requirements on quantification if assayed against FFPE-samples from different donors or normal versus malignant tissues, although optimization remains for validating qualitative measurements.

Although earlier studies have shown HIER to be successful in rendering functional epitopes, the circumstances behind HIER and successful antigen retrieval are unknown. Trial-and-error type of experiments are the exclusive way to know which epitopes are conserved after HIER-treatment (as seen in the early trials for VEGF-D, all with negative results), and a suitable approach is to adapt existing protocols. Although earlier studies and protocols have been

regarded in this study, the mode of procedure has mainly stemmed from what has been available at Olink and reasonable to obtain within set timeframes. One major improvement to the developed protocol for protein extraction from FFPE is needed regarding the homogenization. If solubilized protein concentration levels were to rise in lysates, so could the detected levels of antigen and maybe even antigens that were not detectable with the contemporary protocol. Earlier studies have conducted serial treatments of the pellet during extraction[16], a phase which has been neglected in this study. A straight forward way of increasing the concentration of solubilized protein, would be to use a dispersing aggregate designed for volumes below 100 µl, adding of stabilizers such as SDS[17], or by using reagents which have been validated for FFPE-tissue[16]. Standardizing the antigen retrieval methods for several types of FFPE-tissues and their contained proteins may be troublesome for some antigens or FFPE-samples as dehydration and paraffin embedding procedures differ. However, the sampling techniques can be standardized by a more practically convenient method for retaining equal amount of tissue and protein in the homogenization protocol, as has been shown in earlier studies[5].

Fresh-frozen tissue, in contrast to FFPE-tissue, have been shown to easily undergo homogenization and protein extraction in a standardized manner, with protein concentration in lysates reaching compatible levels for Proseek assays (>0.021 mg/ml) (Mats Gullberg, personal communication). No greater reasoning is justifiable on possibilities of quantifying protein concentrations in fresh-frozen tissue due to the absence of a valid antigen standard in the experimental assay.

## 4.2 Implications of alternate sample buffer

Considering the undergone methodology for comparing buffer A against buffer B and its effect on $C_t$ for antigens, the results indicate that one or several properties of buffer B either facilitate the binding of antibodies to their respective antigen pairs or simply hinder antigen loss during pipetting. As the methodology only differed in the composition of sample buffer, the only plausible explanation for possible false-positives is a faulty pipetting technique. The scenario of inconsistent handling of the small sample volumes (1 µl) is however unlikely, considering that 1) $C_t$ was lower for different diluting techniques in assays performed with buffer B, 2) certain proteins in CVD I were prone to render better assays with buffer B in multiplex – indicating that deviations were not random, and 3) even if a faulty technique has been used during pipetting, some differences in $C_t$ are too high (for example adrenomedullin) to be explained by excess sample volume caused by different viscosities or pipetting procedures. By substituting the conventional sample buffer used for setting up antigen standards in Proseek with buffer B, measured protein concentrations may come closer to the absolute values – raising opportunities for improved quantifications. Futures studies could however benefit from studying the effect on $C_t$ when assaying antigen standards in the same dilution range, but with different pipetting techniques.

Experiments pertaining to plasma samples diluted 8-fold in buffer A may have had unreliable standard deviations between replicates. Yet the overall results for plasma in buffer B to A overwhelmingly point to plasma being unaffected by buffer B. In the view of equal $C_t$

observed between buffer A and B in plasma samples, buffer B may hold equivalent properties as the biological samples the assay is run in. In that case, by using buffer B in the antigen standard from which sample measurements are related to, one can reach a more reliable quantification of proteins in complex biological samples. However, trials with fresh-frozen human colon showed a $\Delta dC_t>3$ at certain concentrations for detected EpCAM when using sample buffer B compared to A. Further dilution of plasma in buffer B may therefore raise observations of buffer B affecting $C_t$, as the whole dynamic range for plasma was not covered in this study. An equally interesting discussion is related to the before mentioned CV% of 8-fold dilution of plasma in buffer A. I argue that the high CV% has to do with my own malpractice, but while working with this buffer I have had to reassess my own convictions at several occasions due to its exact properties being unknown. Future studies could merit from trying to validate if the disparity in CV% for one particular concentration level diluted with buffer A was a simple misfortune, or if buffer B actually raises precision in diluted plasma samples in comparison to buffer A.

As the study for buffer B emerged as a consequence of the initial research plan regarding the compatibility of FFPE-samples and immunoassays, no extensive literature has been investigated to discuss any theories behind buffer properties on immunoreactivity. Elucidating properties for used buffers becomes even more cumbersome due to the undisclosed nature of components in all commercial buffers used. Although empirical data found in this study point to assays with plasma samples being unaffected by buffer B, the circumstances of how the plasma samples have been obtained and processed are unknown – making it difficult to determine whether the properties of buffer B are intrinsic to plasma or not. Buffer B did however show a strong correlation between lowered $C_t$ with fresh-frozen tissue lysates, yet only assayed with antibodies for EpCAM.

# 5 Conclusions

At this point, the functionality of developing a standardized FFPE-protocol for entire panels of biomarkers is far-fetched. Nonetheless, a proof of concept for assaying FFPE-samples in Olink's PEA-mediated Proseek immunoassay has been established in this study. As shown, some proteins extracted from FFPE-samples are detected in a Proseek assay through the given homogenization and extraction protocol. The homogenization and extraction can thus be examined further for improvement on biomarkers of interest in an ad-hoc manner.

A homogenization protocol and extraction method for fresh-frozen tissue has been adapted to be compatible for immunoassays with Olink's PEA technology. Further validation is needed for including a larger set of biomarkers assayed in different types of tissue with validated antigen standards.

Concluding from the discussion of the affected $C_t$ for the CVD I assay, I argue that the properties for Buffer B are suitable for one or several categories of proteins and that more research on this matter is justifiable. If properly validated and incorporated into the Proseek protocol, Buffer B would provide a powerful and cost-efficient improvement to the

contemporary immunoassay panels by lowering $C_t$. A suggestion for further research is to combine the assessment of buffer B in a wider range of biological samples with other panels of biomarkers and integrating it into earlier steps in the assay protocol to determine possible improvements.

All in all, the results obtained in this study regarding FFPE, fresh-frozen tissue and buffer B provide a vast data pool which can be statistically analysed more extensively if needed.

## 6 Acknowledgments

I would like to convey my special thanks to my supervisor Mats Gullberg at Olink Bioscience, for guiding me through the project without ever compromising on my own creativity, to my scientific reviewer Masood Kamali-Moghaddam at the department of Immunology, Genetics and Pathology at Uppsala University, for reviewing my work, to Ann-Catrin Andersson at Olink Bioscience for going beyond her formal duties and tutoring me during the project's onset, and to Lars-Göran Johansson and Lena Henriksson for reviewing my report.

In addition to all the foregoing, I extend my thanks and appreciation to all employees affiliated with Olink Bioscience, who have every so often facilitated my familiarization of routines and procedures at the company.

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# 8 Appendix

## 8.1 Biomarkers in Olink-panel CVD I

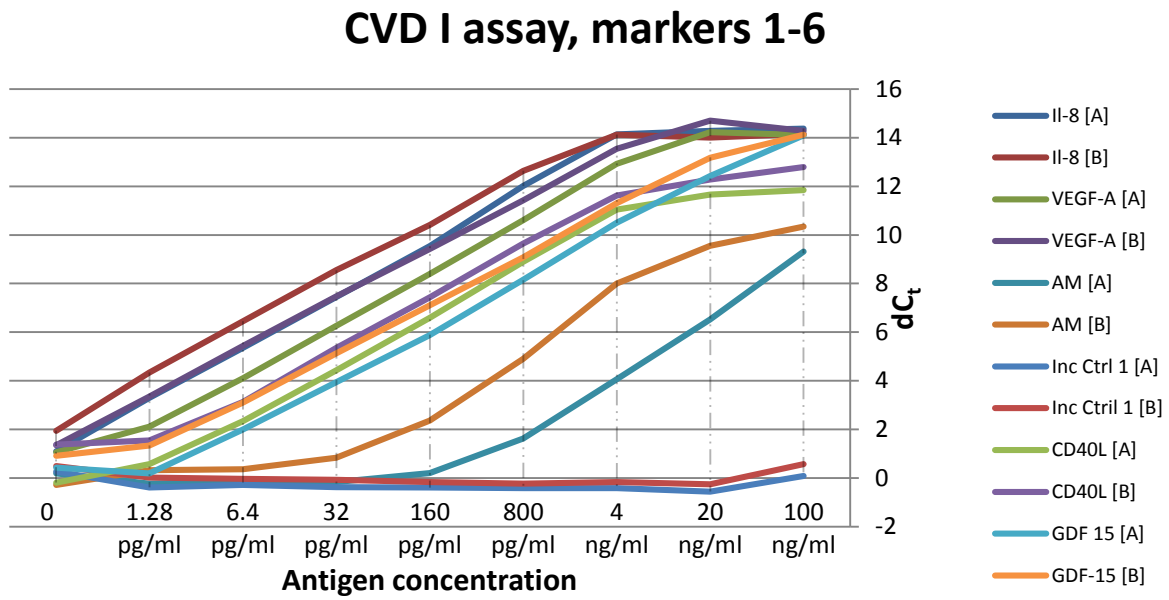
**Table 9 – List of biomarkers in the CVD I panel**

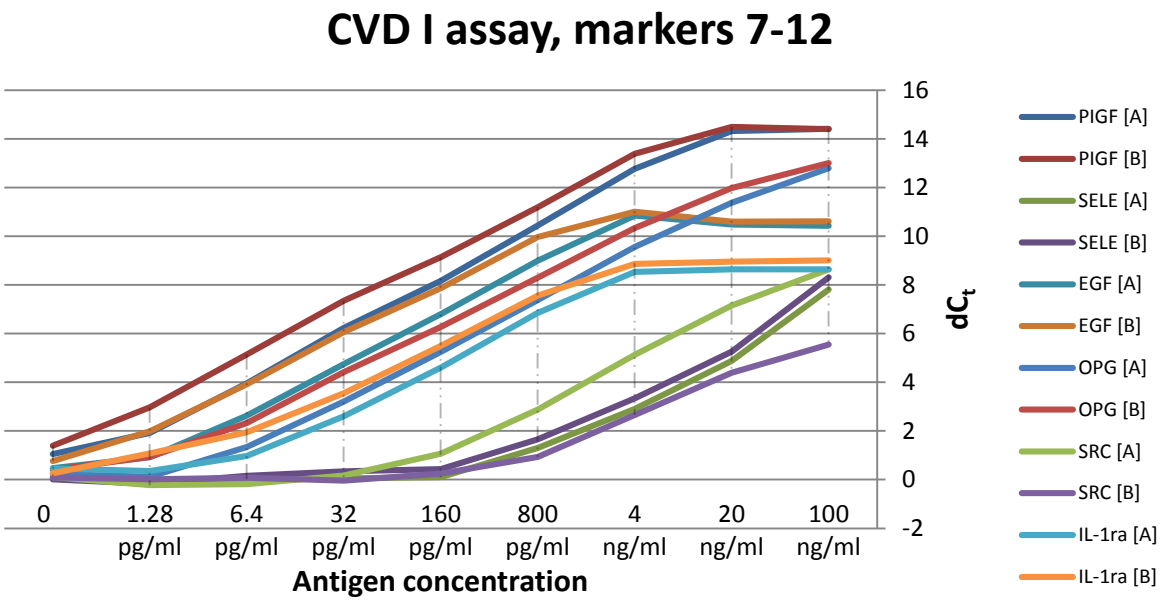
| | |
|---|---|
| **Adrenomedullin (AM)** | Agouti-related protein (AGRP) |
| **Angiopoietin-1 receptor (TIE2)** | Beta-nerve growth factor (Beta-NGF) |
| **Cathepsin D (CTSD)** | Caspase-8 (CASP-8) |
| **Cathepsin L1 (CTSL1)** | C-C motif chemokine 20 (CCL20) |
| **C-C motif chemokine 3 (CCL3)** | C-C motif chemokine 4 (CCL4) |
| **CD40 ligand (CD40L)** | Chitinase-3-like protein 1 (CHI3LI) |
| **C-X-C motif chemokine 1 (CXCL1)** | C-X-C motif chemokine 6 (CXCL6) |
| **C-X-C motif chemokine 16 (CXCL16)** | Cystatin-B (CSTB) |
| **Dickkopf-related protein 1 (Dkk-1)** | Endothelial cell-specific molecule 1 (ESM-1) |
| **Eosinophil cationic protein (ECP)** | Epidermal growth factor (EGF) |
| **E-selectin (SELE)** | Fatty acid-binding protein, adipocyte (FABP4) |
| **Fibroblast growth factor 23 (FGF-23)** | Follistatin (FS) |
| **Fractalkine (CX3CL1)** | Galanin peptides (GAL) |
| **Galectin-3 (Gal-3)** | Growth hormone (GH) |
| **Growth/differentiation factor 15 (GDF-15)** | Heat shock 27 kDa protein (HSP 27) |
| **Heparin-binding EGF-like growth factor (HB-EGF)** | Hepatocyte growth factor (HGF) |
| **Interleukin-1 receptor antagonist protein (IL-1ra)** | Interleukin-18 (IL-18) |
| **Interleukin-27 subunit alpha (IL27-A)** | Interleukin-4 (IL-4) |
| **Interleukin-6 (IL-6)** | Interleukin-6 receptor subunit alpha (IL-6RA) |
| **Interleukin-8 (IL-8)** | Kallikrein-11 (hK11) |
| **Kallikrein-6 (KLK6)** | Lectin-like oxidized LDL receptor 1 (LOX-1) |
| **Leptin (LEP)** | Macrophage colony-stimulating factor 1 (CSF-1) |
| **Matrix metalloproteinase-1 (MMP-1)** | Matrix metalloproteinase-10 (MMP-10) |
| **Matrix metalloproteinase-12 (MMP-12)** | Matrix metalloproteinase-3 (MMP-3) |
| **Matrix metalloproteinase-7 (MMP-7)** | Melusin (ITGB1BP2) |
| **Membrane-bound aminopeptidase P (mAmP)** | Monocyte chemotactic protein 1 (MCP-1) |
| **Myeloperoxidase (MPO)** | Myoglobin (MB) |
| **Natriuretic peptides B (BNP)** | NF-kappa-B essential modulator (NEMO) |
| **N-terminal pro-B-type natriuretic peptide (NT-pro-BNP)** | Osteoprotegerin (OPG) |
| **Ovanrian cancer-related tumor marker CA 125 (CA-125)** | Pappalysin-1 (PAPPA) |
| **Pentraxin-related protein PTX3 (PTX3)** | Placenta growth factor (PlGF) |
| **Platelet endothelial cell adhesion molecule (PECAM-1)** | Platelet-derived growth factor subunit B (PDGF subunit B) |
| **Interleukin-16 (IL16)** | Prolactin (PRL) |
| **Protein S100-A12 (EN-RAGE)** | Proteinase-activated receptor 1 (PAR-1) |
| **Proto-oncogene tyrosine-protein kinase Src (SRC)** | P-selectin glycoprotein ligand 1 (PSGL-1) |
| **Receptor for advanced glycosylation end products (RAGE)** | Renin (REN) |

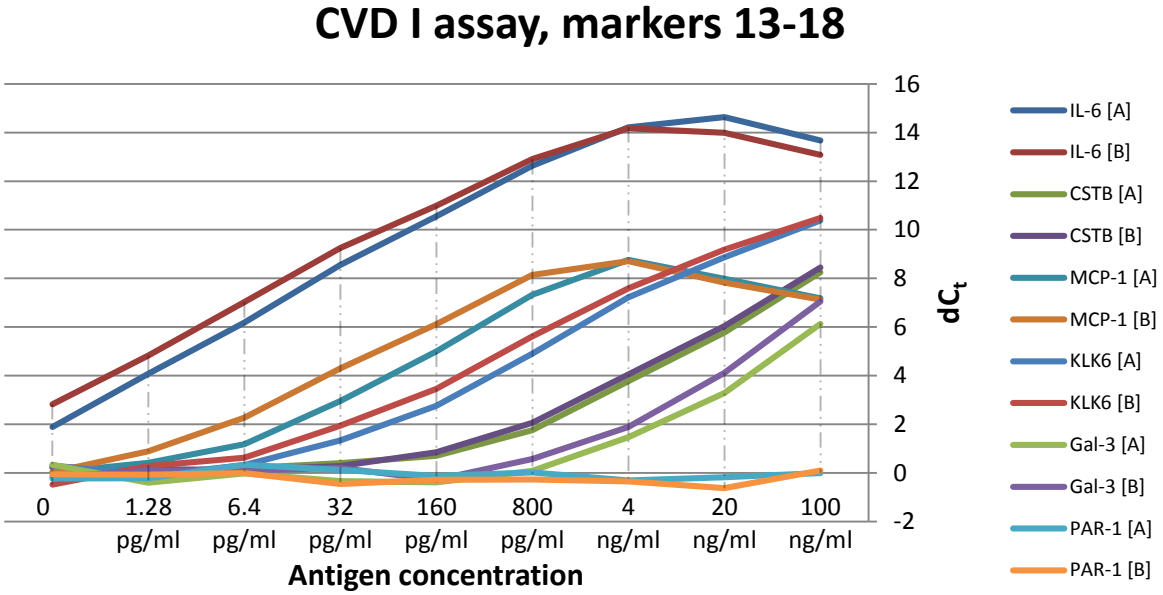
| | |
|---|---|
| **Resistin (RETN)** | SIR2-like protein (SIRT2) |
| **Spondin-1 (SPON1)** | ST2 protein (ST2) |
| **Stem cell factor (SCF)** | Thrombomodulin I |
| **TIM-1 (TIM)** | Tissue factor (TF) |
| **Tissue-type plasminogen activator (t-PA)** | TNF-related activation-induced cytokine (TRANCE) |
| **TNF-related apoptosis-inducing ligand (TRAIL)** | TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) |
| **Tumor necrosis factor ligand superfamily member 14 (TNFSF14)** | Tumor necrosis factor receptor 1 (TNF-R1) |
| **Tumor necrosis factor receptor 2 (TNF-R2)** | Tumor necrosis factor receptor superfamily member 5 (CD40) |
| **Tumor necrosis factor receptor superfamily member 6 (FAS)** | Urokinase plasminogen activator surface receptor (U-PAR) |
| **Vascular endothelial growth factor A (VEGF-A)** | Vascular endothelial growth factor D (VEGF-D) |

## 8.2 Results from MUX-assay of CVD I antigen standard, buffer A to buffer B

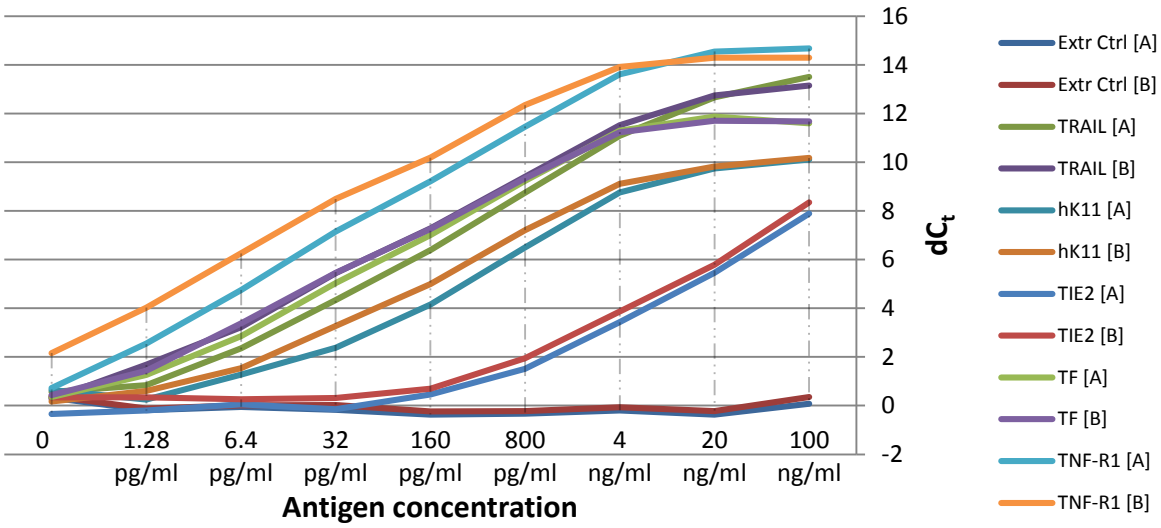
Capital letter within brackets indicates sample buffer.



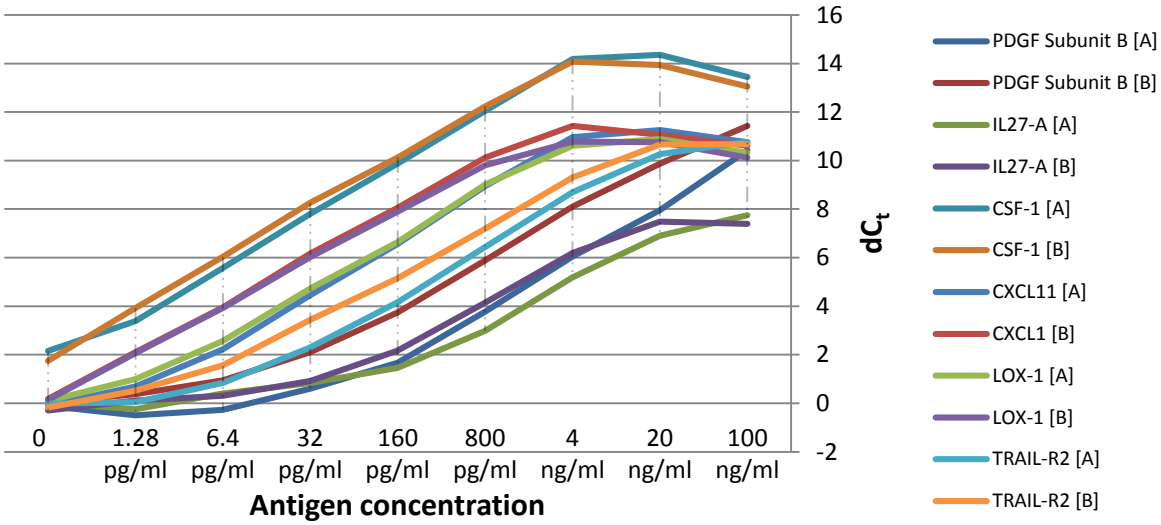

CVD I assay, markers 7-12
16
14
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
PIGF [A]
PIGF [B]
SELE [A]
SELE [B]
EGF [A]
EGF [B]
OPG [A]
OPG [B]
SRC [A]
SRC [B]
IL-1ra [A]
IL-1ra [B]


CVD I assay, markers 13-18
16
14
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
IL-6 [A]
IL-6 [B]
CSTB [A]
CSTB [B]
MCP-1 [A]
MCP-1 [B]
KLK6 [A]
KLK6 [B]
Gal-3 [A]
Gal-3 [B]
PAR-1 [A]
PAR-1 [B]

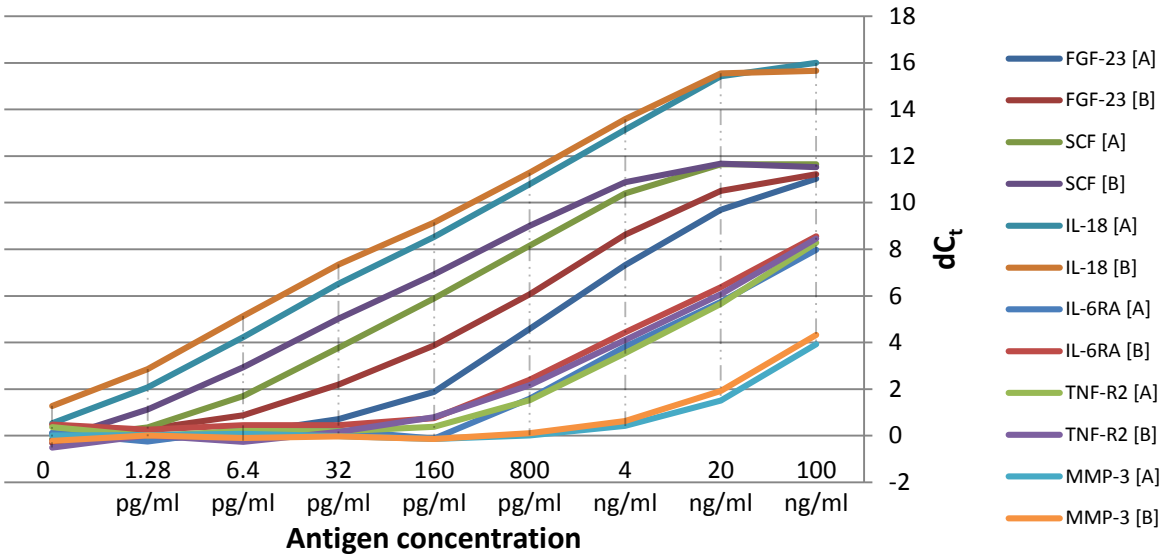
## CVD I assay, markers 19-24



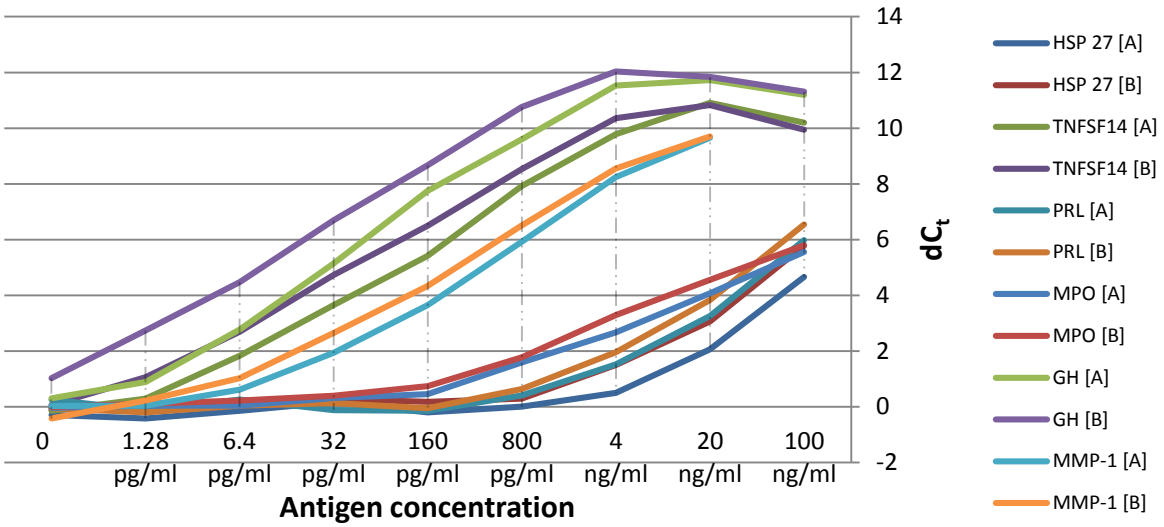

## CVD I assay, markers 25-30

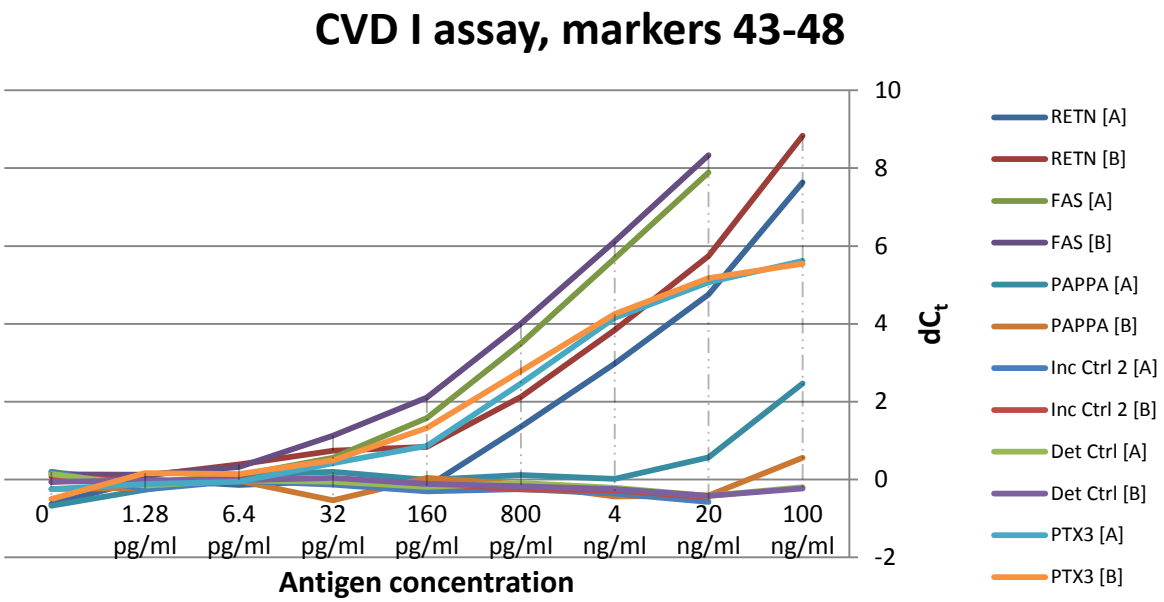


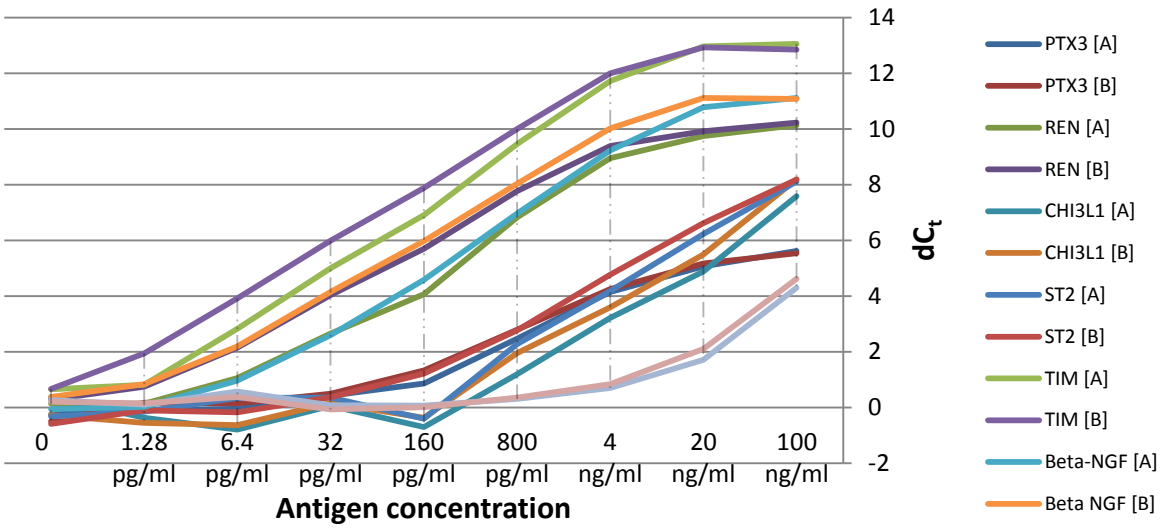
## CVD I assay, markers 31-36



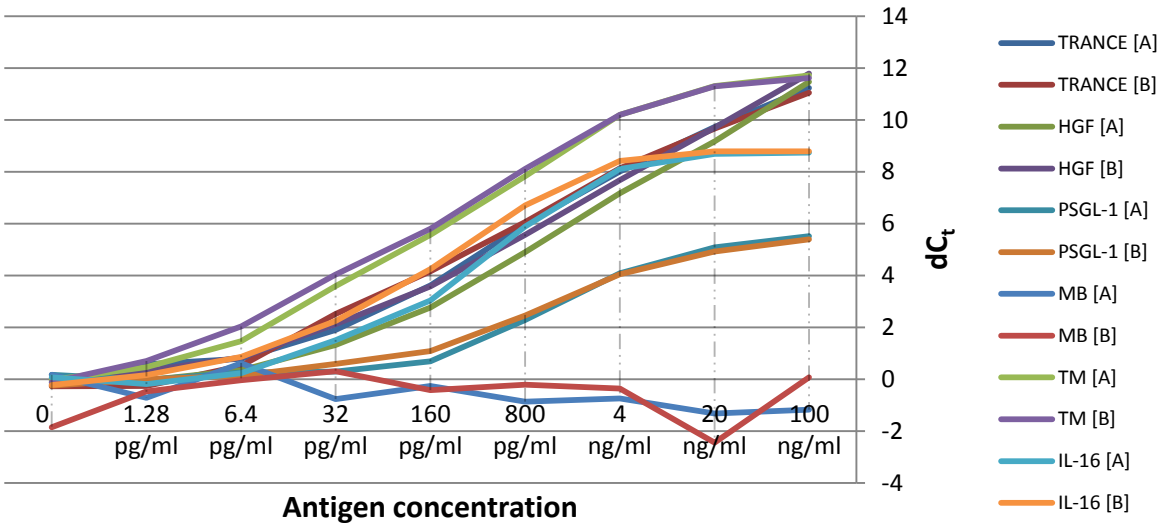

## CVD I assay, markers 37-42



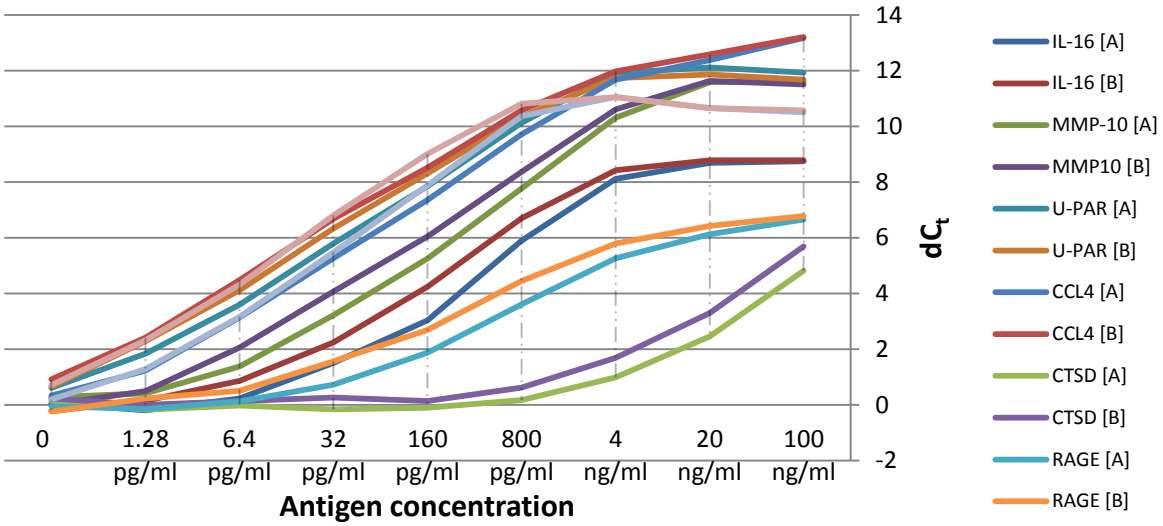

CVD I assay, markers 43-48
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
RETN [A]
RETN [B]
FAS [A]
FAS [B]
PAPPA [A]
PAPPA [B]
Inc Ctrl 2 [A]
Inc Ctrl 2 [B]
Det Ctrl [A]
Det Ctrl [B]
PTX3 [A]
PTX3 [B]

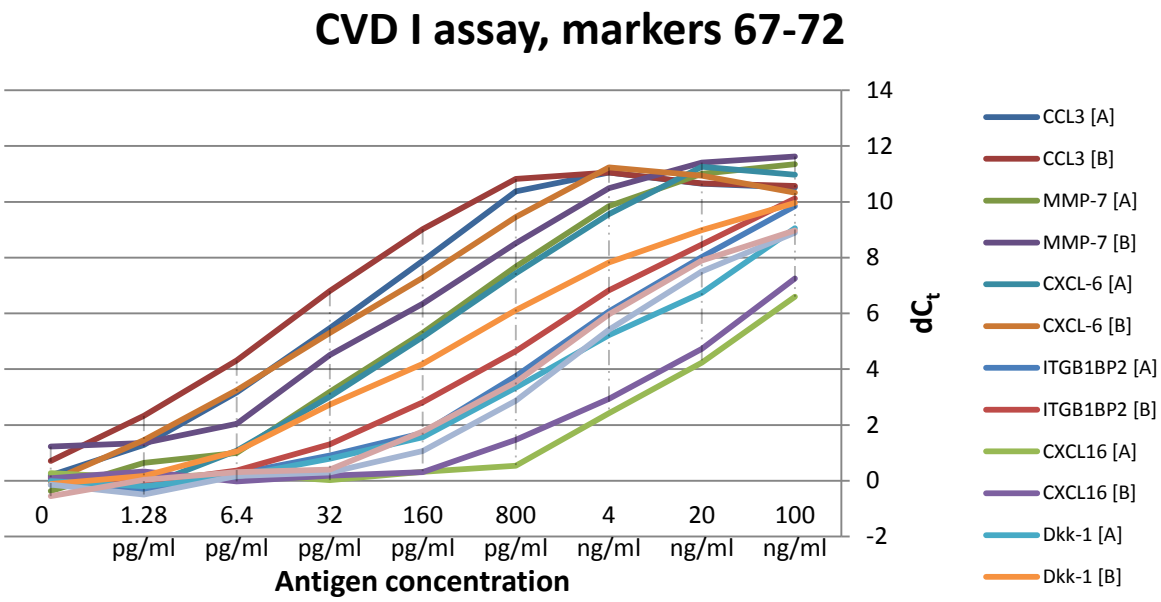

CVD I assay, markers 49-54
14
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
PTX3 [A]
PTX3 [B]
REN [A]
REN [B]
CHI3L1 [A]
CHI3L1 [B]
ST2 [A]
ST2 [B]
TIM [A]
TIM [B]
Beta-NGF [A]
Beta NGF [B]

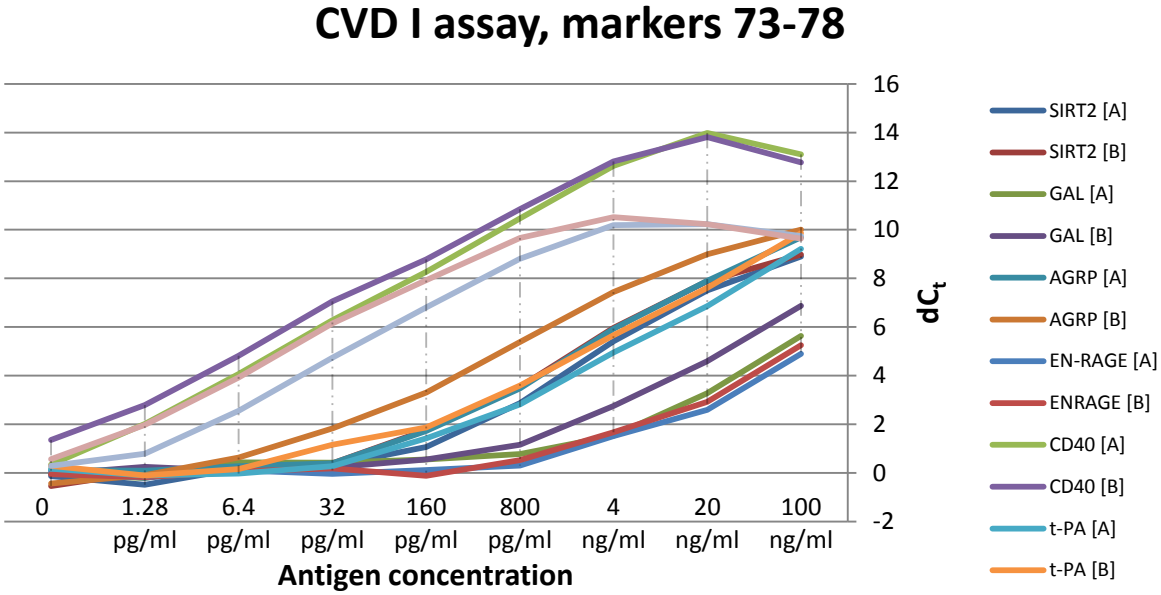
## CVD I assay, markers 55-60



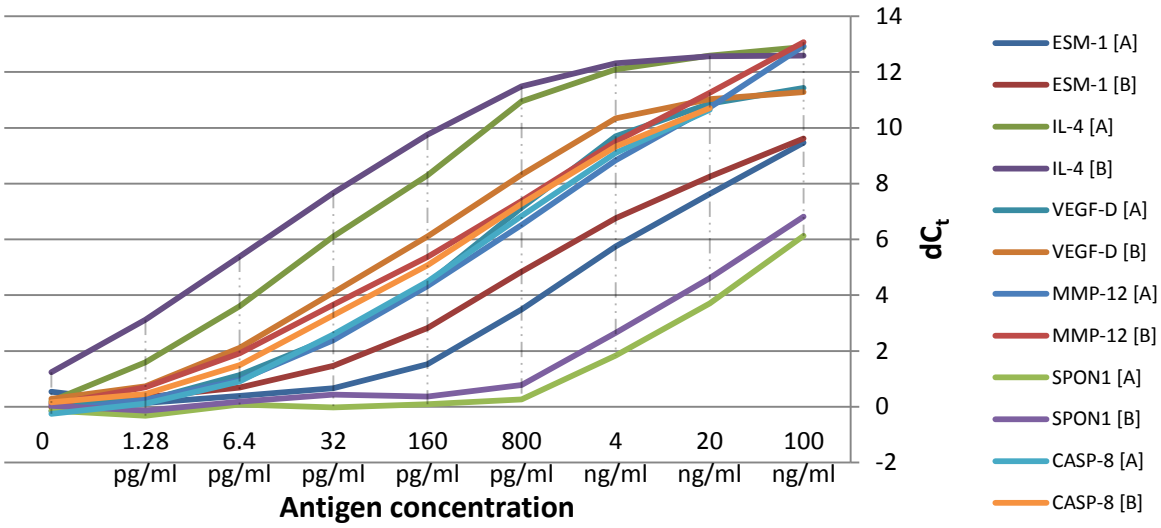

## CVD I assay, markers 61-66



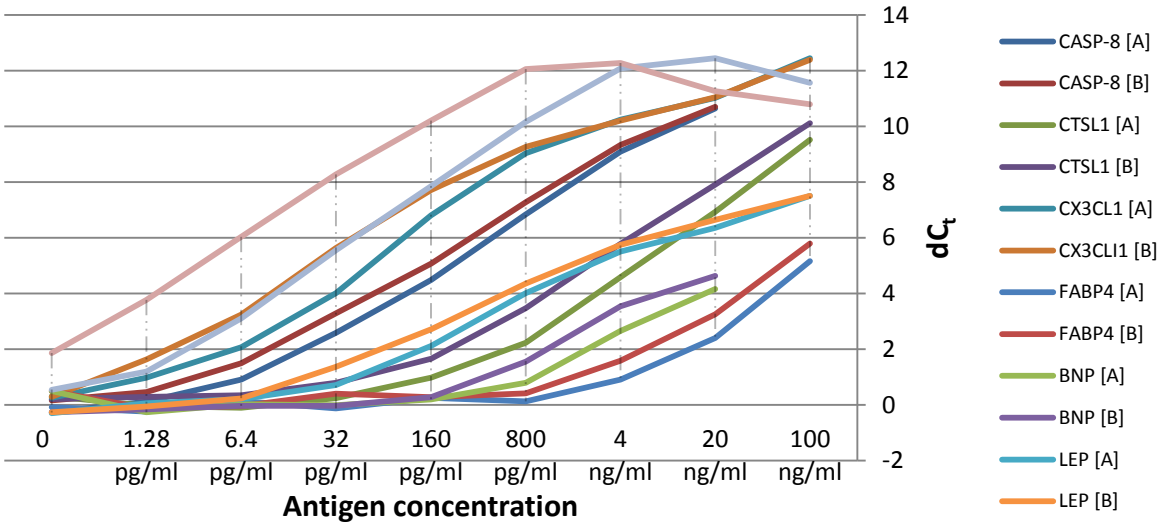

CVD I assay, markers 67-72
14
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
CCL3 [A]
CCL3 [B]
MMP-7 [A]
MMP-7 [B]
CXCL-6 [A]
CXCL-6 [B]
ITGB1BP2 [A]
ITGB1BP2 [B]
CXCL16 [A]
CXCL16 [B]
Dkk-1 [A]
Dkk-1 [B]

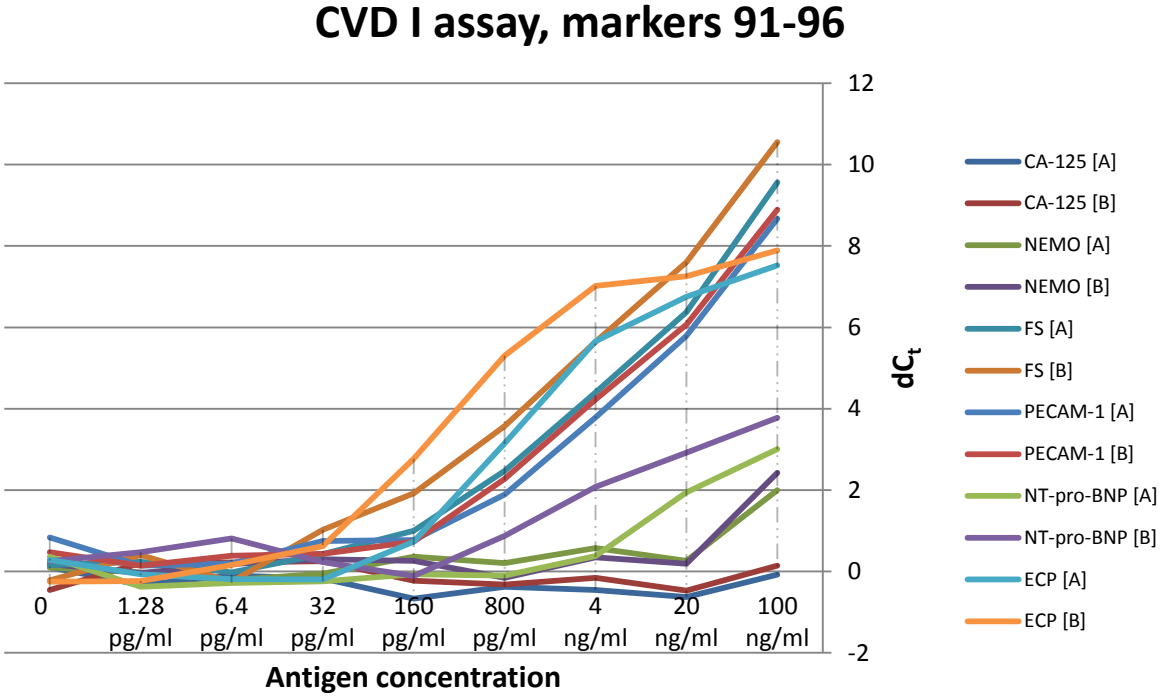

CVD I assay, markers 73-78
16
14
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
SIRT2 [A]
SIRT2 [B]
GAL [A]
GAL [B]
AGRP [A]
AGRP [B]
EN-RAGE [A]
ENRAGE [B]
CD40 [A]
CD40 [B]
t-PA [A]
t-PA [B]

## CVD I assay, markers 79-84




## CVD I assay, markers 85-90




CVD I assay, markers 91-96
12
10
8
6
4
2
0
-2
dCt
0
1.28 pg/ml
6.4 pg/ml
32 pg/ml
160 pg/ml
800 pg/ml
4 ng/ml
20 ng/ml
100 ng/ml
Antigen concentration
CA-125 [A]
CA-125 [B]
NEMO [A]
NEMO [B]
FS [A]
FS [B]
PECAM-1 [A]
PECAM-1 [B]
NT-pro-BNP [A]
NT-pro-BNP [B]
ECP [A]
ECP [B]