UPPSALA
UNIVERSITET

Development and evaluation of procedures and reagents for extraction of proteins from dried blood spots for analysis using Proseek

Johan Björkesten

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 14 001	Date of issue 2014-02

Author

Johan Björkesten

Title (English)

Development and evaluation of procedures and reagents for extraction of proteins from dried blood spots for analysis using Proseek

Title (Swedish)

Abstract

A method for extraction of proteins from dried blood spots (DBS) for analysis using Proseek is developed and evaluated. DBS, as sample format, possesses a number of desirable advantages over for example plasma samples. These advantages include for example minimal patient invasiveness, sampling simplicity and non regulated sample transportation. Highly reproducible quantitative detection of 92 proteins is demonstrated from a 1.2 mm in diameter DBS disk. The DBS inter spot analysis precision (7% coefficient of variance) is comparable to plasma inter assay precision (6% coefficient of variance). Coefficient of variance is the ratio between standard deviation to mean value for the analysed replicates. Proseek analysis of DBS could possibly reveal a unique opportunity to examine health related issues in extremely premature infants hopefully resulting in increased survival rates in the future.

Keywords

DBS, dried blood spots, PEA, Proseek, Proseek Multiplex, qPCR, Protein detection, Olink

Supervisors

Dr. Mats Gullberg
Olink Bioscience

Scientific reviewer

Dr. Masood Kamali-Moghaddam
Uppsala University

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **64**

Development and evaluation of procedures and reagents for extraction of proteins from dried blood spots for analysis using Proseek

Johan Björkesten

Populärvetenskaplig sammanfattning

Etiska studier av för tidigt födda barns hälsotillstånd kan idag inte genomföras eftersom vanliga venösa blodprover innebär en för stor påfrestning på de mycket känsliga barnen. Att istället sticka barnet med en tunn nål i hälen och droppa några få droppar blod på ett filterpapper skulle kunna anses vara etiskt korrekt om många olika hälsorelaterade proteiner kan mätas med hög precision från ett sådant prov. Andra fördelar med att använda sig av torkade bloddroppar, som substitut till vanliga blodprover och plasmaframställning, är enkel provtagningsprocedur, inget krav på avancerad utrustning, okomplicerade förvaringsbetingelser och simpla transportbestämmelser. Dessa fördelar kan visa sig vara mycket betydelsefulla inom områden som diagnostik och screening av folksjukdomar i låginkomstländer och utveckling av hemtester.

Detta arbete beskriver hur metoder för att extrahera ett stort antal olika proteiner från bloddroppar intorkade på filterpapper har utvecklats och testats. Dessa proteiner har sedan analyserats med en proteindetektionsmetod som heter Proseek®. Proseek® har utvecklats av ett företag i Uppsala som heter Olink Bioscience. Arbetet visar att ett stort antal olika proteiner kan analyseras med hög precision från en enda enskild intorkad bloddroppe. Detta resultat skulle kunna leda till att etiska studier på för tidigt födda barn skulle kunna genomföras. Sådana studier skulle i förlängningen kunna leda till ökad förståelse för barnens hälsotillstånd och förhoppningsvis ökad överlevnadsgrad i framtiden.

Examensarbete 30 hp
Civilingenjörsprogrammet Molekylär bioteknik

Uppsala universitet, januari 2014

Table of contents

1 Abbreviations

AAPS	American Association of Pharmaceutical Scientists
Ct value	Cycle threshold value
CV	Coefficient of variance
DBS	Dried blood spots
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	U.S. Food and drug administration
HIV	Human immunodeficiency virus
HS	Heat stabilization
IFC	Integrated fluidic circuit
IL-6	Interleukin 6
IL-8	Interleukin 8
IPC	Internal positive control
LBA	Ligand-binding assay
LBABFG	Ligand binding assay bioanalytical focus group
LLOQ	Lower limit of quantification
LOD	Limit of detection
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEA	Proximity extension assay
PLA	Proximity ligation assay
qPCR	Quantitative real-time PCR
RT	Room temperature
S/N	Signal to noise
STD	Standard deviation
TK	Toxicokinetic

2 Introduction

2.1 Historical and present use of DBS

Dried blood spots (DBS) are the use of filter paper as matrix for storage of droplets of whole blood for different kinds of later analysis. DBS was first introduced in the 1960's for the analysis of phenylalanine levels in blood related to the disease phenylketonuria [1]. DBS have since then been used routinely in newborn screening programs for an increasing number if inherent diseases in over 20 countries [2]. DBS usage was for a long time restricted by the small amount of blood stored in the spots. The usage increased significantly with the development of the polymerase chain reaction (PCR) and the production of monoclonal antibodies [2]. The current neonatal screening program in Sweden includes 24 different inherent diseases [3]. DBS samples from this screening program have been stored in a biobank for all individuals born after 1975 making the samples available for re-analysis or other kinds of future studies [3].

The major advantages with standard DBS sampling (a few droplets of capillary blood collected from a finger or heel stick) compared to venepuncture are the minimal invasiveness for the patient, the requirement of minimally trained personnel, the low cost, no need for specific storage conditions and the simple regulations regarding transportation [4]. These advantages make DBS sampling suitable for a number of applications:

- Screening programs of infants and elderly because of the minimal invasiveness for the patient
- Screening in low income countries because of simplicity, low cost and no need for specific storage and transportation conditions
- Home testing due to ease of sampling
- Pre-clinical toxicokinetic (TK) studies (determination of drug exposure) in animals due to small sample volume

As proof of the DBS usefulness in these areas it can be highlighted that:

- Extensive newborn screening programs using DBS have been performed around the world since the 1960's [2].
- DBS assays targeting human immunodeficiency virus (HIV) RNA has been shown equally sensitive and much more suitable for limited field-based conditions, for example in Africa, as traditional plasma-assays [5].
- Consumers in the United States can buy DBS home sampling kits and send the self sampled DBS by mail to a laboratory for analysis of for example Vitamin D, estrogen or testosterone [6].
- A number of studies showing the usefulness of DBS with TK studies in humans have been reported [7]. The use of DBS sampling in pre-clinical TK studies in animals can lead to a reduction in the number of animals needed and to more accurate TK data due to the possibility of single animal sampling (instead of composite sampling) [7, 8]. This is leading to significant benefits in the 3R's (Reduction, Refinement and Replacement) for the use of animals in drug development [7, 8].

2.2 DBS sampling

DBS sampling are performed by applying a small amount (15-50 µl) of peripheral whole blood from a heel stick (infants), a finger stick (adults) or from venepunctured whole blood with or without anticoagulants such as EDTA, citrate or heparin [9]. The application of blood to a pre-printed area on a specific filter paper card is carried out by letting a droplet of blood from for example the finger or a pipette tip touch the filter card without touching the card with the finger or pipette tip itself [9]. The blood is by this approach forming an approximately circular and relatively homogenous DBS. The DBS card should be dried for at least three hours up to overnight depending on the current humidity. The DBS should when dried be stored in individual gas-impermeable zip-lock bags with an added desiccant pack. Proteins and nucleic acids have been shown stable when DBS have been stored in this manner both in fridge and at room temperature for up to 1 year [9]. For long periods (more than 90 days) -20 °C freezing is however the most convenient storage condition [9].

2.3 Immunoassays

Immunoassays are methods using antibodies to recognize specific proteins. The immunoassay accuracy depends on both the specificity in reconnaissance between the antibody and its antigen and the specificity in the detection system. Many different kinds of detection systems have been developed for example coupled enzymatic reactions, radioactive isotopes and DNA reporters. The one, at least historically, most well known type of immunoassay is the enzyme-linked immunosorbent assay (ELISA) [10]. ELISAs often uses an antibody-coupled enzyme that catalyzes a conformational change of compounds leading to a change in visible color. Frequently used enzymes in ELISAs are for example horseradish peroxidase or alkaline phosphatase. One major disadvantage with ELISAs are the cross reactivity occurring when multiplexed assays are to be developed [11]. The increasing understanding of the biological complexity of many diseases increases the need of efficient, highly multiplexed, protein detection systems. ELISA-based methods fail due to insufficient specificity in the antibodies leading to cross-reactivity when highly multiplexed assays are to be performed. Cross reactivity issues increases quadratically with the degree of multiplexing (number of targets) [11]. Fortunately there are other types of immunoassays which are specific enough to be highly multiplexed. The proximity ligation assay (PLA) and the proximity extension assay (PEA) developed by Olink Bioscience in Uppsala Sweden have been shown to be very sensitive and very specific making them suitable for multiplexing [12, 13, 14]. The PEA technique has successfully been developed into a commercial 96-plex assay called Proseek® Multiplex with protein panels related to oncology and cardiovascular diseases.

2.4 Validation of immunoassays

Immunoassays belong to ligand-binding assays (LBA) due to utilization of antibodies specifically binding target antigens. The binding between an antibody and its antigen constitutes the core of the immunoassay principle. Immunoassays may therefore be less precise than chromatographic assays and the accepted validation criterion regarding accuracy and precision for immunoassays should therefore be more lenient compared to chromatographic assays [15]. In 2003 a subcommittee from the American Association of Pharmaceutical Scientists (AAPS), called Ligand Binding Assay Bioanalytical Focus Group

(LBABFG), established a set of validation directives for immunoassays used in pharmacokinetics [16].These accepted and widely used criteria for LBAs on accuracy and precision are, by mentioned reason, less demanding than the criteria for bioanalytical methods, proposed by the United States Food and Drug Administration (FDA) [17] (Table 1). The validations of LBAs are divided in three phases, method development, pre-study validation and in study validation. The parameters to be established or evaluated in the method development phase are assay reagents, selectivity, specificity, matrix selection, standard curves, precision, accuracy, range of quantification, sample stability, dilution linearity and robustness [16].

Table 1. Validation parameter values recommended for the validation of LBAs established by LBABFG[16] and for bioanalytical methods established by FDA[17].

Acceptance criteria	Accepted limit for LBA	Limit by FDA
Accuracy (relative error)	< 20 % (25 % at LLOQ*)	< 15 % (20 % at LLOQ)
Precision (coefficient of variance, CV)	< 20 % (25 % at LLOQ)	< 15 % (20 % at LLOQ)
Total error	< 30 %	N.A.

*Lower limit of quantification

The criterion in Table 1 should be valid for both inter- and intra-assay runs. Formulas for the calculations of the different criteria are found in the supplementary material (9.1 Formulas). In addition to the standard validation criteria used for immunoassays some more experiments should be considered for the specific use of immunoassays with DBS. These additions include analyte stability in human whole blood, analyte stability in DBS, effect of blood volume spotted onto matrix, device used for spotting and temperature of the blood spotted [18].

2.5 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is an accurate and sensitive method for determination of nucleic acid concentrations in different kinds of samples. The qPCR progressed from the PCR invented by Kary Mullis in 1983 (Nobel Prize in 1993) [19]. The number of performed PCR assays increased enormously with the commercialization of heat stable *Taq* polymerase three years after the invention [19]. qPCR amplifies a specific sequence of DNA from a non detectable starting concentration and the increasing amount of DNA is measured continuously. The measured signal is fluorescence, originating from a fluorescing agent. The fluorescence emitted from the agent, proportionally increasing with the DNA amplification, is recorded in each PCR cycle generating an amplification plot of the target DNA [19, 20]. Important concepts involved in qPCR are *baseline*, *threshold*, *Cycle threshold (Ct) value*, *ROX* and *Rn* [19, 20].

The **baseline** is the average background noise (fluorescence) in the tube in the early cycles of the process when no increase in signal, due to target amplification is seen [19, 20].

The **threshold** is a signal level, often defined as a fixed number of background noise standard deviations above the background, where the signal is not considered background [19, 20].

The **Ct value** is defined as the cycle number where there is a significant increase in signal above the threshold level. The Ct value can either be determined as where the reporter signal crosses the threshold or with a signal second derivative method where the Ct is defined as the cycle above the threshold where the signal increase reaches its maximum. The Ct value is inversely proportional to the initial amplicon concentration, meaning that a low (early) Ct value correspond to a high initial amplicon concentration. The Ct values are logarithmically representing the difference between initial amplicon concentrations due to duplication of the total number of amplicons in each qPCR cycle. The difference of one Ct between two samples therefore states that the initial concentration of one sample (lower Ct) was twice as high as the other (higher Ct) [19, 20].

ROX™ is a passive reference dye that can be added to the qPCR master mix in many, but not all, qPCR thermal cyclers. ROX™ is used for normalization purposes [19, 20].

The **Rn** is a normalized version of the signal, simply calculated by dividing the reporter signal to the ROX™ signal. The signal is in this way normalized for for example master mix pipetting errors, air bubbles in the tube and covering film opacity [19, 20].

A qPCR amplification plot example, visually explaining most of the mentioned qPCR concepts, is seen in Figure 1.
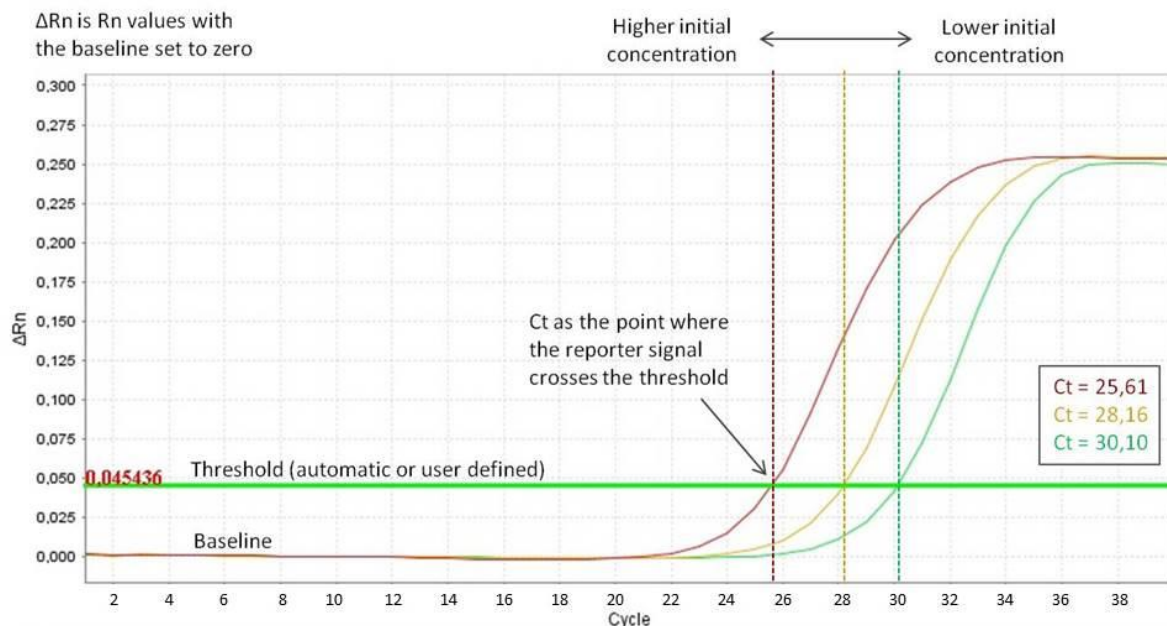
Figure 1. Example of an amplification plot (qPCR output) from the analysis of three samples (red, yellow and green continuous line). The sample signal (ΔRn), measured in each cycle, is the ROX™ normalized reporter fluorescence (Rn) with the baseline (average background fluorescence in early cycles) set to zero. Sample Ct value, used for evaluation of initial concentration ratios, is the cycle where the signal crosses the either automatic or user defined signal threshold. Lower Ct value corresponds to higher initial concentration and vice versa meaning that the initial concentration of the red sample is the highest. The difference of one Ct between two samples, due to amplicon duplication in each PCR cycle, states that the initial concentration of one sample (lower Ct) was twice as high as the other (higher Ct). The red samples (Ct = 25.61) initial concentration is therefore 5.9 times higher than the yellow samples (Ct = 28.16) initial concentration (2 to the power Ct difference).

11

2.6 Proximity extension assay

In biomedical research there are large demands for robust and accurate methods enabling protein detection and quantitation in complex biological samples [14], for example plasma or eluted DBS. There are many technical challenges involved in detecting proteins in complex samples. Genomics and DNA detection are a more developed genre of bioanalysis than proteomics and protein detection due to earlier interventions and routine usage. The proximity extension assay (PEA) method converts the protein detection problem into a, more easily solved, DNA detection problem [14]. This is achieved using two PEA-probes (A and B) aiming a specific protein of interest. The PEA-probes are made up of antibodies with conjugated, partly complementary, DNA oligos [14]. The antibody part of the probes is aiming different epitopes on a specific protein (Figure 2 a). The probes are, upon target antigen recognition, ending up in close proximity to each other allowing their complementary DNA oligos to hybridize [14] (Figure 2 b). The DNA oligos are designed to only hybridize slightly and efficient hybridization therefore requires close proximity [14]. Extension of one oligo along the other creates a double stranded DNA amplicon (Figure 2 c). The amplicon can be targeted with specific primers and detected through qPCR. The increase in total amount of DNA in the qPCR can accurately be measured by the increase in fluorescence of for example SYBR green (agent fluorescing upon binding double stranded DNA). The qPCR results can be analyzed by software (Figure 2 d).

2.7 Proseek® and Proseek® Multiplex

Olink Bioscience in Uppsala Sweden have developed and commercialized two methods based on the PEA technique for accurate and sensitive protein detection in only a 1 µl sample. The first one, Proseek®, is a singleplex assay that can measure protein markers down to femtomolar (fM) level and has a 3-4 log linear concentration range. The second one, Proseek® Multiplex^{96x96} is capable of measuring quantitative levels of 92 different biomarkers in 1 µl of sample, with maintained accuracy and sensitivity. The very small amount of sample needed in Proseek® and Proseek® Multiplex^{96x96} make DBS analysis, constraint to a very limited sample amount, a very interesting implementation.

The reason for successful development of the PEA technique into a 96-plex assay (Proseek® Multiplex^{96x96}), where many other techniques fail, is through the double protein recognition (two probes aiming one target protein) combined with specific oligo hybridization (DNA ligated to the probes need to match), needed for signal generation. This entirely eliminates cross reactivity, due to unspecific binding of antibodies and unspecific signal generation, which is the limiting factor of many techniques to below 10-plex assays. Proseek® Multiplex^{96x96} creates quantitative amounts of 96 unique amplicons through sample incubation with 96 target specific PEA-probe pairs. These amplicons are pre-amplified in a PCR step to generate prerequisite amounts for 96 separate qPCR reactions. Individual Proseek® Multiplex^{96x96} qPCR reactions are performed on a Dynamic Array^{TM} Integrated Fluidic Circuits (IFCs) 96.96 chip, developed and manufactured by Fluidigm in San Francisco. The IFC chip is equipped with chambers, valves and microfluidic channels that automatically combines the 96 added samples with the 96 added primer pairs (specifically targeting the 96 pre-amplified amplicons) for a total of 9216 individual qPCR reactions. The

progress of all individual qPCRs are detected by the instrument Bio Mark[TM] HD System, also developed and manufactured by Fluidigm, generating 9216 data points in a single run.
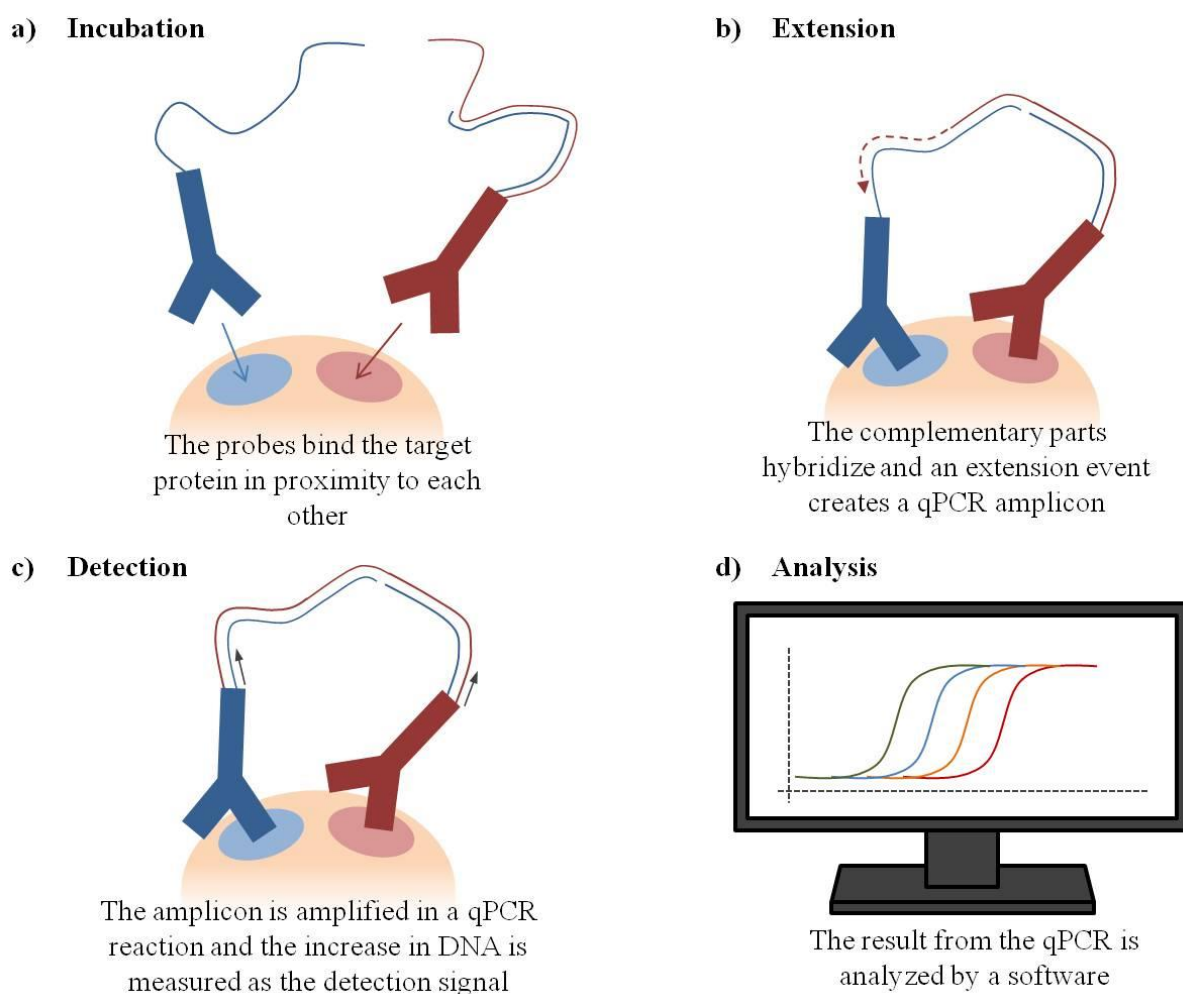
Figure 2. The PEA process for sensitive and specific protein detection is based on (**a**) two PEA-probes (dark red and dark blue) recognizing individual epitopes (light red and light blue) on their target protein, ending up in close proximity. Partly complementary DNA oligos hybridize and an extension event, forming a qPCR amplicon, occurs (**b**). The amplicon is amplified in a qPCR reaction continuously measuring the total amount of DNA (**c**). The resulting amplification plot is visualized by computer software (**d**).

2.8 Heat stabilization

Heat stabilization (HS) is an additive free preservation technology. Many different kinds of samples, for example DBS [21], are easily heat stabilized with the instrument Stabilizor[TM], developed and manufactured by Denator AB in Gothenburg Sweden. Sample stability is crucial for all kinds of accurate bioanalysis. The DBS drying process, approximately 2 hour long, is a probable cause of enzymatic degradation of certain analytes. The use of chemicals to eradicate enzymatic activity possesses limitations, for example loss of certain analytes, where rapid high-temperature heating, used in HS, does not [21]. The mechanism behind the HS preserving property is active enzyme unfolding, and refolding to inactive states, maintained by the quick high-temperature heating. An experiment, analyzing quantitative levels of six commercial drugs from DBS, was performed with and without HS showing significant

positive HS (maintained drug level) for three drugs where non-stabilized samples showed significant losses [21]. One of the drugs in the experiment showed consistent levels for both HS and non-stabilized samples and two drugs were found to be very heat sensitive, decomposing already at 60 $^\circ$C [21]. HS is though a viable technique to stop degradation of some, but not all, compounds analyzed from DBS. The heat stabilized DBS elution capacity of larger molecules, for example proteins, are though somewhat obscure.

2.9 Aim of the project

This degree project aims to develop and evaluate procedures and reagents for extraction of proteins from DBS for analysis using Olink Bioscience PEA technique applications Proseek® and Proseek® Multiplex. General conclusions regarding DBS and Proseek® compatibility and capacity are to be made rather than development of a specific assay. These conclusions are supposed to concern for example convenient DBS extraction reagents and conditions, choice of DBS spotting card and DBS inter spot analysis accuracy.

3 Materials and Methods

3.1 Materials

3.1.1 Consumables
PE Grade 226 filter paper in bulk format and a Stabilizor T1 (DST 0001) was kindly provided by Denator (Uppsala, Sweden). Whatman DMPK-C filter paper carsd (WB129243), Whatman DMPK-C indicating filter paper cards (WB120224), Harris Micro Punch, 1.2 mm and 3 mm with cutting mat (WB100005 and WB1000389), plastic ziploc storage bags (10548232) and a Whatman 903 Dry Rak without Velcro (10537173) were purchased from GE Healthcare, former Whatman (Uppsala, Sweden). Agilent bond elut DMS filter paper cards (A400150) were purchased from Agilent Technologies. MicroAmp® optical 96-well reaction plate (N8010560), MicroAmp™ optical adhesive film (4311971) and MicroAmp™ clear adhesive film (4306311) were obtained from Applied Biosystems (U.K.). Dynamic Array™ Integrated Fluidic Circuits (IFCs) 96.96 chips for Proseek® Multiplex analysis were purchased from Fluidigm (San Francisco, U.S.). Minipax® absorbent packets (Z163619-100EA) were obtained from Sigma-Aldrich. Pipette tips with filter were used for all pipetting.

3.1.2 Reagents
All reagents needed for buffer preparation, Proseek® incubation for example Proseek® probes, Proseek® extension for example PCR Polymerase and Proseek® detection for example PEA solution was handled by Olink Bioscience (Uppsala, Sweden). Also reagent kits for Proseek® Multiplex^{96x96} analysis and Probe maker kits were supplied by Olink Bioscience.

3.1.3 Systems
The 7500 qPCR system was obtained from Applied Biosystems (U.K.). The BioMark™ HD system was obtained from Fluidigm (San Francisco, U.S.). The centrifuges used were Eppendorf Centrifuge 5418 and Hettich zentrifugen Universal 320. The vortex used were vortex-genie® 2 from Scientific Industries.

3.1.4 Samples
Human EDTA blood, plasma and serum samples were obtained from laboratory blood donors through purchase from Uppsala University hospital (Uppsala, Sweden). Human capillary DBS, venous DBS, EDTA blood, Citrate blood and Heparin blood from five individuals were obtained from Örebro University hospital (Örebro, Sweden).

3.2 Methods

3.2.1 Elution buffer preparation
A set of nine different conceivable elution buffers were prepared for evaluation of Proseek® analysis compatibility. All buffers were prepared by adding reagents into Millipore water (70 % of final volume), adjusting the pH by adding sodium hydroxide or hydrochloric acid, and adjusting the final volume by addition of Millipore water. The nine buffers prepared are presented in Table 2.

Table 2. Conceivable DBS elution buffers prepared for evaluation of Proseek analysis compatibility and DBS protein elution efficiency.

Buffer name	Content	pH
Calibrator diluent	Confidential by Olink	
Buffer 1	PBS* with 0.05 % Tween 20	7.4
Buffer 2	Tris buffer with 0.05 % Tween 20	7.0
Buffer 3	Tris buffer with 10 % Glycerol (V/V) and 2 % (W/V) SDS**	7.0
Buffer 4	Tris buffer with 8.0 M Urea	7.4
Buffer 5	Hanks balanced salt solution (only salt, small amounts)	7.3
Buffer 6	PBS with 0.5 % Triton X-100	7.4
Buffer 7	Buffer 2 with 500 mM NaCl	6.8
Buffer 8	Hanks balanced salt solution without $CaCl_2$	7.5

*PBS = Phosphate buffered saline, **SDS = Sodium Dodecyl Sulphate

3.2.2 Standard curve and spiked sample preparation

Standard curves were typically prepared by adding 1 µl sample, or antigen standard, to 9 µl buffer (Tube 1), brief vortex followed by a few seconds centrifugation to secure good mixture, before 1 µl of the Tube 1 content was transferred to 9 µl buffer in Tube 2, and so on. The 1 µl transfers were performed by ordinary pipetting, and sample release through pipetting up and down a few times in the buffer before pushing the pipette ejector to the bottom. The dilutions were made in PCR strips. Spiking of antigen to samples was performed in the same way as one step in the standard curve preparation procedure. When blood (to be spotted onto filter cards) was prepared with these procedures the volumes were though larger, typically 5 µl antigen to 45 µl blood.

3.2.3 DBS spotting, drying and storage

DBS was typically prepared by reverse pipetting of 15 µl of Ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood to a filter paper. The 15 µl blood was allowed to hang from the pipette tip before gentle application by touching the blood droplet, but not the pipette tip, to the filter paper surface generating an evenly spread DBS. Reverse pipetting refers to a technique where the pipette tip is filled with a little larger than desired volume by pushing the pipette ejector down just below the first stop before filling it up and eject to the first stop only. DBS were at all times allowed to dry horizontally in a Whatman drying rack for at least 3 h in room temperature (RT) before use or storage in a zip-lock bag with a desiccant pack (in RT).

3.2.4 DBS punching

To achieve reproducible results from DBS analysis, a fixed sized DBS disk is punched out from the entire DBS. To manage this, a Micro Puncher (3 or 1.2 mm in diameter) and a cutting mat developed for DBS usage, was used. The Micro Puncher is very similar to a ballpoint pen. The puncher tip is manually pressed through the DBS and the disk stays in the puncher tip until ejection in an appropriate place. DBS disks were never punched from the very edge of the DBS and a blank punch (filter without blood) was always performed in between DBS punches to avoid transfer between samples. After usage the mat and the puncher were carefully cleaned with 70% ethanol.

3.2.5 DBS elution

Different parameters related to elution efficiency were evaluated with respect to % recovery of a certain analyte spiked to the blood before DBS preparation. The parameter optimization included elution time, elution temperature and elution buffer volume. The optimized elution procedures for 1.2 or 3 mm in diameter DBS disks from Whatman DMPK-C or Agilent Bond Elut DMS cards are presented in Table 3.

Table 3. Elution procedures optimized for 1.2 or 3 mm in diameter DBS disks from Whatman DMPK-C or Agilent Bond Elut DMS cards.

Elution procedure	Whatman (1.2 mm)	Whatman (3 mm)	Agilent (1.2 mm)	Agilent (3 mm)
Step 1	Put disk in a PCR tube	Put disk in an 1.5 ml eppendorf tube	Put disk in a PCR tube	Put disk in an 1.5 ml eppendorf tube
Step 2	Add 3,2 µl elution buffer	Add 20 µl elution buffer	Add 6,4 µl elution buffer	Add 40 µl elution buffer
Step 3	Elute 1 h RT on vortex set to intensity "5"	Elute 1 h RT on shaker plate set to 540 rpm	Elute 1 h RT on vortex set to intensity "5"	Elute 1 h RT on shaker plate set to 540 rpm
Step 4	Carefully pipette 1 µl eluate to be used as sample	Carefully pipette 1 µl eluate to be used as sample	Carefully pipette 1 µl eluate to be used as sample	Carefully pipette 1 µl eluate to be used as sample

3.2.6 Proseek® analysis

Proseek® singleplex analysis is carried out in a 96 well plate with qPCR detection for example by a 7500 Real Time PCR instrument from Applied biosystems. The overall Proseek® protocol is very simple:

1. Add 3 µl of probe mix (A- and B-probes aiming one specific marker) to the bottom of the wells in the 96-well plate by reverse pipetting using an 8-channel pipette.
2. Add 1 µl of sample to the upper part of the well wall by reverse pipetting.
3. Seal the plate with a protective plastic film to prohibit evaporation.
4. Spin the plate 1 minute at 1000 rpm.
5. Incubate the sealed plate 1 hour at 37 °C.
6. Remove the plastic film and add 47 µl detection buffer.
7. Seal the plate with an optical adhesive plastic film.
8. Spin plate 1 minute at 1000 rpm.
9. Place the plate in the 7500 qPCR instrument and run the detection program (2.5 h).

The Proseek® singleplex analysis takes about 4 hours to perform for a complete 96 well plate.

3.2.7 Proseek® Multiplex^96x96 analysis

The Proseek® Multiplex^96x96 analysis is carried out in three separate steps; incubation, extension and detection.

1. Incubation

Add 3 µl incubation mix to the wells in a 96-well PCR plate (the incubation plate) and thereafter 1 µl sample to 92 of the wells followed by positive or negative control to the remaining four wells. Seal the plate with an adhesive plastic film to prevent evaporation and centrifuge 1 min, 1000 rpm at RT. This part is very similar to step 1-4 for the singleplex analysis except that the incubation mix contains 96 Proseek® probe pairs for multiplex analysis and only a single probe pair for the singleplex analysis. Incubate the plate overnight at +4 °C.

2. Extension

Bring the incubation plate to RT and spin 1 min at 1000 rpm. Carefully remove the plastic film and add 96 µl extension mix to the wells. Seal the plate with an adhesive plastic film, place in the thermal cycler and start the PEA program.

3. Detection

Prepare and prime a 96.96 Dynamic Array™ IFC according to the manufacturer´s instructions. Thaw a frozen 96-well plate with primers aiming the 96 Proseek probe pair oligos. Add 7.2 µl of detection mix to each well of a 96-well PCR plate (the sample plate) and 2.8 µl from the corresponding wells on the incubation plate. Seal the sample plate and spin down 1 min at 1000 rpm before transfer of 5 µl from each well to the 96.96 Dynamic Array IFCs right inlets. 5 µl from each well of the primer plate are transferred to the 96.96 Dynamic Array IFC chips left inlets. Remove any visible bubbles with a sterile lancet load the chip in the Fluidigm IFC Controller HX according to the manufacturer´s instructions. Run the Olink Protein Expression 96x96 Program in the Fluidigm Biomarker Reader according to the manufacturer´s instructions.

3.2.8 Data analysis

Data from the Proseek® qPCR runs were analyzed with the software *7500 Software v2.0.5*. Raw amplification plots and melt curves were visually analyzed for all performed experiments and Ct values were exported from the 7500 Software to Microsoft Excel for further analysis, making different plots and graphs for simple visualization. Data from the Proseek® Multiplex^{96x96} runs were analyzed with the Fluidigm Real-Time PCR analysis software. A heat map view was first analyzed for rough evaluation of run performance. The 9216 data points (Ct values for a certain assay and sample) were exported from the Fluidigm Real-Time PCR analysis software and imported in excel for further analysis, making different plots and graphs for simple visualization. The Ct values (raw data from the experiment) are on log2 scale and were thus linearized before % CV was calculated for the samples. The Ct values from the singleplex analysis were only normalized with background values for for example the buffer used generating signal to noise (S/N) differences, dCT. The Ct values from the multiplex analysis were normalized by subtraction of values for extension control, internal positive control (IPC) and negative control (background noise) generating dddCt values. Linearization was for the singleplex analysis performed by calculations of two to the power of dCt and for multiplex analysis as two to the power of dddCt.

A very widespread and straight forward way to visualize differences and similarities in complex datasets, is by means of principal component analysis (PCA). PCA is a linear orthogonal transformation, generating transformed dimensions without covariance. PCA is typically calculated from the multivariate data covariance matrix, finding a linear transform in such a way that the covariance matrix becomes diagonal (the directions were the data varies the most). PCA is most often used to transform multivariate data into two or three dimensions (principal components). The first principal component is the dimension where the data varies the most. The second principal component is the orthogonal dimensions to the first principal component that keeps as much variation as possible in the data. The graphical statistical tool for PCA calculations and visualization used in this project is a Microsoft Excel add-in called Multibase 2014. Multibase 2014 is a freeware developed by Numerical Dynamics.

3.2.9 Heat stabilization

Heat stabilization (HS) with the Denator AB (Gothenburg, Sweden) Heat Stabilizer[TM] was performed using the by Denator AB developed DBS HS procedure. The DBS was shortly after application of blood to the HS filter card placed in the Heat Stabilizer[TM] and a short DBS HS program was run. HS does not dry the DBS but is thought to stop enzymatic processes by unfolding end refolding protein structures. After HS the DBS are dried and stored according to the procedure described in 3.2.3.

4 Results

4.1 Selection of model system

4.1.1 Assay choice

The first assay to be used for the evaluation of Proseek[®] compatibility with proteins extracted from DBS was interleukin 8 (IL-8). IL-8 is an 11 kDa chemokine produced by for example macrophages and epithelial cells [22]. IL-8 was chosen as a first evaluation assay because Proseek[®] A- and B-probes aiming IL-8 were already prepared and ready to be used before this project started. IL-8 had also been shown to be a very well working assay with high sensitivity and a large dynamic range.

4.1.2 Compatibility between elution buffers and Proseek[®]

The first parameter to be evaluated was compatibility with the different proposed conceivable elution buffers and Proseek[®] analysis. The first experiment was designed as a test where blank buffer and buffer spiked with 100 pM IL-8 antigen were analyzed simply to exclude the buffers that ruined the Proseek[®] capability to distinguish the spiked and non-spiked samples. Most of the buffers in Table 2 (section 3.2.1) performed equally well in this primary test and did not interfere notably with the Proseek[®] analysis. All buffers except buffer 7 and 8 were tested in this experiment. The buffers were analyzed in triplicate runs with both 100 and 25 % concentration (titrated in Millipore water). A graph with the individual Ct-values are found in the supplementary material (9.2.1). Most buffers performed equally both concentrated and titrated. Concentrated Buffer 3 and 4 killed the qPCR detection completely resulting in no amplification signal at all. Concentrated versions of these two buffers were therefore excluded

19

from further testing. Titrated versions of Buffer 3 and 4 generated signals that could be used to distinguish the spike. The span between spiked and non-spiked sample was however very small for Buffer 3 leading to rejection of titrated Buffer 3 but acceptance of titrated Buffer 4. The rest of the buffers were all qualified for further testing already at concentrated versions.

The next compatibility evaluation experiment was generation of standard curves of IL-8 in the qualified buffers (this was also done for buffer 7 and 8). Calibrator diluent and Buffer 1, 2, 6 and 7 performed approximately equally well in terms of sensitivity and linear range (Supplemetary material 9.2.2). All these buffers had a limit of detection (LOD) of approximately 100 fM, similar linear detection range and approximately 3.32 Ct-values increase with 10 fold increase in IL-8 concentration (in the linear interval) which is the same as duplication of amplicon number in each PCR cycle ($2^{3.32} = 10$). A value close to 3.32 corresponds to an efficiently working PCR reaction. All these buffers were by this performance classified as suitable elution buffers regarding compatibility with Proseek[®] analysis. Titrated Buffer 4 were not as sensitive as the others and had a very steep slope of the linear part of the standard curve but was still qualified because of the denaturing properties (Buffer 4 includes Urea) which eventually could be beneficial in the elution step if the proteins are tightly stuck to the filters.

4.1.3 DBS filter card evaluation

The project plan was to evaluate two different types of filter papers. One made of cotton, Whatman DMPK-C, which has been used in many studies, and one made of glass fibre, Agilent Bond Elut DMS, which is a new and fairly unproven filter. Because of delivery reasons of the Whatman DMPK-C (~2 months delivery time), another filter paper was used instead until Whatman DMPK-C arrived. This paper, PE Grade 226, was provided by Denator and should in practice be the same as Whatman DMPK-C.

The most striking difference between the two kinds of filter papers was started to be revealed already before the first evaluating experiment when 20 µl Assay diluent were added to the filters to examine the spot-area. The spreading on the filter papers were very different and resulted in 14.5 mm spots on PE Grade 226 and 4.5 mm spots on Agilent Bond Elut DMS. The spot diameter was determined as the mean value of the measuring of two orthogonal diameters from each spot from multiple spots.

The first experiment was designed to evaluate the extraction efficiency of IL-8 from the two filter papers, and also the performance of some of the proposed elution buffers. This was managed by spotting 20 µl Assay diluent with different concentrations of IL-8 on the two filter types. 3 mm disks were punched from the dried spots and analyzed with Proseek[®]. The samples presented in Table 4 were prepared with different initial concentrations of IL-8 for the different filters resulting in equal expected maximum concentrations after extraction. The final concentrations of the eluate with assumed 100 % elution efficiency were calculated according to Equation 1 and Equation 2 where V is volume, r is radius, d is diameter and c is concentration using the presumption that the concentration of the analyte of interest is homogenously spread over the spot.

$$V_{disk} = \frac{Area_{disk}}{Area_{spot}} \cdot V_{spot} = \frac{\pi r_{disk}^2}{\pi r_{spot}^2} \cdot V_{spot} = \frac{d_{disk}^2}{d_{spot}^2} \cdot V_{spot} \qquad (1)$$

$$c_{eluate} = \frac{V_{disk}}{V_{buffer}} \cdot c_{spot} \qquad (2)$$

Table 4. Concentrations of IL-8 in the Assay diluent added to the two types of filter papers evaluated and theoretical maximum concentration of IL-8 in the eluate after elution of a 3 mm in diameter spot with 40 µl elution buffer. The formulas in Equation 1 and 2 were used for the calculations.

Conc. IL-8 in Assay diluent added to PE Grade 226 (20 µl → 14,5 mm diameter)	Theoretical max conc. IL-8 for a 3 mm PE Grade 226 disk eluted in 40 µl Buffer	Conc. IL-8 in Assay diluent added to Agilent Bond Elut DMS (20 µl → 4,5 mm diameter)	Theoretical max conc. IL-8 for a 3 mm Agilent Bond Elut DMS disk eluted in 40 µl Buffer
2.33 nM	50 pM	233 pM	51 pM (~50 pM)
233 pM	5 pM	23.3 pM	5.1 pM (~5 pM)
23.3 pM	0.5 pM	2.33 pM	0.51 pM (~0.5 pM)
None	None	None	None

The 3 mm disks were eluted in 40 µl Calibrator diluent, Buffer 1, 6 or 7 for 1 h at RT on a shaker plate set to 400 rpm. 1 µl of the eluates and buffers spiked with IL-8, corresponding to the maximum expected concentrations of IL-8 in the eluates, were analyzed with Proseek® (Table 4). The Ct values from the experiment showing good correspondence between theoretical elution maximum and actual measured concentrations (similar curves between eluted disks and references) are presented in Figure 3. All the buffers tested performed acceptably.

Also, % recovery was calculated between the different eluates and the corresponding spiked buffers. The % recovery values were calculated according to the formula in Equation 3 where *Eluate* and *Buffer* refers to linearized Ct values.

$$\% \, Recovery = \frac{Eluate \, (in \, Buffer \, x)_{non-spiked} - Eluate \, (in \, Buffer \, x)_{spiked}}{Buffer \, x_{non-spiked} - Buffer \, x_{spiked}} \cdot 100 \quad (3)$$
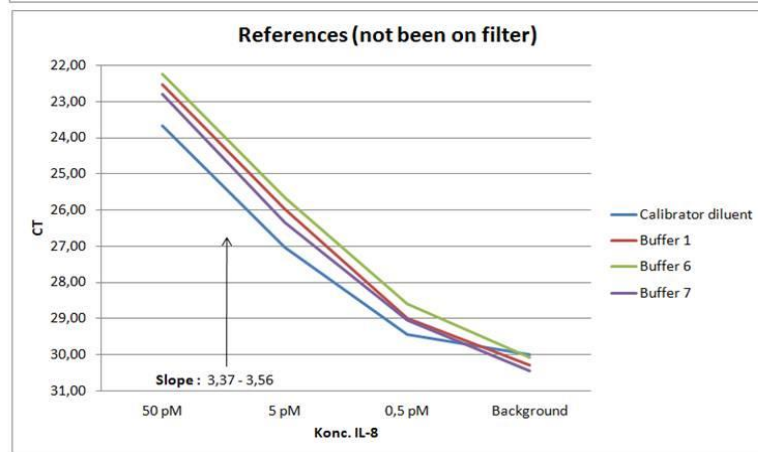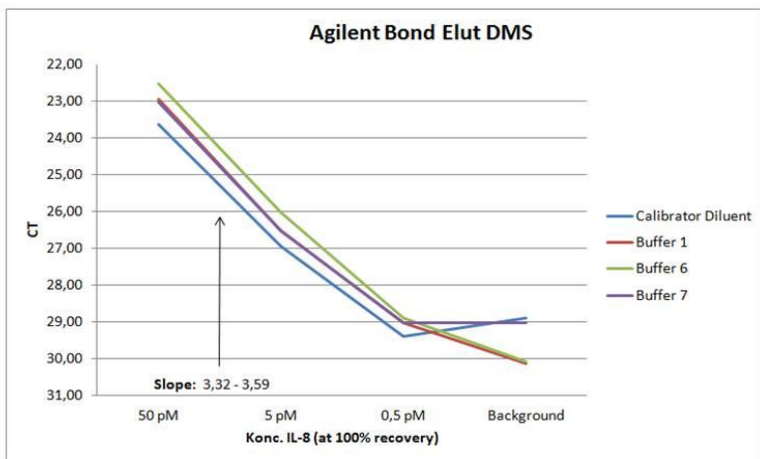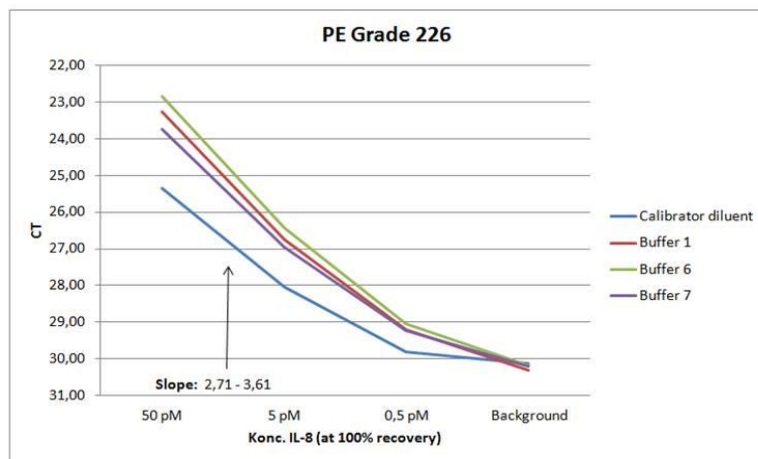
Figure 3. The graphs are showing raw Ct values for the Proseek® analysis of IL-8 eluted from 3 mm filter disks (upper two) and from analyzed spiked buffers (lower one). The concentrations on the x-axis for the upper two graphs are the theoretical maximum for the elution of the 3 mm disk and the concentrations on the x-axis for the lower one is the actual concentration of IL-8 in the buffer. A low Ct value corresponds to a high concentration of IL-8. The Ct values are, due to the nature of PCR, on log2 scale.

The calculated % recovery values (Figure 4) are fairly stable for the different concentrations analyzed and for the different elution buffers, except for the calibrator diluent generating the lowest values for the elution from PE Grade 226, but the highest values for the elution from Agilent Bond Elut DMS. The % recovery seems to be consequently higher for Agilent Bond Elut DMS than for PE Grade 226. This difference could though be due to imprecise measurement of spot area and not necessarily be due to favorable properties of the glass fiber material in Agilent Bond Elut DMS.
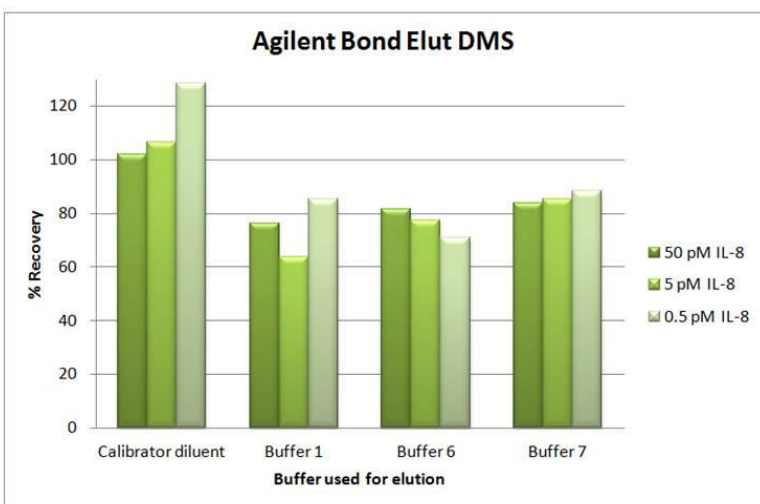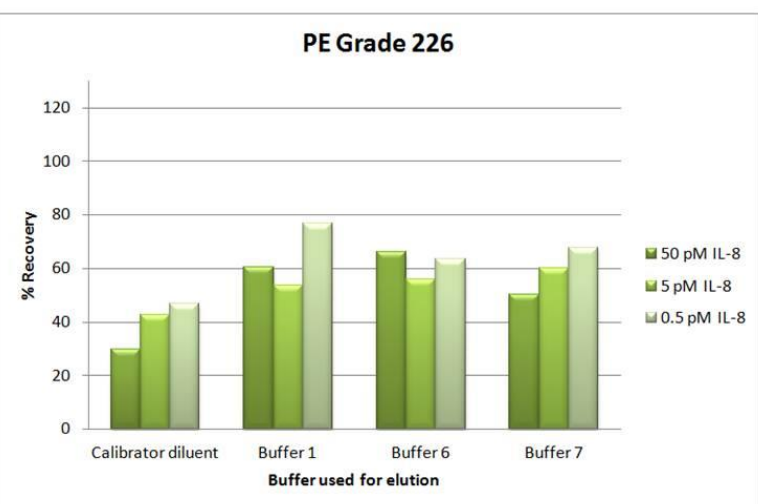

Figure 4. Calculated % recovery values for the extraction of IL-8 spiked buffers from two kinds of filter paper, PE Grade 226 (cotton) and Agilent Bond Elut DMS (glass fiber). The % recovery values are calculated as 100 times the ratio between the differences of non-spiked and spiked eluates and buffers (Equation 3). Linearized Ct values were used for the % recovery calculations.

4.2 DBS and Proseek®: procedure development and performance evaluation

4.2.1 % Recovery for IL-8 in different matrixes

The experiments with elution from IL-8 spiked buffers from filter cards generated satisfying results. The project therefore headed on to validate the properties of titrated EDTA blood as matrix for Proseek® analysis. This was achieved by a comparison between % recovery of spiked IL-8 in different matrixes. The matrixes tested were calibrator Buffer 1 (used as reference), eluted EDTA DBS, 6 % EDTA whole blood (titrated in Buffer 1) and serum. To generate the eluted EDTA DBS matrix 3, mm PE Grade 226 DBS disks were prepared, as described in the methods section, and eluted in 100 µl Buffer 1 in a 1.5 ml Eppendorf tube on a 340 rpm shaker plate for 1 hour at RT. 6 % EDTA whole blood was used to mimic the blood concentration in the eluted DBS disk. 100 pM IL-8 spikes were used and made just before the Proseek® analysis was initiated. Duplicates were made on all samples. The results (Table 5) indicates the usefulness of both eluted EDTA DBS and 6 % EDTA whole blood as Proseek® matrixes, not devastating IL-8 recovery. The *amplicon* values are linearized and calculated as 2 to the power of the difference between 40 and mean Ct. Amplicon values were used for the % recovery and % CV calculations.

Table 5. % recovery evaluation of 100 pM IL-8 spikes in different matrixes analyzed with Proseek®. All samples were analyzed in duplicates. Amplicon values are linearized Ct values (2 to the power of the difference between 40 and mean Ct) used to calculate % recovery and % CV values. ΔCt are the difference between non-spiked and spiked sample.

Sample	mean Ct	ΔCt	Amplicon	% Recovery	% CV
Buffer 1	29.16	-	1833	-	5.68
Buffer 1 + 100 pM IL-8	20.35	8.81	820205	100 %	1.52
Eluted EDTA DBS	27.61	-	5385	-	11.23
Eluted EDTA DBS + 100 pM IL-8	20.16	7.45	935428	114 %	0.86
6 % EDTA blood	27.12	-	7580	-	13.66
6 % EDTA blood + 100 pM IL-8	20.50	6.62	741894	90 %	8.57
Serum	26.88	-	8902	-	1.74
Serum + 100 pM IL-8	21.07	5.81	500478	60 %	7.28

4.2.2 DBS standard curve for IL-6 and IL-8

Since % recovery from both eluted DBS and 6 % EDTA blood matrixes seems satisfying, the next aspect to evaluate was DBS standard curve shapes. Standard curves of IL-8 and Interleukin 6 (IL-6) were prepared in EDTA whole blood, and spotted onto both PE Grade 226 and Agilent Bond Elut DMS according to the procedures in the methods section. 3 mm punches were eluted with 40 µl Buffer 1 (75 minutes, RT) on a shaker plate set to 560 rpm and the eluates were analyzed in duplicates with Proseek®. The generated standard curves (Figure 4) indicate endogenous levels of both IL-6 and IL-8 in the eluates. The shape of the standard curves though seems reasonable. The reason to the missing values for 10 nM antigen on Agilent Bond Elut DMS is that no such DBS were prepared.
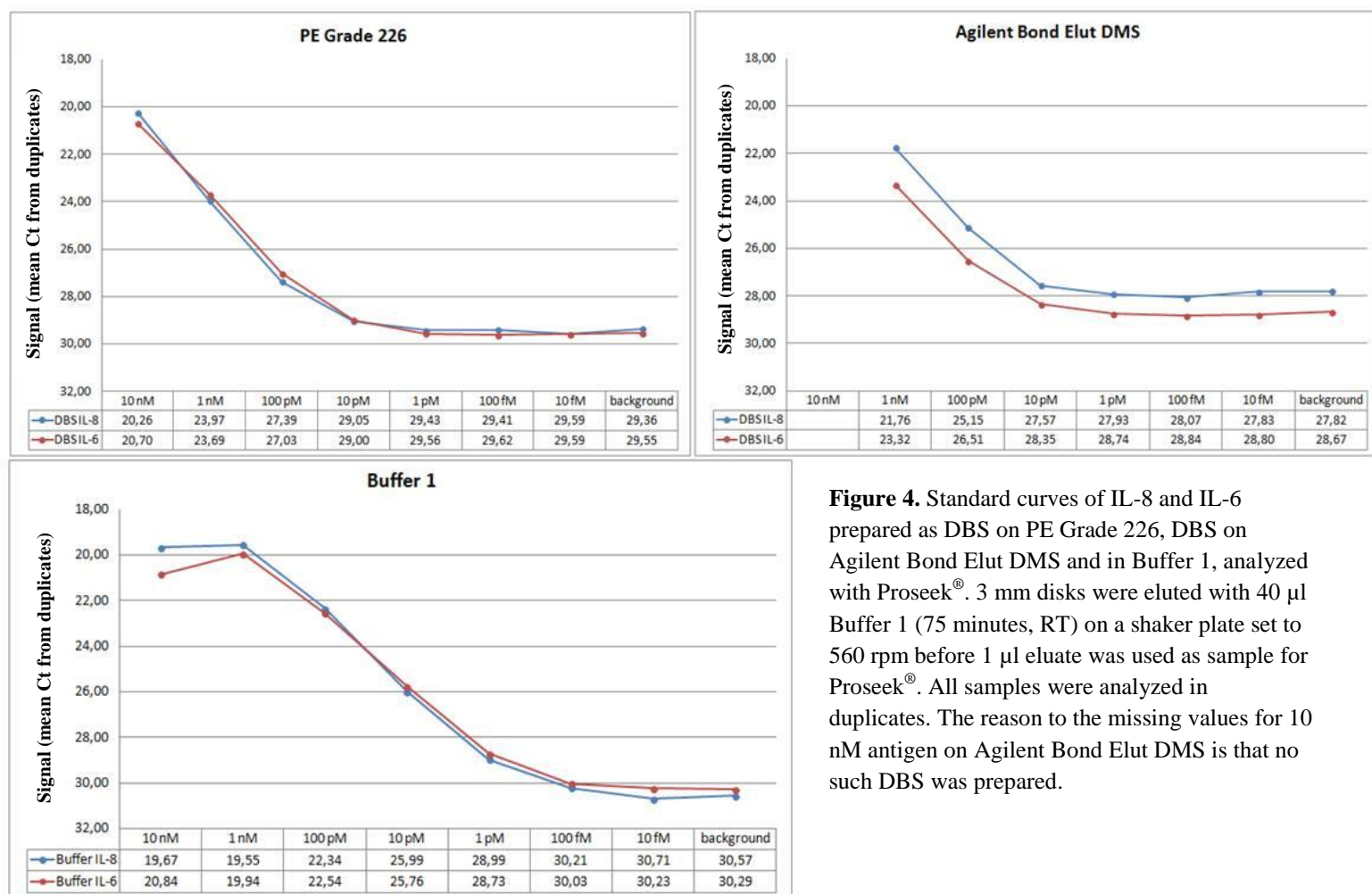
PE Grade 226

	10 nM	1 nM	100 pM	10 pM	1 pM	100 fM	10 fM	background
DBSIL-8	20,26	23,97	27,39	29,05	29,43	29,41	29,59	29,36
DBSIL-6	20,70	23,69	27,03	29,00	29,56	29,62	29,59	29,55

Agilent Bond Elut DMS

	10 nM	1 nM	100 pM	10 pM	1 pM	100 fM	10 fM	background
DBSIL-8		21,76	25,15	27,57	27,93	28,07	27,83	27,82
DBSIL-6		23,32	26,51	28,35	28,74	28,84	28,80	28,67

Buffer 1

	10 nM	1 nM	100 pM	10 pM	1 pM	100 fM	10 fM	background
Buffer IL-8	19,67	19,55	22,34	25,99	28,99	30,21	30,71	30,57
Buffer IL-6	20,84	19,94	22,54	25,76	28,73	30,03	30,23	30,29

Figure 4. Standard curves of IL-8 and IL-6 prepared as DBS on PE Grade 226, DBS on Agilent Bond Elut DMS and in Buffer 1, analyzed with Proseek®. 3 mm disks were eluted with 40 µl Buffer 1 (75 minutes, RT) on a shaker plate set to 560 rpm before 1 µl eluate was used as sample for Proseek®. All samples were analyzed in duplicates. The reason to the missing values for 10 nM antigen on Agilent Bond Elut DMS is that no such DBS was prepared.

The background levels of both IL-8 and IL-6 from DBS analysis are, considering the dilutions made in the elution step, corresponding to concentrations of approximately 10 pM endogenous levels when comparing to the standard curve of IL-8 and IL-6 in Buffer 1. This is true for DBS on both PE Grade 226 and Agilent Bond Elut DMS. An visualization of this are seen in Figure 5 where the two Buffer 1 standard curves are plotted together with modified Ct values for the DBS PE Grade 226 analysis, where 3.92 are subtracted from the original Ct values to compensate for the elution titration. The value 3.92 have been used because a 3 mm disk from PE Grade 226 contains 2.64 µl original blood which when eluted in 40 µl buffer equals 15.14 times dilution (40/2.64) which equals 2^3.92. The calculations are made with the assumption that the elution is 100 % efficient and that the PCR is 100 % efficient (duplication of total amplicon in every cycle). An endogenous level of about 10 pM makes a spike of 1 pM approximately the smallest possible detectable spiked concentration. When defining the endogenous level as 10 pM the actual final concentrations, after standard curve preparations, in Table 6 are achieved. Table 5 also presents the actual concentrations achieved after dilution in the elution step for PE Grade 226 and Agilent Bond Elut DMS assuming 100 % elution efficiency. Those theoretical actual concentrations in the eluates are similar to those achieved when comparing the Ct values of the eluted DBS with standard curve in Buffer 1 (Figure 4).
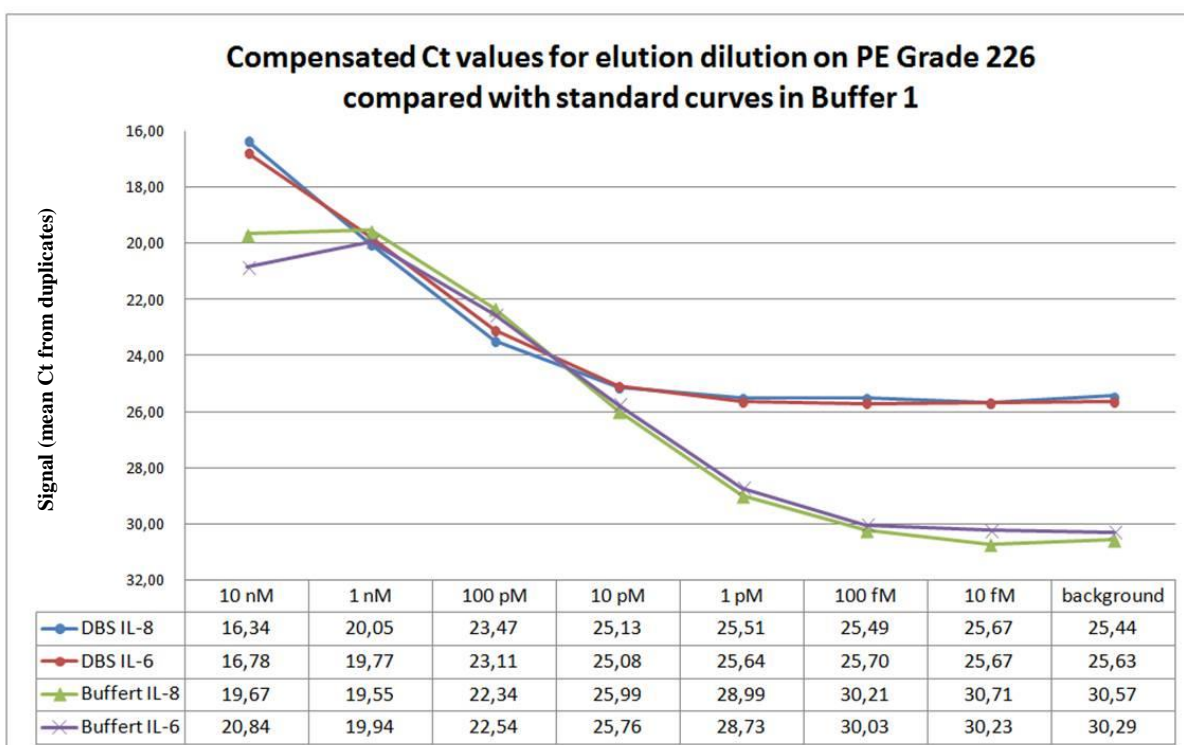
Figure 5. IL-8 and IL-6 standard curves in Buffer 1 plotted together with modified Ct values for the DBS PE Grade 226 analysis where 3.92 are subtracted from the original Ct values to compensate for the elution titration.

Table 6. Actual final concentrations achieved, after standard curve preparation, assuming an endogenous antigen level of 10 pM. Also, actual concentrations of antigen in the eluates, assuming 100 % elution efficiency, from 3 mm DBS disks from PE Grade 226 and Agilent Bond Elut DMS, are presented.

Target concentration in standard curve	Actual concentrations with 10 pM endogenous level	Actual concentration after elution from PE Grade 226	Actual concentration after elution from Agilent Bond Elut DMS
10 nM	10.0 nM	660.5 pM	1.2 nM
1 nM	1.0 nM	66.1 pM	123.0 pM
100 pM	110.0 pM	7.3 pM	13.5 pM
10 pM	20.0 pM	1.3 pM	2.5 pM
1 pM	11.0 pM	0.7 pM	1.4 pM
100 fM	10.1 pM	0.7 pM	1.2 pM
10 fM	10.0 pM	0.7 pM	1.2 pM
none	10.0 pM	0.7 pM	1.2 pM

4.2.3 Inter-spot elution accuracy

A very important property for the use of DBS as sampling model is inter-spot elution accuracy, the precision in the spotting, punching and elution steps. To obtain a first inkling of the inter-spot elution accuracy an experiment with punching and simultaneous elution of three different DBS from PE Grade 226 and Agilent Bond Elut DMS was set up. The raw Ct values from the experiment are presented in the supplementary material (9.3) and the calculated inter-spot elution % CV´s are found in Table 7. The inter elution % CV values were calculated as 100 times the ratio of the standard deviation between the amplicon (linearized

Ct) mean values of the triplicate analysis from elution A, B and C divided by the total amplicon mean value. PE Grade 226 seems to generate more reproducible spotting and elution than Agilent Bond Elut DMS. The inter elution % CV are great for PE Grade 226 and rather week for Agilent Bond Elut DMS. As written in the introduction (2.4 Validation of immunoassays) a % CV less than 20 % should be accepted. DBS analysis from PE grade 226 easily passes this limit but DBS analysis from Agilent Bond Elut DMS fails.

Table 7. Inter-spot elution accuracy from three different DBS on PE Grade 226 and Agilent Bond Elut DMS, eluted with calibrator diluent and Buffer 2, analyzed with Proseek®. The inter elution % CV values were calculated as 100 times the ratio of the standard deviation between the amplicon (linearized Ct), mean values of the triplicate analysis from elution A, B and C, divided with the total amplicon mean value. The mean values at the bottom of the table are means from PE Grade 226 and Agilent Bond Elut DMS regardless of elution buffer used.

Buffer	% CV, PE Grade 226	% CV, Agilent Bond Elut DMS
Calibrator diluent	9.7	33.8
Buffer 2	2.8	24.0
Mean	6.3	28.9

4.2.4 Elution optimization

To evaluate the best performing Buffer 1 elution conditions regarding temperature, elution volume and elution time a combined experiment of some of those parameters were performed. Two different temperature/time settings were used, 4 °C over night and RT 1 hour on a 560 rpm shaker plate. These two settings were combined with 4 different elution volumes; 8, 20, 40 and 100 µl. These 8 experiments were performed on both PE Grade 226 and Agilent Bond Elut DMS. The performance evaluation for the different elution settings was based on the recovery of 1 nM spiked EDTA DBS. The % recovery values were calculated from a standard curve of IL-8 in calibrator diluent. The best recoveries were achieved when eluting a 3 mm disk from PE Grade 226 (1 h, RT) with 20 µl Buffer 1 and a 3 mm disk from Agilent Bond Elut DMS (1 h, RT) with 40 µl Buffer 1 (Figure 6). Both these recoveries were 85 %, which can be viewed as the sum of matrix effects and actual elution efficiency.

4.2.5 Correlation between hematocrit and DBS spot size

The primary reason why Agilent Bond Elut DMS cards, based on a glass fiber material, are evaluated in this degree project is because they are proposed to maintain a more consistent DBS size with different hematocrit levels (volumetric amount of blood cells in the blood) compared to standard cellulose based filters (for example Whatman DMPK-C) [23]. This is though claimed in a publication note for the Agilent Bond Elut DMS itself, written by the manufacturer, and therefore a conformational experiment was performed to evaluate the validity in the statement. The experiment was performed in the way that 2.0 ml EDTA blood was centrifuged at 2000 g for 10 min in a small centrifuge tube. The resulting plasma (supernatant) was transferred to a new tube by careful pipetting. The amount transferred plasma was 925 µl and the hematocrit level in the blood was thus 54 % (Equation 4). Different amounts of blood cells or plasma was then added to seven tubes containing 100 µl original blood (54 % hematocrit), generating different hematocrit levels according to Table 8.
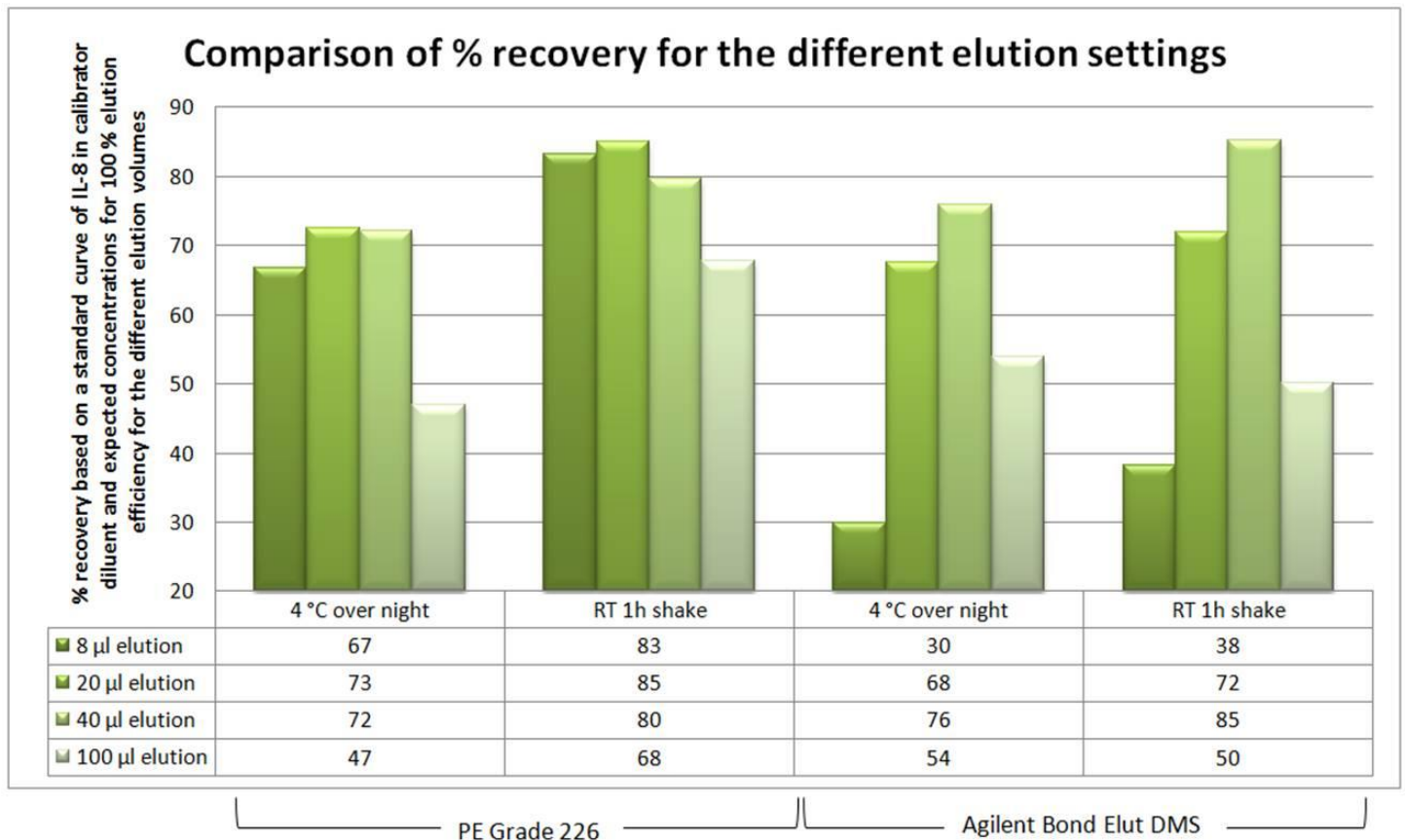
Figure 6. Comparison of 1 nM IL-8 % recovery for 8 different elution settings on PE Grade 226 and Agilent Bond Elut DMS. The different temperature/time settings used were, 4 °C over night and RT 1 hour on a 560 rpm shaker plate. These two settings were combined with 4 different elution volumes, 8, 20, 40 and 100 µl Buffer 1. The % recovery values were calculated from a standard curve of IL-8 in calibrator diluents.

$$\% \, Hematocrit = 100 \cdot \frac{Volume_{blood \, cells}}{Volume_{blood}} = 100 \cdot \frac{(2000 - 925)[\mu l]}{2000 \, [\mu l]} \approx 54 \qquad (4)$$

Table 8. Hematocrit levels prepared by addition of blood cells or plasma to original 54 % hematocrit EDTA whole blood. 7 different hematocrit levels in the interval 27 – 77 % were prepared.

Tube number	#1	#2	#3	#4	#5	#6	#7
µl original blood	100	100	100	100	100	100	100
µl added blood cells	100	50	20	0	0	0	0
µl added plasma	0	0	0	0	20	50	100
Final % hematocrit	77	69	62	54	45	36	27

15 µl blood from each of the 7 tubes (Table 8) were spotted by reverse pipetting on two Agilent Bond Elut DMS cards and two 15 µl spots from each tube was spotted by reverse pipetting on PE Grade 226 generating the DBS in Figure 7.

The DBS area was calculated from the mean value of two orthogonally measured diameters and the equation of a circle with the presumption that this generates a good estimation of the actual area. The measurements were performed with a mm scaled ruler. The values of the calculated spot areas are much larger for PE Grade 226 than Agilent Bond Elut DMS (Table 9). Notably is also the increasing DBS area with decreasing hematocrit seen for PE Grade 226 but the opposite for Agilent Bond Elut DMS, showing decreasing DBS area with decreasing

hematocrit. The obtained values are similar to those presented in the publication note for Agilent Bond Elut DMS supporting the statement that hematocrit dependence on spot size is less pronounced for Agilent Bond Elut DMS than for for example Whatman DMPK-C[23].
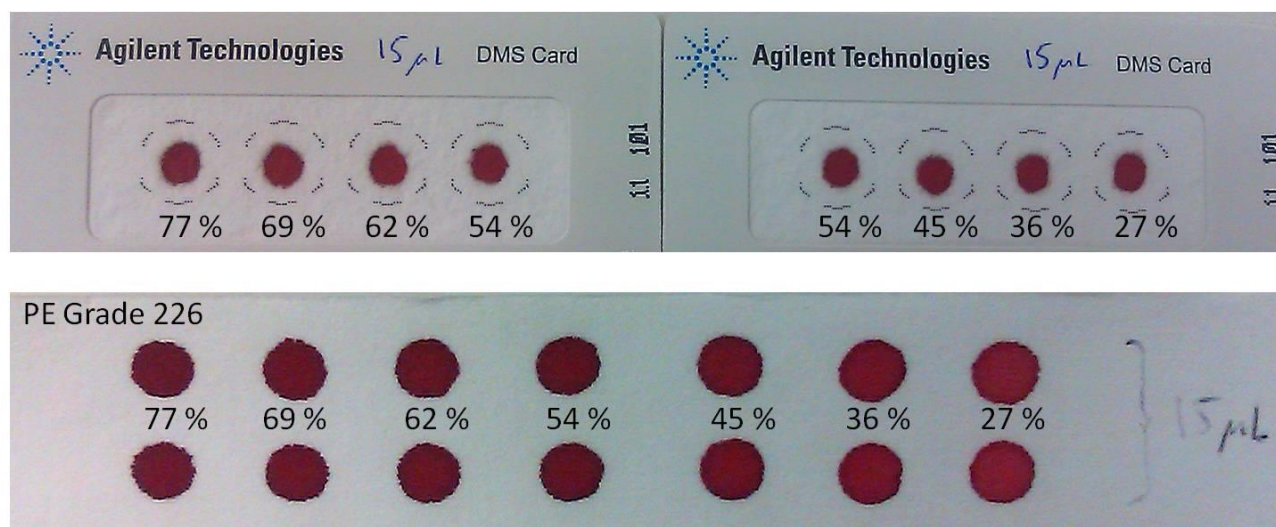

Figure 7. DBS on Agilent Bond Elut DMS and PE Grade 226 from EDTA blood prepared to 7 different hemtocrit levels in the interval 27 – 77 %.

Table 9. The calculated 15 µl DBS areas on PE Grade 226 and Agilent Bond Elut DMS for EDTA blood prepared to 7 different hematocrit levels. The areas were calculated from the mean value of two orthogonally measured diameters and the equation of a circle with the presumption that this generates a good estimation of the actual area.

% hematocrit	77	69	62	54	45	36	27
Area (mm$^{2)}$ on PE Grade 226	28.3	28.3	28.3	33.2	33.2	38.5	38.5
Area (mm$^{2)}$ on Agilent Bond Elut DMS	22.1	19.6	19.6	19.6	19.6	15.9	15.9

4.2.6   Cytokine level consistency in the DBS drying process

Levels of inflammatory markers can vary a lot between DBS and plasma/serum samples[24]. A possible reason could be that these proteins are natively present in hematocytes, bursting in the DBS drying process, releasing these proteins. Another scenario involves inflammatory reactions between the fibers in the filter cards and certain blood components, possibly stimulating production and release of inflammatory related cytokines. To study these theories collaboration with the Uppsala situated company Denator AB was introduced. Denator AB has developed a method called heat stabilization (HS) to snap freeze samples in their sampling state by stopping enzymatic activities (for example degradation of proteins). The collaboration was performed in the way that Denator AB was responsible for the sampling, antigen spiking and HS and this project performed the DBS Proseek® analysis. The samples prepared by Denator AB included DBS HS at 4 different time points after spotting or not at all (Table 10). Also samples with delayed drying times, accomplished by storage in sealed plastic bags, were prepared for both HS and non HS samples (Table 10). The signal to noise (S/N) Ct values from the experiment are seen in Figure 8. The error bars in Figure 8 are the standard

deviations from triplicate analysis (three individual DBS) performed for each sample type. What could be seen is that IL-6 is not efficiently eluted from HS DBS and that the HS is very efficient in keeping the IL-8 level down with delayed drying compared to the delayed non HS sample. This effect is though not seen for the standard drying procedure.

Table 10. DBS samples prepared with and without HS by Denator AB to evaluate IL-8 and IL-6 stability in the drying process.

| Sample name	Description
HS T=0	EDTA blood stabilized directly after addition to filter paper
HS T=4	EDTA blood stabilized 4 minutes after addition to filter paper
HS T=10	EDTA blood stabilized 10 minutes after addition to filter paper
HS T=15	EDTA blood stabilized 15 minutes after addition to filter paper
SoP	EDTA blood added to filter paper (not stabilized)
HS T=1 + bag 2 h	EDTA blood stabilized 1 minute after addition to filter paper and then put in an sealed plastic bag for 2 hours
SoP + bag 2 h	EDTA blood added to filter paper (not stabilized) and put in an sealed plastic bag for 2 hours
Spiked HS T=0	EDTA blood spiked with 100 pM IL-6 and IL-8 stabilized directly after addition to filter paper
Spiked SoP	EDTA blood spiked with 100 pM IL-6 and IL-8 added to filter paper (not stabilized)
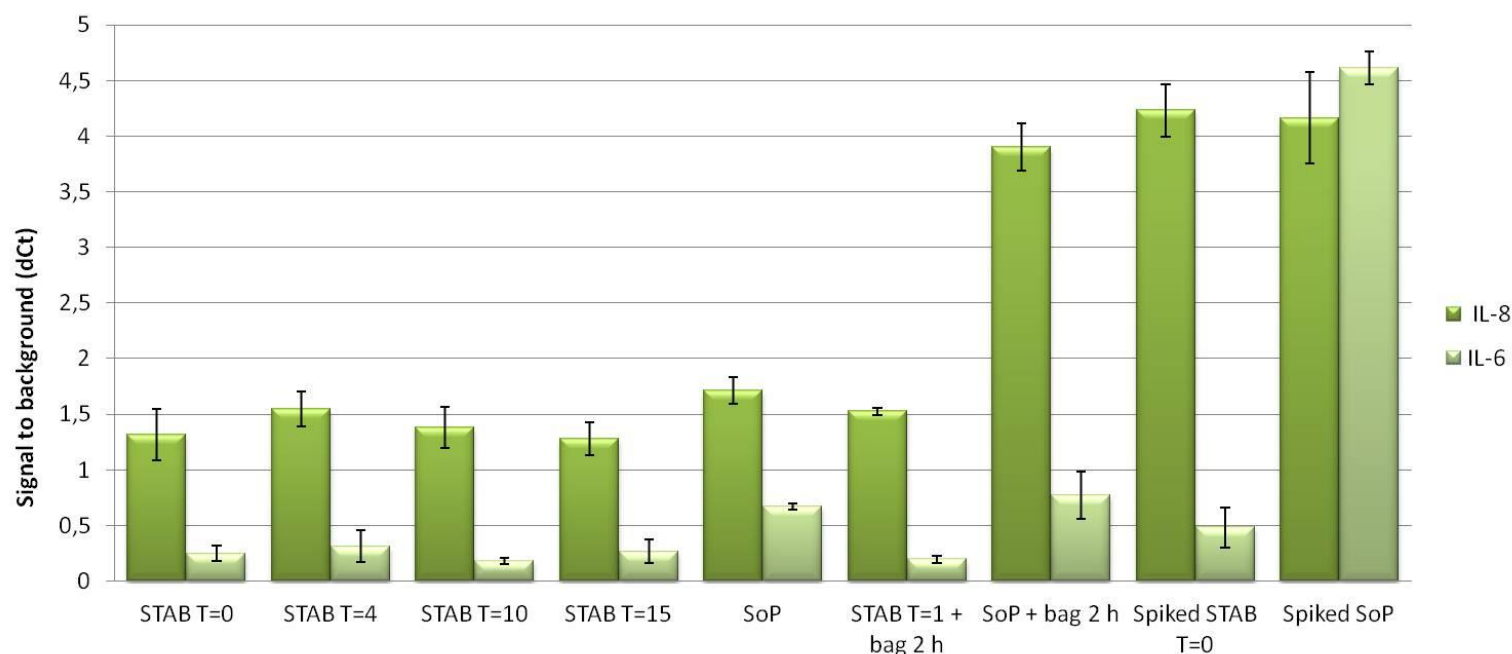
Figure 8. S/B Ct values from the Proseek® analysis of the samples prepared by Denator AB to evaluate HS properties in the DBS drying process. Explanations of the samples analyzed are seen in Table 10. The error bars are the standard deviations from triplicate analysis (three individual DBS) performed for each sample type.

4.2.7 Possibly faster protocol combining elution and probe incubation

An idea to make the total DBS - Proseek® analysis protocol faster is to combine the elution and probe incubation steps. This idea was tested in an experiment comparing the % recovery of 100 pM IL-8 spiked DBS with the 40 µl Buffer 1 standard elution procedure, an 8 µl Buffer 1 elution procedure and an 8 µl probe/Buffer 1 mix (6 µl probe-mix and 2 µl buffer 1). 100 pM IL-8 spiked Buffer 1 and non spiked Buffer 1 were used as reference for the recovery comparison. 3 mm in diameter 100 pM IL-8 spiked and non-spiked EDTA DBS disks from both PE Grade 226 and Agilent Bond Elut DMS were eluted in the standard way for the 40 and 8 µl Buffer 1. The combined elution and probe incubation was performed in the way that 8 µl probe/Buffer 1 mix were added to tubes with 3 mm DBS disks, centrifuged 1 h at 1000 rpm before combined elution and probe incubation in the 37 °C heat chamber, 1 h. After the combined elution and probe incubation, 4 µl of the sample were used for the Proseek® qPCR detection for PE Grade 226 and 2 µl for Agilent Bond Elut DMS. A smaller volume was taken from the Agilent Bond Elut DMS sample because no more than 2 µl could be obtained due to filter disk absorption. The % recovery values are calculated from linearized Ct values with respect to elution dilution (Table 11).

Table 11. % recovery values from the experiment comparing standard elution with combined elution and probe incubation. The % recovery are based on reference values from 100 pM IL-8 spiked and non sipked Buffer 1 values and are calculated from linearized Ct values with respect to elution dilution.

Analysis procedure	% recovery, PE Grade 226	% recovery, Agilent Bond Elut DMS
40 µl standard elution with Buffer 1	94	99
8 µl standard elution with Buffer 1	57	70
8 µl elution/incubation (2/4 µl sample from PE Grade 226/Agilent Bond Elut DMS)	8	12

The very low recoveries from the combined elution and probe incubation are not promising. One hypothesis to the low values is that the charged DNA in the probes tightly interacts with the filter fibers. The probes could in this way be absorbed to a large extent by the filter. This would lead to a large amount of the probes and probe-antigen complexes subsequently not transferred as sample to the detection step. This hypothesis is based on the fact that DBS disks are routinely boiled to elute DNA when DNA is the molecule of interest[25]. The combination of the experiment result and this hypothesis lead to the decision not to do follow ups on combined elution and probe incubation. The 8 µl standard elution procedure does not either work as sufficient as the 40 µl elution in terms of % recovery. When the elution volume is lowered from 40 µl to 8 µl (5 times) the actual level of measured analyte is about 3 times higher (for PE Grade 226) as seen in the raw data presented in the supplementary material (9.4). This lowered elution volume is a little bit more sufficient for Agilent Bond Elut DMS with approximately 4 times higher signal for 8 µl elution compared to 40 µl.

4.2.8 Possibly faster and more sensitive protocol with DBS disk in well through qPCR detection

If assuming that the hypothesis in section 4.2.7 is correct (the DNA in the probes are absorbed by the filter), what would then happen if the filter disks are left in the wells in the 96-well

PCR plate through the qPCR detection? Could the temperature increases in the PCR cycles release the probes from the filter pores? To evaluate this possibility, 3 mm DBS disks, both spiked with 100 pM IL-8 and non-spiked from both PE Grade 226 and Agilent Bond Elut DMS, were left in the wells through the qPCR detection. The % recovery values from this experiment was very low (mean ~ 10 %) and the background levels in the amplification plots seemed influenced by random events (Supplementary material 9.5). The amplification plots tough, at least have a more or less clear and steady raise in fluorescence due to the amplification of a specific amplicon verified by studying the corresponding melting curves (Supplementary material 9.5 DBS disks in wells through qPCR detection). Possible reasons why a 3 mm DBS disk in the well through the qPCR detection did not work sufficiently are mainly focused on the actual DBS disk size:

- 4 µl (and also 8 µl) elution/incubation volume is not enough for a 3 mm in diameter disk
- The disk is physically too large to fit in the bottom of the wells in a 96-well plate leading to elution/incubation problems simply due to insufficient contact with the probe mix
- The disk ends up in different orientations in the tube before the qPCR detection leading to large variation in background levels
- The disk can generate two separate layers in the well with different component concentrations leading to variance

A smaller disk fitting the bottom of a well in a 96-well PCR plate could possibly solve these, probably disk size related, problems. A 1.2 mm Harris micro puncher was purchased to examine if a 1.2 mm in diameter DBS could solve these size related problems and also to verify that a 1.2 mm in diameter DBS disk could be used with the same efficiency as a 3 mm disk, simply by linear scaling down of the elution volume with the disk area.

4.2.9 1.2 mm in diameter DBS disk performance

To evaluate if similar elution efficiency could be obtained from a 1.2 mm disk as a 3 mm disk, an experiment with linear scale down of the elution volume to the disk area was performed. 1.2 and 3 mm DBS disks from both native and 1 nM spiked DBS from both PE Grade 226 and Agilent Bond Elut DMS filters were eluted with different elution buffer volumes. The 1.2 mm PE Grade 226 disks were eluted in 3.2 µl Buffer 1 and the reference 3 mm PE Grade 226 disks were eluted in 20 µl Buffer 1 according to the optimized procedure. The 1.2 mm Agilent Bond Elut DMS disks were eluted in 6.4 µl Buffer 1 and the reference 3 mm Agilent Bond Elut DMS disks were eluted in 40 µl Buffer 1 according to the optimized procedure. The 3 mm disks were eluted in 1.5 ml Eppendorf tubes on a shaker plate set to 560 rpm and the 1.2 mm disks were eluted in 0.2 ml PCR strip tubes on a vortex set to intensity "5" (middle intensity). All the disks were eluted simultaneously after 1 hour at room temperature. 1 µl eluate from each elution (duplicates for all samples) was used as samples in the IL-8 Proseek® analysis. The resulting mean Ct values for the 3 mm and 1.2 mm DBS disks from both PE Grade 226 and Agilent Bond Elut DMS perfectly corresponds for both 1 nM IL-8 spiked and non-spiked DBS (Table 12).

Table 12. Comparison of linear scale down of elution volume to disk area for 1 nM IL-8 spiked and non-spiked DBS from both PE Grade 226 and Agilent Bond Elut DMS. The 1.2 mm DBS disks from PE Grade 226 and Agilent Bond Elut DMS were eluted with 3.2 and 6.4 µl Buffer 1, respectively. The 3 mm DBS disks from PE Grade 226 and Agilent Bond Elut DMS were eluted with 20 and 40 µl Buffer 1, respectively.

DBS disk properties	Mean Ct (duplicates), PE Grade 226	Mean Ct (duplicates), Agilent Bond Elut DMS
1.2 mm native DBS	28.25	27.78
1.2 mm 1 nM IL-8 spiked DBS	22.65	22.04
3 mm native DBS	28.30	27.85
3 mm 1 nM IL-8 spiked DBS	22.51	22.07

The next experiment was to evaluate the possibility to keep a 1.2 mm DBS disk in the well through the qPCR detection. This was designed as a comparison of a four step IL-8 DBS standard curve between a 1.2 mm DBS disk elution (according to the method section) and a 1.2 mm DBS disk staying in the well through the qPCR. Both DBS disks from PE Grade 226 and Agilent Bond Elut DMS were analyzed. All samples were analyzed in duplicates. The 1.2 mm DBS disks that were in the wells through the qPCR were combined eluted and incubated with Proseek® probes. 3 µl probe mix (Proseek® standard amount and concentration) and 1 µl Buffer 1 were used for this purpose. The combined elution and probe incubation was in this experiment performed with "intensity 5" vortex (1 h, RT) followed by 37 °C incubation (1 h). Before the 37 °C incubation started, 1 µl sample (eluate) from the reference 1.2 µl standard elutions were transferred to empty wells on the 96-well PCR plate and 3 µl probe mix was added to these wells. After the 37 °C incubation, 46 µl detection mix was added to all wells and the qPCR detection program started. The resulting Ct values from the experiment strongly support the possibility to keep 1.2 mm DBS disks in the well through the qPCR detection (Figure 9). The actual Ct levels are higher for the DBS disks left in the wells, than standard elution. This could be due to either the increase in actual sample amount in the analysis (there are more original blood in a complete DBS disk than in 1 µl eluate), increase in background level due to the filter disk in the well, or a combination of both.

The next experiment evaluated what happens to the background level when running the Proseek® analysis with 1.2 mm filter disks left in the wells, compared to standard elution. This was performed as a comparison between ordinary background (Buffer 1), background with 1.2 mm blank filter disk in the well through qPCR, and 1.2 mm native EDTA DBS disk left in the well through qPCR. The experiment was performed with addition of 3 µl probe mix and 1 µl Buffer 1 to the wells, with and without disks, in an optical 96-well PCR plate. The plate was incubated 1 hour at 37 °C, before addition of 46 µl detection mix and initiation of the qPCR detection. Background levels for both PE Grade 226 and Agilent Bond Elut DMS were analyzed. All samples were analyzed in duplicates. The background was notably higher with blank filter disks in the well than without for both PE Grade 226 and Agilent Bond Elut DMS (Table 13).
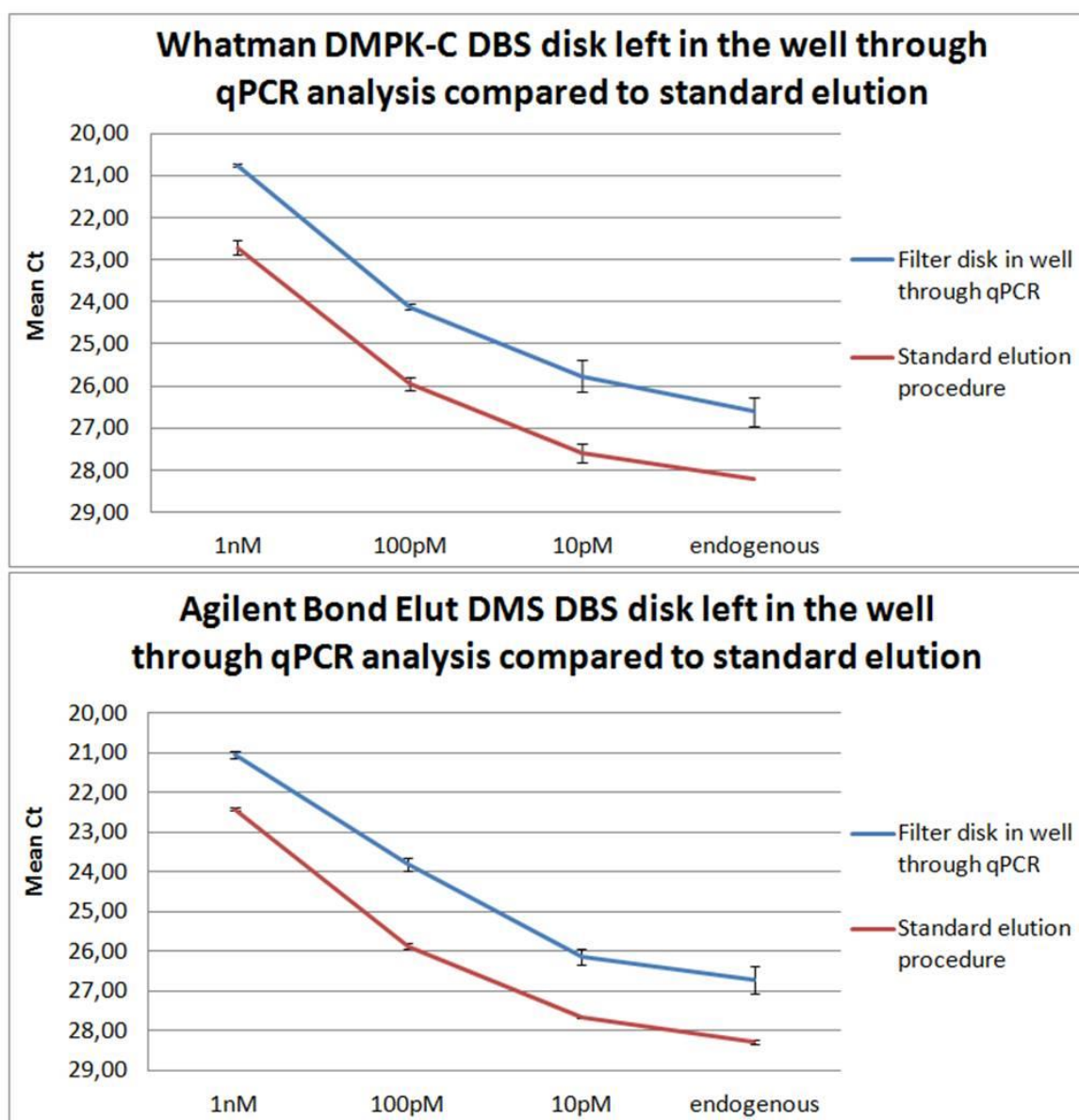
Figure 9. Ct values from the Proseek® comparison of a four step IL-8 DBS standard curve between 1.2 mm DBS disk elution (according to the method section) and 1.2 mm DBS disk staying in the well through the qPCR. DBS disks from both PE Grade 226 and Agilent Bond Elut DMS were analyzed. All samples were analyzed in duplicates and the mean value Ct between the duplicates are presented in the figure together with standard deviation error bars.

Table 13. Mean Ct values and standard deviations (STD) from duplicates from the Proseek® analysis of blank 1.2 mm filter disks in the wells through the qPCR, and ordinary Proseek® background. Blank filter disks from PE Grade 226 and Agilent Bond Elut DMS were analyzed.

Sample	Mean Ct (STD), PE Grade 226	Mean Ct (STD), Agilent Bond Elut DMS
1.2 mm blank disk in well through qPCR	36.11 (0.25)	34.95 (0.03)
Reference background (Probe mix)	37.47 (0.06)	37.47 (0.06)

33

4.3 DBS analysis compatibility between Proseek® and Proseek® Multiplex[96x96]

The aim of the project was besides evaluation and development of DBS - Proseek® singleplex analysis to evaluate the compatibility with Proseek® Multiplex[96x96]. A first trial with the 1.2 mm DBS disk standard elution procedure and a non-released Proseek® Multiplex[96x96] panel, with inflammation markers, were performed with native EDTA DBS from PE Grade 226 and Agilent Bond Elut DMS and plasma. A protease inhibitor cocktail was evaluated as a possible additive to Buffer 1 in the elution step to prohibit possible enzymatic degradation of proteins in the eluate. Presence of protease inhibitors when eluting DBS can lead to significantly higher levels of most analytes[26]. The fact that the panel used is under development and not finally validated is to be considered while evaluating the results from the experiment. Another thing to consider when analyzing the data is that the plasma in the experiment and the EDTA DBS used not originates from the same person's blood. A direct comparison between plasma and DBS samples are though not possible. A comparison that is not really possible anyways because of the fundamental difference that whole blood contains cells that break in the drying process [27] and eventually releases certain proteins resulting in higher levels for DBS [24].
Ct values from the experiment were very similar between DBS eluted PE Grade 226 and DBS eluted from Agilent Bond Elut DMS for almost all assays (Supplementary material 9.6). The mean absolute value difference between the two filter types for normalized data for all analyzed analytes were 0.5 Ct and the mean difference between them was 0.004 Ct. This can be interpreted as a variance of 0.5 Ct up and down around a level that are the same for both filters with no general trends. No effect of the protease inhibitors added in the elution buffer was seen. The mean difference between normalized endogenous levels for elution with and without protease inhibitors were 0.03 Ct when analyzing DBS from PE Grade 226 and -0.03 Ct for DBS from Agilent Bond Elut DMS. This is interpreted as though the protease inhibitor additive in the elution buffer did not generate significantly higher general levels. The difference between elution with and without protease inhibitors for individual analytes can be represented by the standard deviation value that was 0.26 Ct for DBS from PE Grade 226 and 0.46 Ct for DBS from Agilent Bond Elut DMS. The largest individual differences in Ct was 0.7 Ct for PE Grade 226 and 1.8 Ct for Agilent Bond Elut DMS. The analyte with the highest difference from Agilent Bond Elut DMS had a level of -1.6 Ct below background, making it unreliable. When comparing the endogenous marker levels between eluted DBS and Plasma it is obvious that plasma generated generally higher signals (Figure 10). This is true with only a few exceptions. Not to rash conclusion should though, as mentioned before, be drawn from these results. The generally higher signals in plasma could be explained with the approximately 7 times dilution of the blood occurring in the elution step. 7 times dilution corresponds to a decrease with 2.8 Ct values (2^2.8 = 7).
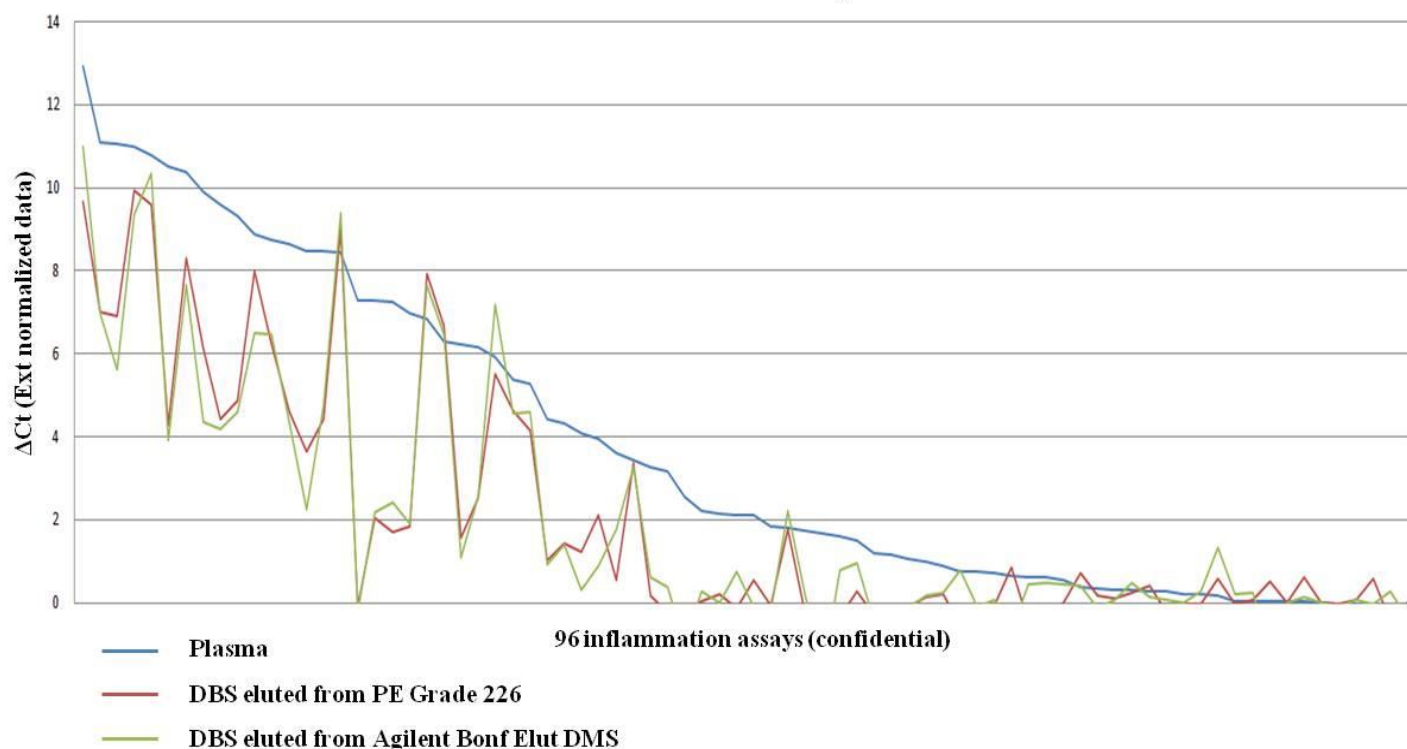
Figure 10. S/N values from Proseek® Multiplex[96x96] inflammation panel analysis of native plasma and native DBS from PE Grade 226 and Agilent Bond Elut DMS. The inflammation panel is not yet released and the 96 individual assays are therefore not shown.

4.4 DBS - Proseek® Multiplex[96x96] Oncology I: performance evaluation

4.4.1 Samples and experimental setup

For the final experiments, to conclude the practical DBS performance with Proseek® Multiplex[96x96], 8 different types of samples were collected from 5 different individuals. DBS were sampled on both Whatman DMPK-C and Agilent Bond Elut DMS cards. This yielded a total of 65 different samples to be evaluated in different ways. The different sample types were:

- Capillary DBS from finger stick with lancet directly to filter card
- Venous DBS drawn from venepuncture in arm to a non-anticoagulated tube and within 1 minute applied to filter card with a pipette
- DBS from venous EDTA blood
- DBS from venous Heparin blood
- DBS from venous Citrate blood
- EDTA plasma
- Heparin plasma
- Citrate plasma

These samples were evaluated in three different Proseek® Multiplex[96x96] Oncology I runs. The plate setups are found in the supplementary material (9.7.1 Experiment setups). The first run

included all 8 different sample types from individual 1 and 2 on Whatman DMPK-C cards. The samples were prepared by the standard elution procedure from a 1.2 mm punched DBS disk. All samples were run in duplicates with punches from different DBS spots. All samples were also run in three eluate titrations: 1:1, 1:10 and 1:100 to evaluate eventually hooked assays. This is done because the Oncology I panel assay is set up to work well with native plasma levels, and some levels could potentially be much greater when DBS are analyzed. Positive and negative control (used for between run normalization and S/N calculations) were run in triplicates. The second 96-well run was identical to the first with the only exception that individual 1 and 2 were replaced with individual 3 and 4. The third run included all the 5 individuals and these samples were prepared by letting a 1.2 mm DBS disk incubate with the probe mix over night and then stay in the well during the pre-amplification step. The samples in this third run were capillary, venous and EDTA DBS. To evaluate hooked assays for this procedure, two replicates from different spots of an entire 1.2 mm disks and two replicates from different spot of 1.2 mm disks, divided into approximately halves, were analyzed. All analysis were performed with DBS from Whatman DMPK-C cards and only capillary DBS were analyzed from Agilent Bond Elut DMS cards. Also here, 3 positive and 3 negative controls were run and used in the same way as in the other two runs.

4.4.2 Overall performance

Probably the most crucial parameter to evaluate with regards to DBS analysis performance is inter-spot accuracy, measured as % CV. Mean inter-spot % CV values for the different sample types and extraction procedures from the three performed Proseek® Multiplex^{96x96} Oncology I runs are all very low (Table 14).

Table 14. Mean inter-spot accuracy from all assays for different sample types and extraction procedures from the three performed Proseek® Multiplex^{96x96} Oncology I runs. Also the number of samples underlying the different accuracies is presented.

Sample and extraction procedure	% CV	Number of samples
Standard elution (Whatman DMPK-C)	7	40 spots (20 duplicate samples)
Standard elution (Agilent Bond Elut DMS)	8	8 spots (4 duplicate samples)
DBS disk through pre-amplification (Whatman DMPK-C)	5	40 spots (20 duplicates samples)
DBS disk through pre-amplification (Agilent Bond Elut DMS)	8	8 spots (4 duplicate samples)
Plasma	6	24 analysis (12 duplicate samples)

Another important aspect is assay dependent accuracy. To visualize the variation of % CV for the 92 different assays the individual assay % CV are grouped into different % CV spans and counted. This discretized distribution of % CV values for the 92 different assays, based on separate assay % CV mean values for DBS and plasma, is seen in Figure 11.
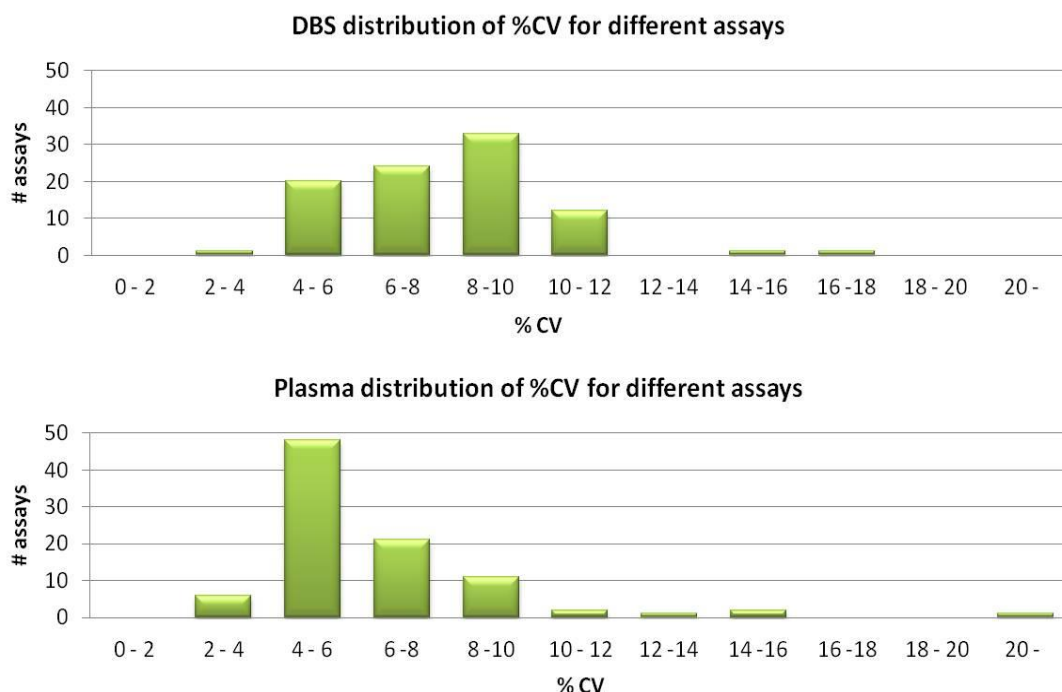

Figure 11. Discretized distribution of Proseek® Multiplex^{96x96} Oncology I assay mean value % CV for DBS and plasma samples. Numbers of assays falling into different % CV spans are visualized.

The fact that the % CV only slightly increases for the DBS samples despite the extra moments in the DBS procedures compared to plasma analysis seams really promising for the usage of DBS to generate accurate results with simpler sampling and simpler sample handling.

The analyte concentration of the sample that is put into the Proseek Multiplex analysis procedure has a maximum limit before a hook effect is introduced. The Proseek Multiplex Oncology I kit is adapted to meet the demands on measurable endogenous analyte levels in plasma samples. The adaptation was made by titration of the different probes to transform the endogenous levels to measurable levels by increasing the amount of probes for low abundant markers and vice versa. All the Oncology I kit assays are thus not necessarily working sufficiently with DBS analysis. Evaluation of hooked assays was performed for the different DBS sample analysis procedures. The eluate from the DBS standard elution procedure was titrated in negative control to the final concentrations 1:1, 1:10 and 1:100. Approximately half of the DBS disks were analyzed as complement to the complete DBS disks for the disk in well through pre-amplification procedure. The mean dddCt values from the duplicates for the different sample types were for every assay plotted according to the Cystatin B assay example in Figure 12. This is an example of an assay that is hooked for DBS samples but not for plasma samples. For accurate usage of this assay with DBS samples the Cystatin B Proseek® Multiplex^{96x96} probe pair concentration is needed to be lowered. Plots like this one was for all

92 assays in the Oncology I panel manually inspected for hook effects and the results of the inspection are presented in Table 15.
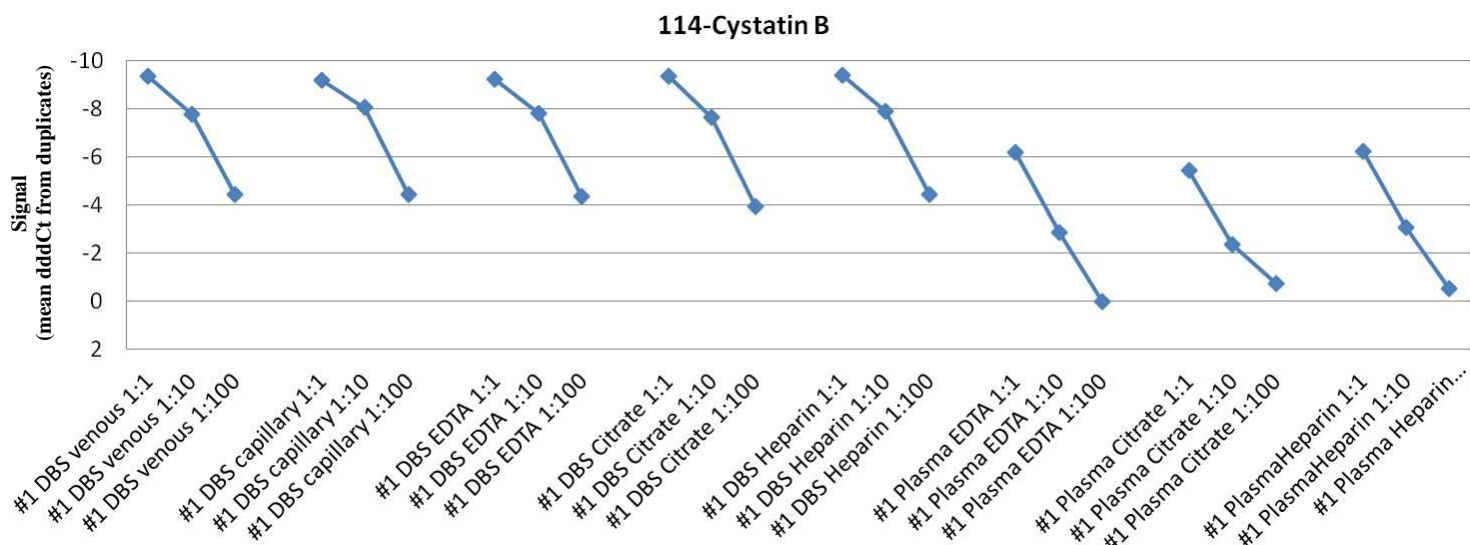

Figure 12. Mean dddCt values from duplicates for different titrations from Whatman DMPK-C DBS and plasma Proseek® Multiplex^{96x96} Oncology I analysis of individual 1. A hook effect is seen for DBS samples but not plasma.

Table 15. Number of hooked assays for different sample types analyzed with Proseek® Multiplex^{96x96} Oncology I. The numbers were obtained from manual inspection of titration curves equal to the example in Figure 12. An assay was defined as hooked if a majority of sample types hooked.

Sample type	No. of hooked assays	Hooked assays
DBS standard elution	3	CD30L, Cystatin B, IFNg
DBS filter left through pre-amplification step	6	CD30L, Cystatin B, CD31, Caspase 3, Myeloperoxidase, EMMPRIN
Plasma	2	CD30L, HGFR

The reason why more assays hooked when analyzing the DBS disk when keeping them in the wells through the pre-amplification compared to the standard elution procedure is probably due to the larger volume of sample added to the pre-amplification step, approximately 7 times more. Another possible reason could be that hardly eluted analytes eventually could be recognized and amplified even though the analyte is not released from the filter pores. The difference in sensitivity for the different procedures and samples is tightly connected to the normalized S/N (dddCt). Mean dddCt values for all individuals and all assays for the different sample and analysis procedures are presented in Table 16 together with mean dddCt corrected for the relative amount of sample introduced in the analysis.

Plasma sensitivity is, according to expectations, higher than DBS left in the well through pre-amplification, which in turn is higher than DBS standard elution. This is probably due to the difference in amount of sample introduced in the analysis where plasma is highest (1 µl) followed by DBS in the well through pre-amplification (0.43 µl) followed by standard elution (0.14 µl). dddCt values corrected for these different amounts of sample initiated in the analysis indicates highest signal to sample value for DBS standard elution and lowest for

plasma samples. When working with DBS it is important to always consider plasma and DBS as distinctly separated sample types, not easily compared to each other.

Table 16. Mean dddCt values for all individuals and all assays for the different sample and analysis procedures together with mean dddCt corrected for the relative amount of sample introduced in the analysis.

Sample/analysis procedure	Mean dddCt for all individuals, all samples and all assays	Mean dddCt corrected for the amount introduced sample
DBS standard elution	2.4	5.2
DBS filter disk left through pre-amplification step	3.7	4.9
Plasma	4.1	4.1

4.4.3 Background changes with DBS disk in well through pre-amplification

The attempt to make the DBS analysis procedure faster, simpler and even more sensitive was to skip the separate elution step of the DBS disk and instead add the probe mix directly to the DBS disk. The DBS disk is then subsequently kept in the well through the pre-amplification. A filter disk has a capacity to absorb substances and fluids and could therefore interfere with the substances used in the pre-amplification PCR reaction. Tendencies that different assays are not affected equally have already been seen in the project. The background levels for the assays in the Oncology I panel are to be evaluated and used if generated DBS signals (S/N) are to be compared to signals generated by the standard elution procedure. An empty 1.2 mm Whatman DMPK-C disk was therefore analyzed in the same way as the DBS disks staying in the well through the pre-amplification. Duplicate analyses were made. dddCt values, with negative control as background, were calculated and it is obvious that all assays are not equally affected of the blank filter (Figure 13). The reason to these differences are not clearly understood but these backgrounds have to be used for accurate procedure overstep comparisons. The mostly affected assays were Caspase-3 and EMMPRIN, showing signals more than 1.5 Ct higher than the negative control (the ordinary background). This alternative background, obtained with a blank 1.2 mm Whatman DMPK-C filter disk in the well through the pre amplification, has been used for all S/N calculations for DBS disks analyzed by this procedure.
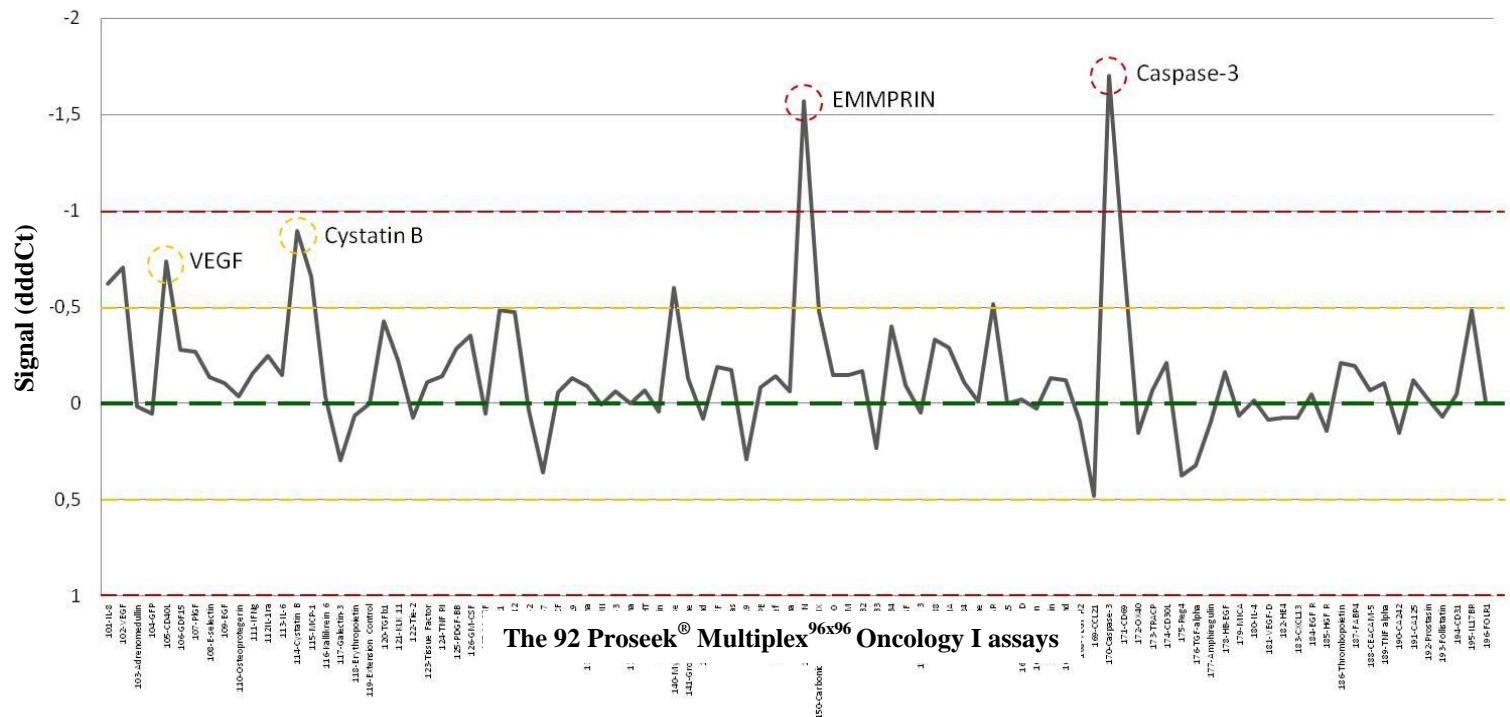
Figure 13. S/N values from the Proseek® Multiplex^96x96 Oncology I analysis of a blank 1.2 mm Whatman DMPK-C disk in the well through the pre-amplification step. Negative control (the ordinary Proseek® Multiplex^96x96 background) was used as background for the S/N calculations. Mean values for duplicate analysis were used. The four most affected assays are Caspase-3, EMMPRIN, Cystatin B and VEGF. This is the background of choice when S/N values are to be calculated for the DBS disks in well through the pre-amplification procedure.

4.4.4 EDTA DBS versus capillary DBS from finger stick with lancet

The easiest and least invasive way to sample DBS is to add a blood droplet to a filter paper directly from a finger stick with a lancet. This procedures simplicity makes it suitable for home sampling by patients themselves. It is though a fact that capillary blood from for example a finger stick and venous blood from venepuncture is not identical in all aspects. It is also not necessarily true that blood with an added anticoagulant for example EDTA behaves equally in terms of for example DBS spreading and detectable analyte levels. In cases where venous blood is used for DBS sampling it will probably most often be anticoagulated. Based on this reasoning it would be interesting to compare the results from EDTA venous DBS and capillary DBS from finger stick. The correlation between EDTA venous DBS and capillary DBS from fingerstick in terms of normalized S/N (dddCt) levels for the different assays in the Proseek® Multiplex^96x96 Oncology I panel from 4 different individuals is presented in Figure 14. The filter paper card used was Whatman DMPK-C and both the standard elution procedure and filter in well through pre-amplification results are combined in the datasets for individual #1, #2, #3 and #4. The marked striped line is not a trend line but the line the observations should follow if the two sample types would be identical. It is obviously no major general differences between the sample types when analyzing them with the the Proseek® Multiplex^96x96 Oncology I panel.
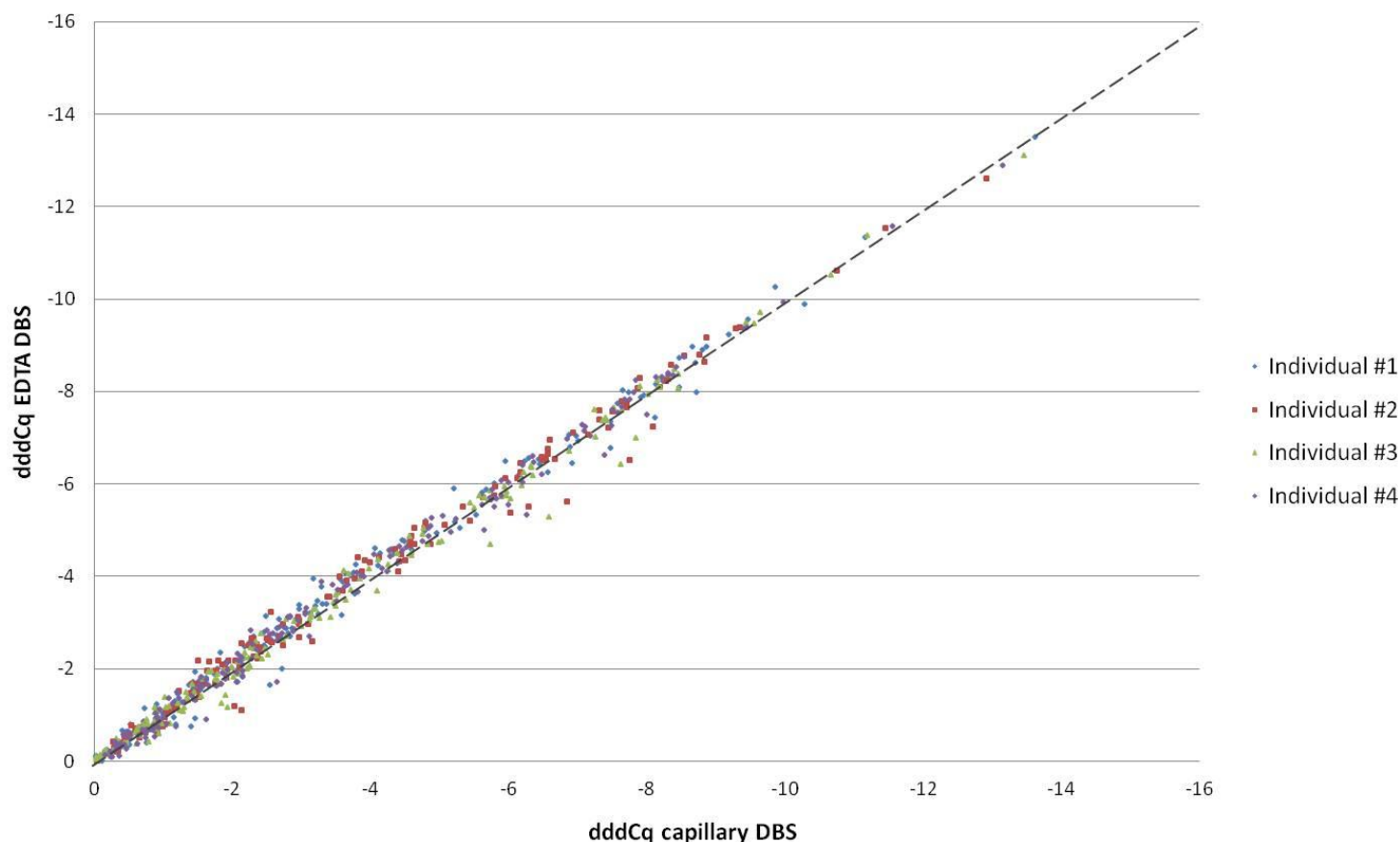
40

Figure 14. Correlation between EDTA venous DBS and capillary DBS from fingerstick in terms of normalized S/N (dddCt) levels for the different assays in the Proseek® Multiplex[96x96] Oncology I panel. The figure include data from four different individuals analyzed with both standard elution and filter disk in well through pre-amplification. The striped line marks were the assays are supposed to be located if the sample types are identical. Dots in the plot are individual assays.

4.4.5 EDTA DBS versus EDTA plasma

Another import aspect to investigate regarding the availability of DBS as sampling method for any clinical use is the correlation to plasma samples for marker level differences between individuals. Direct comparison between DBS and plasma results cannot easily be done because of the different types of samples. DBS is containing blood cells as opposed to plasma. Most blood cells probably burst in the DBS drying process probably releasing cellular proteins not seen in plasma. This arguing leads to the hypothesis that at least some of the markers in the Oncology I panel that are expressed in or on the surface of blood cells should be elevated in DBS compared to plasma. At least when the results are normalized for the different sample volumes initiated into the analysis. A plot, trying to shed some light over this hypothesis, with normalized S/N values for EDTA plasma and EDTA DBS is seen in Figure 15. The DBS values are corrected for the dilution in the elution procedure and it could be seen that the plasma levels are at least equivalent in DBS. The DBS signals are for a number of assays increased compared to plasma, probably due to the blood cells present in the DBS. The striped line is not a trend line but the theoretical perfect correlation between the two sample types. The data used in Figure 15 are mean values for duplicates of EDTA DBS and EDTA plasma from individuals 1-4. All 92 assays are used and results from both DBS standard elution and pre-amplification procedures.
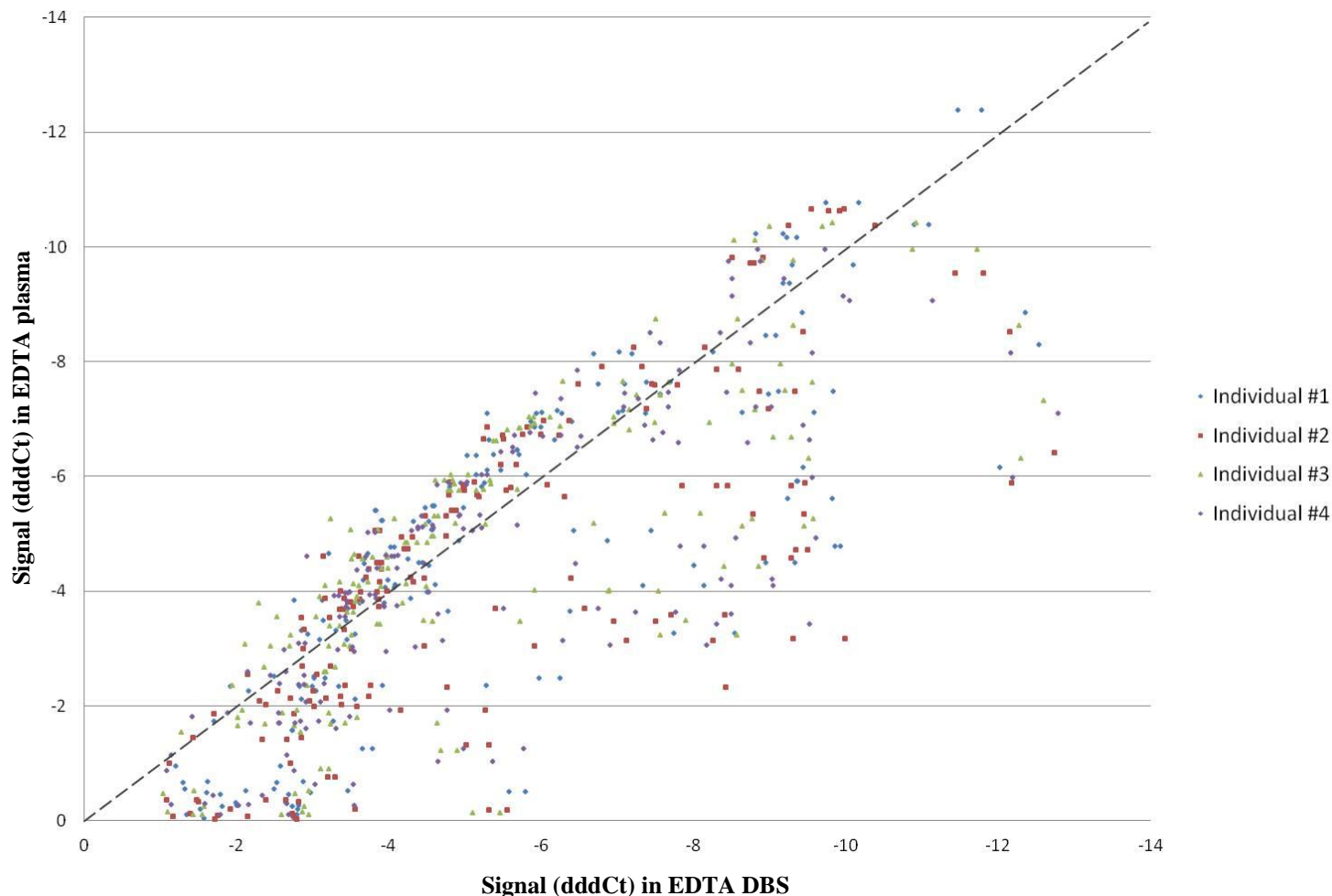
Figure 15. Correlation of normalized S/N values between EDTA plasma and EDTA DBS. Data from individual 1-4 and DBS analyzed with both standard elution and filter disk through pre-amplification are used. The DBS S/N values are corrected to equate the initiated amount of sample between DBS and plasma. The striped line is not a trend line but the theoretical perfect correlation between the two sample types. Dots in the plot are individual assays.

Another attempt to evaluate differences between the sample types is to compare how differences in marker levels between individuals manifest by the sample types (Figure 16). It should though be very clear that the noise in the data is raised in this type of comparison leading to lowered precision in the plot. Data with small differences between the individuals for both plasma and DBS are therefore unreliable. The striped line is not a trend line but the line the data should follow if there would be a perfectly equal marker difference between individuals for the different sample types. The upper right and lower left quadrant in Figure 16 are where observations (S/N difference between two individuals for a certain assay) with equal differences between individuals for the two sample types gather. The other two quadrants are areas where one sample type shows a higher level of a certain marker for one individual but the other sample type indicates the opposite. The two sample types agree regarding differences between individuals in 73 % of the observations. The data used are all combinations of differences among individual 1-4 for EDTA plasma and EDTA DBS. The mean values of duplicates for all 92 Oncology I assays are used and the results from both DBS standard elution and filter through pre-amplification procedures are used. One dot in the

plot is thus the difference for a certain marker between two individuals for plasma on the y-axis and for DBS on the x-axis.
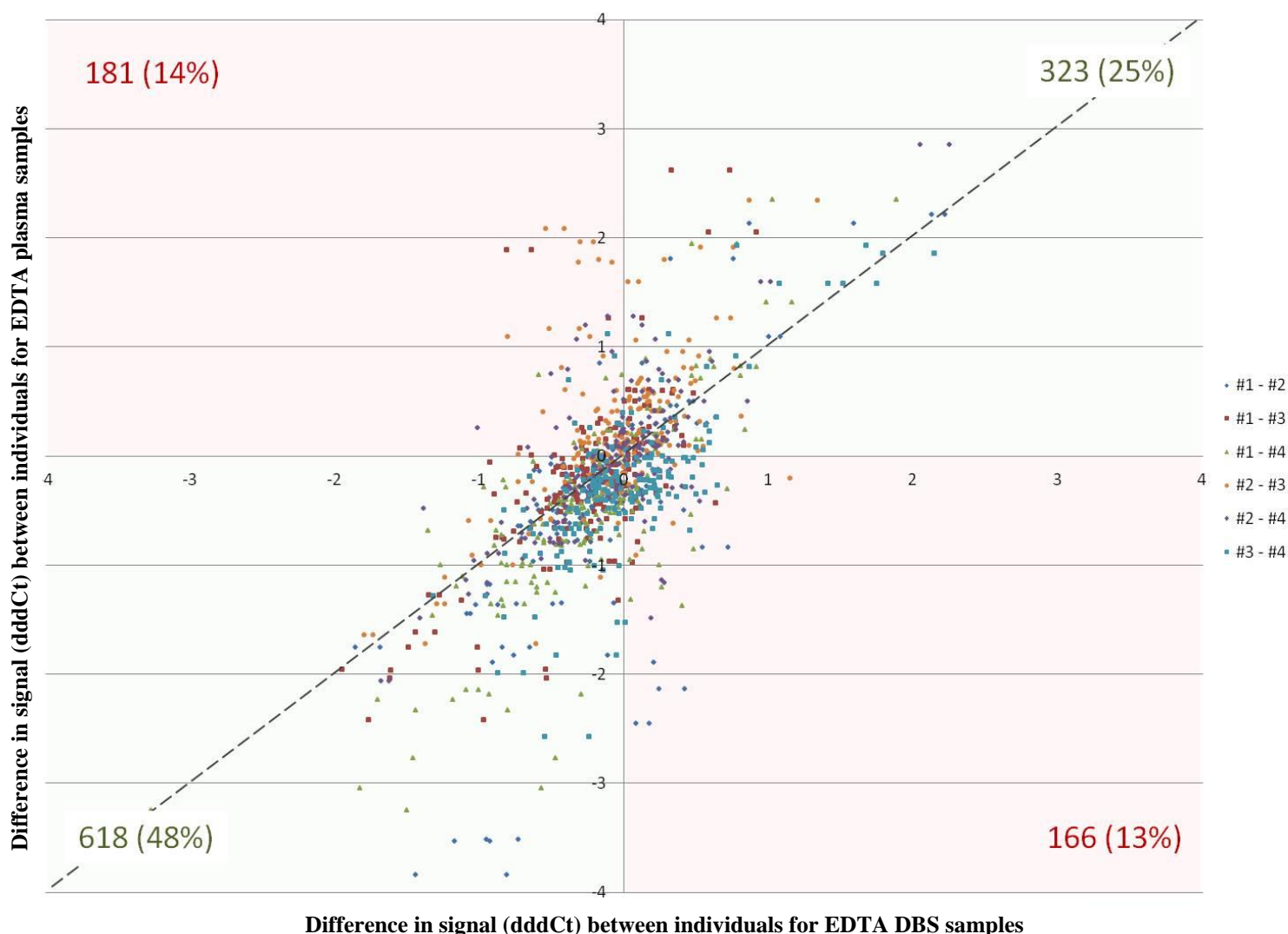

Figure 16. Correlation in S/N difference between individuals for certain Proseek® Multiplex[96x96] Oncology I assays for two individuals between EDTA DBS and EDTA plasma. All combinations of differences among individuals 1-4 for DBS analyzed with both standard elution and filter in well through pre-amplification. Mean normalized S/N values for all 92 Oncology I assays are used. The upper right and lower left quadrant are where observations (S/N difference between two individuals for a certain assay) with equal differences between individuals for the two sample types gather. The other two quadrants are areas where one sample type shows a higher level of a certain marker and individual but the other sample type indicates the opposite. Numbers in the corners are the number of observations in the corresponding quadrant and in parenthesis the percentage of all observations in that corresponding quadrant.

4.4.6 DBS standard elution versus plasma

Many different PCA visualizations can be made from the 92 dimensional observations achieved from the three Proseek® Multiplex[96x96] Oncology I experiments. One aspect to evaluate is how standard eluted DBS relates to plasma, regardless choice of anticoagulant (EDTA, Citrate or Heparin), or no anticoagulant at all (Figure 17). A distinct separation between DBS and plasma is seen regardless anticoagulant used. The difference between DBS

and plasma are thus greater than the difference between the anticoagulant used. The assays used in Figure 16 are based on data from the 20 Oncology I assays that showed distinct endogenous levels without hook-effects for all the different sample types. These assays are also the ones used in Figure 17 and Figure 18 and they are presented in the supplementary material (9.7.2). The raw data used in Figure 17 are mean values of normalized S/N values from duplicates from individuals 1-4 for all sample types categorized as DBS or plasma. Only data from the DBS standard elution procedure is used.

The PCA visualization of how the different individuals (number 1 - 4) compare to each other when analyzed by different kinds of DBS (capillary, venous, EDTA, citrate and heparin) with the standard elution procedure are seen in Figure 18. Clearly separated clusters for the different individuals are achieved indicating that there is more difference between the individuals than type of DBS. This distinct separation is achieved even though all analyzed individuals are healthy adult women. When the same PCA analysis is made for the plasma samples it is hard discern if the individual analyzed or the plasma type (EDTA, citrate or heparin) varies the most (Figure 19). This claims DBS as more robust than plasma in terms of sample subtype.
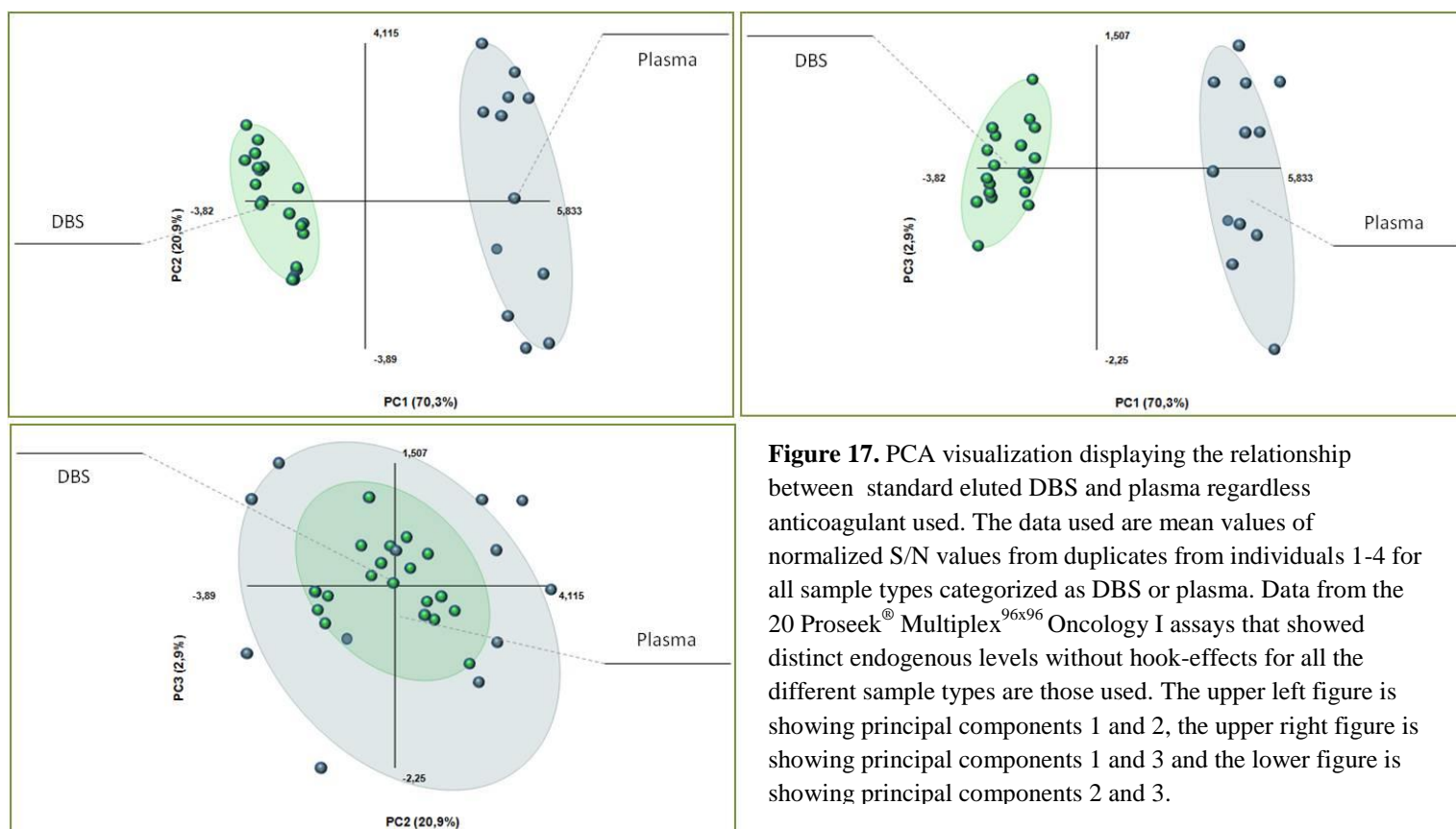

Figure 17. PCA visualization displaying the relationship between standard eluted DBS and plasma regardless anticoagulant used. The data used are mean values of normalized S/N values from duplicates from individuals 1-4 for all sample types categorized as DBS or plasma. Data from the 20 Proseek® Multiplex^{96x96} Oncology I assays that showed distinct endogenous levels without hook-effects for all the different sample types are those used. The upper left figure is showing principal components 1 and 2, the upper right figure is showing principal components 1 and 3 and the lower figure is showing principal components 2 and 3.

Figure 18. PCA visualization displaying the relationship among individuals 1-4 for Proseek® Multiplex[96x96] Oncology I standard eluted DBS analysis, regardless sample subtype. The data used are mean values of normalized S/N values. Data from the 20 Oncology I assays that showed distinct endogenous levels without hook-effects for all the different sample types are those used. The upper left figure is showing principal components 1 and 2, the upper right figure is showing principal components 1 and 3 and the lower figure is showing principal components 2 and 3.
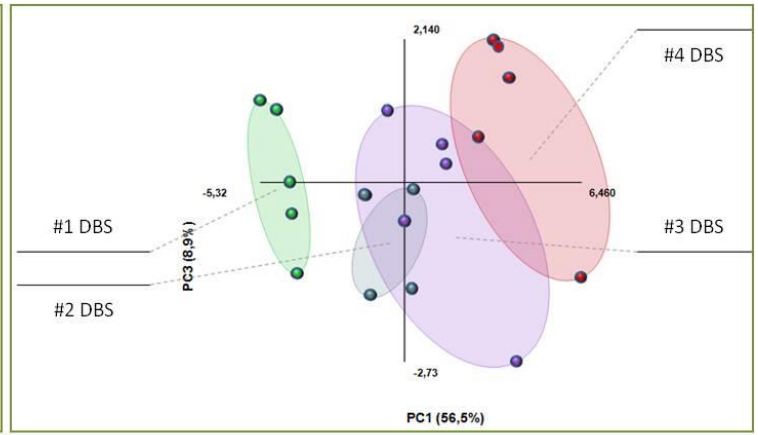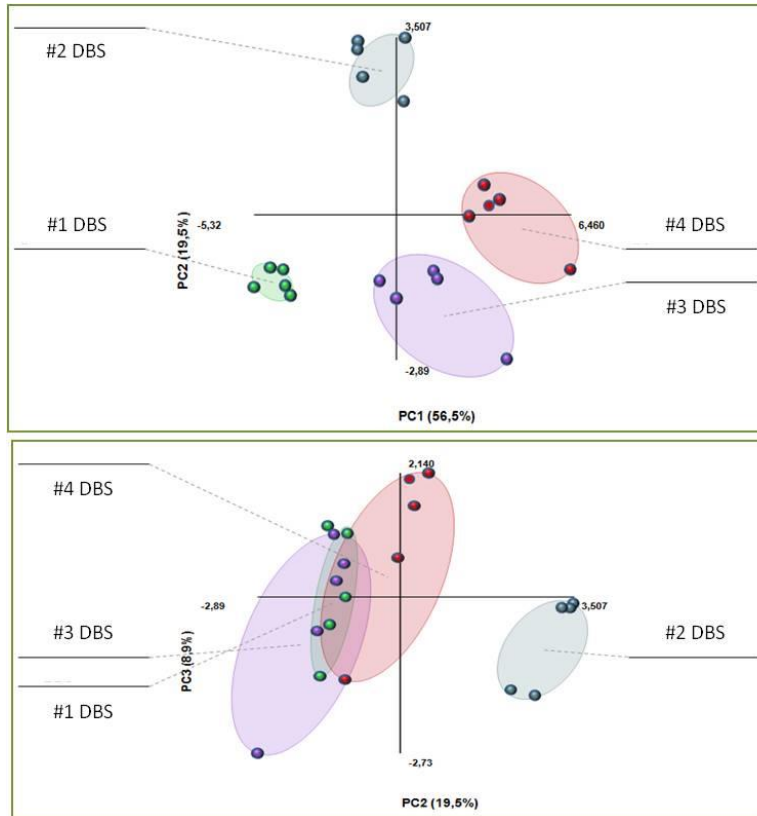

Figure 19. PCA visualization displaying the relationship among individuals 1-4 for Proseek® Multiplex[96x96] Oncology I plasma analysis, regardless sample subtype. The data used are mean values of normalized S/N values. Data from the 20 Oncology I assays that showed distinct endogenous levels without hook-effects for all the different sample types are those used. The upper left figure is showing principal components 1 and 2, the upper right figure is showing principal components 1 and 3 and the lower figure is showing principal components 2 and 3.
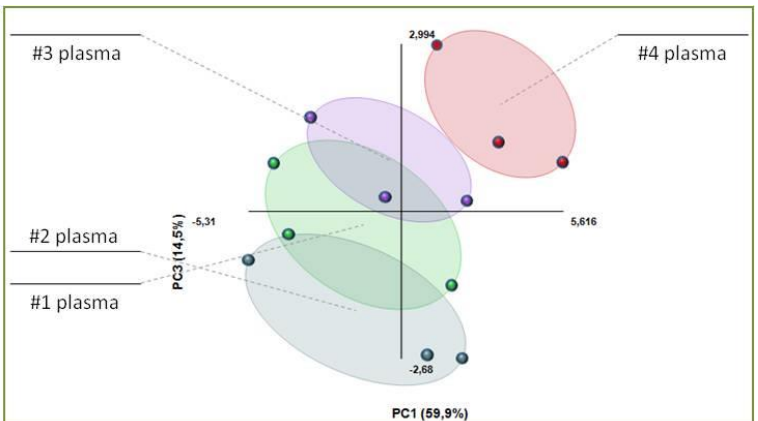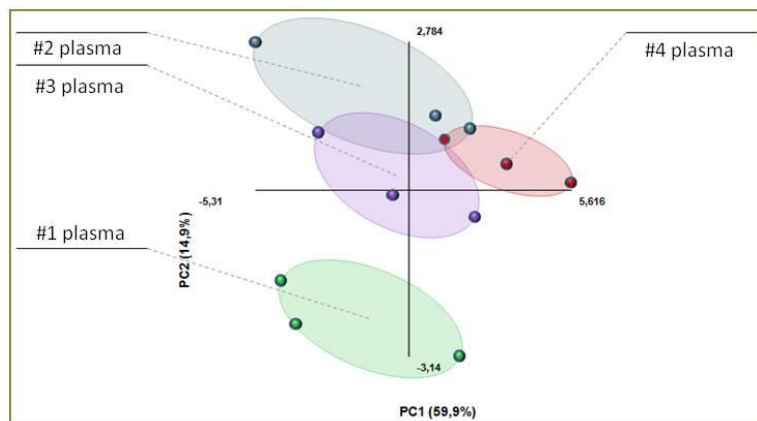
4.4.7 Comparison between DBS std. elution, DBS disk through pre-amplification and plasma

The final presented comparison from the experiments with Proseek® Multiplex[96x96] Oncology I panel is mean normalized S/N values for all individuals and sample types falling into the categories DBS standard elution, DBS disk in well through pre amplification and plasma (Figure 20). Data from all 92 assays were used. The assays are sorted in descending S/N order for plasma, the reason why the plasma line is almost straight. These DBS signals are not compensated for the dilution occurring in the elution step. When the signals are not compensated, which they probably should not be in practical clinical use, the plasma signals are generally highest. Some assays are though peaking for DBS, over the plasma signal. The S/N for DBS disks left through pre amplification is almost exclusively higher than the S/N for DBS standard elution. This is not astonishing because the blood in the standard procedure is diluted approximately 7 times when filter left through pre amplification is not diluted at all. This is true even though DBS left in the pre amplification S/N is normalized with the higher blank filter background, which is the case in the Figure 20.

Comparison of mean signal (dddCt) from individual #1, 2, 3 and 4 for all sample types categorized into DBS standard elution, DBS disk in well through pre-amplification, and plasma
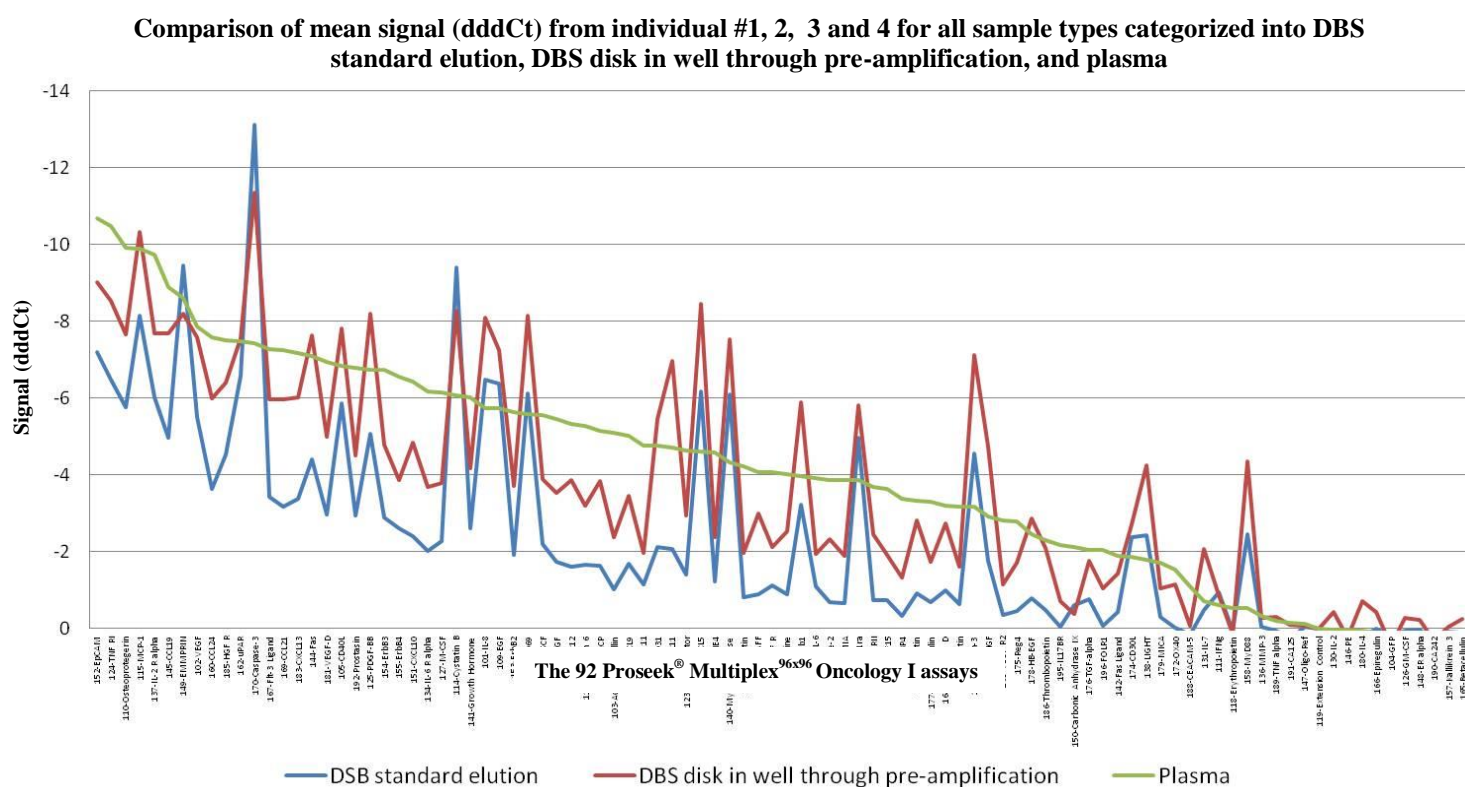

Figure 20. Mean normalized S/N values for duplicates for all individuals and sample types falling into the categories DBS standard elution, DBS disk in well through pre amplification and plasma. All Proseek® Multiplex[96x96] Oncology I assays are used and sorted in descending S/N order for plasma.

5 Discussion

5.1 DBS sampling advantages and difficulties

DBS, filter paper as matrix for storage of droplets of whole blood, are nowadays routinely used in extensive newborn screening programs. DBS are very well suited for infant screening due to the small amount of blood needed and the minimal invasiveness by the capillary heel stick. These advantages with DBS sampling, over for example venepuncture and plasma preparation, possibly reveal unique opportunities to examine health related issues in extremely premature infants. This relies to the possibility of accurate, sensitive and highly multiplexed quantitative protein marker analysis from very limited sample amounts. Proseek® Multiplex^{96x96} is, due to high sensitivity and the small sample amount needed, as shown in this degree project, probably very well suited for this demanding task. A well working method to study health status in extremely premature infants could impart knowledge and hopefully result in increased premature infant survival in the future.

DBS sampling is, besides minimal patient invasive, also very simple in terms of sampling, storage and transportation issues. This makes DBS sampling very well suited for home testing for for example long term follow ups after different diseases or more commercially minded health status controls were the patient himself handles the sampling and sends the DBS by regular mail for analysis and medical judgment. Another aspect to the DBS ease of sampling, storage and transportation are screening for ethnical diseases in low income countries were resource settings are limited. A DBS based assay for for example tuberculosis would be a bull's eye hit for developing countries[28]. A further possible area for DBS applications are pre-clinical TK studies in animals enabling improvement in the 3R´s (Reduction, Refinement and Replacement) [7, 8]. The PEA technology is a suitable way to perform quantitative analysis of protein markers from DBS samples, possibly applicable to all these areas of implementation.

History is probably the most contradictory factor to DBS usage. Plasma has for a long time been the sampling format used for most protein diagnostics and all gained experience is thus related to plasma analysis. DBS and plasma are clearly separated sample types and an easy transition between the two formats is therefore not possible. Two alternative ways to overcome these problems exists. The first alternative is to perform simultaneous DBS and plasma analysis for a period of time to translate plasma knowledge into DBS knowledge. The other way is to develop and evaluate DBS assays for specific usage, more or less from scratch using extensive validation studies.

5.2 DBS filter cards

Two different types of filter cards were evaluated. The cotton based Whatman DMPK-C (equal to PE Grade 226) and the glass fiber based Agilent Bond Elut DMS. One major concern regarding quantitative DBS analysis accuracy is the hematocrit level in the blood. Hematocrit level differences between individuals make DBS size not perfectly correlated to blood volume applied. The blood concentration in DBS is unequal for individuals with different hematocrit levels. Equal blood concentrations between all DBS are assumed in the DBS analysis procedure, where a fixed sized DBS disk is supposed to contain a fixed volume

original blood. Hematocrit differences are by this mean lowering the DBS analysis accuracy. The DBS size dependence due to different hematocrit levels is stated to be less for Agilent Bond Elut DMS than Whatman DMPK-C[23]. This statement is supported by experiments performed in this degree project. It has though also been shown that the accuracy in terms of inter spot % CV are better for Whatman DMPK-C. The blood concentration is higher in Agilent Bond Elut DMS DBS but the optimal elution volume (highest % recovery) is larger so the actual sensitivity is approximately equal for the two filters. Both filter types worked sufficiently and were therefore used through the entire project even though Whatman DMPK-C were the main choice for the final experiments due to much more extensive history and proven properties.

5.3 Proseek® - DBS sample compatibility

Proseek® has during this degree project displayed quite robust properties regarding the elution buffer used for DBS elution and also for larger modification for example keeping a filter disk in the well through the complete analysis procedure. From the introducing experiments of Proseek® compatibility with seven different conceivable elution buffers, the majority of the buffers showed no signs of analysis interference. The only buffers totally ruining the analysis, were both concentrations of the buffer containing SDS, and the high concentrated urea buffer. These buffers were originally prepared if brute protein denaturation would be necessary to release the proteins from the filter pores. It was though displayed that at least IL-8 were almost equally easily eluted by all compatibility accepted buffers. This directed the project to go for the buffer found in most DBS protein elution references (Buffer 1) containing PBS and 0.05 % Tween 20 (pH 7.4) which was used for all further experiments. The experiment with Proseek® IL-8 % recovery in different DBS related matrixes compared to serum (matrix accepted for Proseek®) showed irreproachable compatibility between DBS matrixes and Proseek®. Both 6% EDTA whole blood (used to mimic the blood concentration after DBS elution) titrated in Buffer 1 and eluate from a 100 µl 3 mm EDTA DBS elution generated clearly better IL-8 recovery values (90 and 110 %) than serum (60 %).

5.4 Proseek® - DBS analysis performance

An overview of the IL-8 DBS elution performance was gained from DBS IL-8 standard curve analysis performed on both PE Grade 226 and Agilent Bond Elut DMS. Both sensitivity and dynamic range can be examined from a properly prepared standard curve. The maximum IL-8 and IL-6 concentrations in the Proseek® analyzed DBS standard curves were though not high enough to level out the standard curve for determination of the dynamic ranges upper limit. It was also clear that the endogenous levels of IL-8 and IL-6 in the blood made determination of IL-8 and IL-6 sensitivity impossible. When the DBS standard curves were compensated for the elution dilution, they nicely fitted the upper part of the standard curves prepared in Buffer 1 and kept straight when the Buffer 1 standard curve levelled out. This due to the actual lower concentration analyzed in the eluted DBS samples. The standard curve experiment though strengthened the DBS/Proseek® usefulness viewpoint. Both filter paper types were performing approximately equally.

The inter-elution precision, analyses and comparison of two individual DBS from the same blood, were really promising when analyzing DBS from PE Grade 226 (6.3 % CV) and quite mediocre when analyzing DBS from Agilent Bond Elut DMS (28.9 % CV). This is indicating a large benefit for PE Grade 226 (thus also Whatman DMPK-C) over Agilent Bond Elut DMS even though not too much weight are to be laid on this initial small study.

The optimized elution procedures elution volumes were related to the DBS blood concentration. The optimized elution volume for a 3 mm DBS disk for Agilent Bond Elut DMS (40 µl) were twice as large as for PE Grade 226 (20 µl) and DBS from Agilent Bond Elut DMS contains approximately twice as much blood per area than PE Grade 226. It was clear that 1 hour RT elution with shaking was more efficient than 4 °C elution over night, for both Agilent Bond Elut DMS and PE Grade 226. The optimized elution settings generated equal 85 % recoveries of 1 nM IL-8 spiked DBS, which should be considered as satisfying.

There are lots of imaginable reactions possibly occurring in the DBS drying process. One such thing could be inflammatory reactions between blood components and the body foreign filter paper material (cellulose or glass fibre). To examine what happens to the levels of the two inflammatory related cytokines IL-8 and Il-6, an experiment with heat stabilization was performed. The experiment did not work sufficiently for IL-6 because IL-6 was not easily eluted from HS DBS. The IL-8 analysis showed consistent levels of IL-8 for all HS time points and for the non-HS normal drying procedure. The one setting that made the IL-8 distinctly increase (except spiked samples for both HS and non-HS) were 2 hours delayed drying time for non-HS samples. These IL-8 levels were strongly raised compared to HS samples with delayed drying time and non-HS samples with normal drying time. The conclusions from this is that IL-8 levels, and probably also other proteins, could be altered by the drying procedure. For the case with IL-8 this influence were though only seen for prolonged drying times and not standard drying times.

5.5 Protocol development

Two different approaches to improve the DBS analysis in terms of both speed and sensitivity were evaluated. The first attempt was to combine elution and probe incubation by eluting the DBS with the Proseek® probe mix. The second attempt was to treat the DBS disk as an ordinary sample for example plasma and keep it in the PCR plate well through the qPCR detection. The experiment with combined elution and probe incubation generated very low IL-8 % recoveries. One hypothesis to the low values is that the charged DNA in the probes tightly interacts with the filter fibers. The probes could in this way be absorbed to a large extent by the filter. This would lead to a large amount of the probes, and probe-antigen complexes subsequently not transferred as sample to the detection step. This hypothesis is based on the fact that DBS disks are routinely boiled to elute DNA when DNA is the molecule of interest[25]. The combination of the experiment result and this hypothesis lead to the decision not to do follow ups on combined elution and probe incubation. Neither the DBS disk in well through qPCR detection worked sufficiently. The reasons to this was eventually due to that the 3 mm DBS disk size was not small enough to nicely fit in the PCR plate well.

A smaller puncher (1.2 mm in diameter) was obtained and 1.2 mm DBS disks were showed to generate equal results as the 3 mm in diameter disks with linear scale down of elution volume to disk area both for PE Grade 226 and Agilent Bond Elut DMS. This made the 1.2 mm DBS disks the natural choice for the continuing project because of less sample waste. Up to six individual 1.2 mm DBS disks can be punched from a 15 µl DBS compared to only one possible 3 mm punch. This increases the potential amount of data obtained from a single DBS six times. Also to keep a 1.2 mm DBS disk in the well through the qPCR detection worked. The actual signal was clearly higher than an eluted DBS disk but also the background from a blank 1.2 mm filter disk was higher than background from buffer only. The S/N was approximately equal suggesting equal sensitivity properties. The major advantage is instead that no accurate sample pipetting is required. This could potentially be a game-changing advantage for clinical use and for obtaining really reproducible and trustworthy results.

5.6 Proseek$^®$ Multiplex^{96x96} – DBS analysis performance

The final major aspects embraced by the project were DBS analysis performance with Proseek$^®$ Multiplex^{96x96} and differences between different sample types, partly plasma and DBS comparisons but also plasma and DBS subtypes. 8 different sample types were collected from five healthy mid-aged women and analyzed by Proseek$^®$ Multiplex^{96x96} Oncology I panel. Both DBS standard elution and DBS disk in well through pre-amplification procedures were evaluated. Most attention was directed to DBS from Whatman DMPK-C because of more extensive history and proven properties. A total of three Proseek$^®$ Multiplex^{96x96} Oncology I runs were performed and the combined inter elution % CV from these runs indicate DBS analysis accuracy (7 % CV) patently matching plasma analysis accuracy (6 % CV). These values are certainly great and are way under the recommended guidelines stated by the LBABFG. Only very small variations in % CV were seen for DBS standard elution and DBS disk in well through pre-amplification and also between Whatman DMPK-C and Agilent Bond Elut DMS.

Another aspect to evaluate for all Oncology I assays are hook effects. The Proseek Multiplex Oncology I kit is adapted to meet the demands on measurable endogenous analyte levels in plasma samples. The adaptation was made by titration of the different probes to transform the endogenous levels present in plasma to measurable levels by increasing the amount of probes for low abundant markers and vice versa. All the Oncology I kit assays are thus not necessarily working sufficiently with DBS analysis. Evaluation of hooked assays was performed for the different DBS sample analysis procedures. A few more assays hooked for DBS than plasma, and a few more for filter in well through pre-amplification than the separate elution procedure. The reason why more assays hooked when analyzing the DBS disk when keeping them in the wells through the pre-amplification compared to the standard elution procedure is probably due to the larger volume of sample added to the pre-amplification step, approximately 7 times more. Another possible reason could be that hardly eluted analytes eventually could be recognized and amplified even though the analyte is not released from the filter pores. The difference in sensitivity for the different procedures and samples is tightly connected to the normalized S/N (dddCt). Mean dddCt values for all individuals and all assays were highest for plasma followed by DBS disk in well through pre-

amplification and finally DBS analyzed by the separate elution procedure. This is probably completely due to the amount sample initiated in the analysis because when the dddCt values were corrected for the amount sample initiated, the order where the other way around, DBS standard elution first and plasma last. S/N calculations were for plasma and DBS standard elution based on negative control background and the DBS disk in well through pre-amplification background were based on the empty filter disk background. This empty filter disk background is certainly assay specific, but are for most assays higher than the negative control background.

The easiest and least invasive way to sample DBS is to add a blood droplet to a filter paper directly from a finger stick with a lancet. It is though a fact that capillary blood from for example a finger stick and venous blood from venepuncture is not identical in all aspects. It is also not necessarily true that blood with an added anticoagulant for example EDTA behaves equally in terms of for example DBS spreading and detectable analyte levels. Based on the experiment performed in this degree project it is concluded that there are no general differences and only very small differences for individual Oncology I assays. The correlation between the two sample types is very tight.

Another import aspect to investigate regarding the availability of DBS as sampling method for any clinical use, is the correlation to plasma samples for marker level differences between individuals. Direct comparison between DBS and plasma results cannot easily be done because of the different types of samples. DBS is containing blood cells as opposed to plasma. Most blood cells probably burst in the DBS drying process probably releasing cellular proteins not seen in plasma. This arguing leads to the hypothesis that at least some of the markers in the Oncology I panel that are expressed in or on the surface of blood cells should be elevated in DBS compared to plasma. At least when the results are normalized for the different sample volumes initiated into the analysis. This hypothesis is strengthened by the experiments performed showing compensating DBS levels at least equivalent to plasma. The DBS signals are for a number of assays increased compared to plasma, probably due to the blood cells present in the DBS. Another attempt to evaluate differences between the sample types is to compare how differences in marker levels between individuals manifest by the sample types. The two sample types, DBS and plasma, agree regarding differences between individuals in 73 % of the observations.

When differences between the individuals were studied by means of PCA it was evident that DBS had at least equal ability to resolve differences between the individuals as plasma. Sample subtype for example different anticoagulants had a greater impact on plasma samples than DBS samples.

The final presented comparison from the experiments with Proseek® Multiplex^{96x96} Oncology I panel was mean normalized S/N values for all individuals and sample types falling into the categories DBS standard elution, DBS disk in well through pre amplification and plasma. When the DBS signals are not compensated, which they probably should not be in practical clinical use, the plasma signals are generally highest. Some assays are though peaking for

DBS, over the plasma signal. The S/N for DBS disks left through pre amplification is almost exclusively higher than the S/N for DBS standard elution. This is not astonishing because the blood in the standard procedure is diluted approximately 7 times when filter left through pre amplification is not diluted at all. This is true even though S/N for DBS left in the pre amplification, is normalized with the higher blank filter background.

5.7 Future perspectives

DBS sampling possesses many advantages over for example plasma sampling. These advantages include minimal invasiveness of the patient, cheap and simple sampling, non-complex storage and transportation. Disadvantages with DBS have for a long time been the limited amount of sample. The largest restraint nowadays is probably the long history and thus extensive knowledge about plasma sample results. The PEA technology and Proseek[®] have the two crucial abilities, low sample amount needed and high sensitivity, needed for DBS analysis. This degree project highly supports the possibility of DBS/Proseek[®] analysis for generation of highly reproducible and accurate quantitative protein analyses. The next step in the DBS/Proseek[®] procedure development and evaluation, is to find a case where actual diagnostic use of DBS/Proseek[®] can be proven. DBS may in the future be the natural sample choice for all kinds of blood analysis.

6 Conclusions

DBS, as sample format, possesses a number of desirable advantages over for example plasma samples. This degree project has demonstrated that accurate (7 % inter spot CV) DBS analysis is possible with the PEA technology products Proseek® and Proseek® Multiplex^{96x96} developed by Olink Bioscience. All the developed and optimized elution procedures for 3 mm and 1.2 mm DBS disks on Whatman DMPK-C and Agilent Bond Elut DMS generate 85 % IL-8 recovery of 1 nM IL-8 spiked DBS analyzed with Proseek®. No difference in signal is obtained for IL-8 and IL-6 DBS/ Proseek® analysis during normal RT drying. 2 hours prolonged drying increases the IL-8 level drastically (a factor of 5), but not the IL-6 level.

The degree project has also demonstrated the usefulness of both the cotton based Whatman DMPK-C and the glass fiber based Agilent Bond Elut DMS filter cards. DBS size differences, due to hematocrit level variation between patients, influence the accuracy of inter patient comparisons. Agilent Bond Elut DMS DBS is stated to be less influenced by the hematocrit size than Whatman DMPK-C. This statement is supported by the degree project. Whatman DMPK-C though display slightly better inter elution accuracy making the DBS sampling card choice not self-evident.

Two different DBS analysis procedures have been developed and evaluated. The first is based on an initial DBS disk (1.2 or 3 mm in diameter) elution and subsequent Proseek® or Proseek® Multiplex^{96x96} analysis of 1 µl elute. The second procedure lets a 1.2 mm DBS disk stay in the PCR plate well through the qPCR detection (Proseek®) or the pre-amplification (Proseek® Multiplex^{96x96}). It has been showed that both procedures work sufficiently even though an alternative background, due to filter disk influence on the qPCR detection (Proseek®) and the pre-amplification (Proseek® Multiplex^{96x96}), should be used for S/N calculations for the second method. This alternative background is obtained by analysis of an empty 1.2 mm in diameter filter disk. The major advantage with the second procedure is the limited amount of accurate pipetting required.

In the final proof of concept demonstration, of DBS analysis with the PEA technology, the degree project validated the developed procedures on eight sample types (for example capillary DBS, venous EDTA, DBS and EDTA plasma) from five mid-aged healthy women. This validation showed no general but only fractional assay specific differences, between capillary and EDTA DBS. The degree project has also shown that the difference between healthy individuals is greater than the difference in DBS sample subtype. This is very valuable properties for versatile use of DBS as sample format. DBS and plasma are by nature distinct sample formats. Plasma can be interpreted as a fraction of DBS, as shown in this degree project, where at least equivalent signals are obtained from EDTA DBS (compensated for the dilution in the elution step) as in EDTA plasma. This is shown in Proseek® Multiplex^{96x96} Oncology I analyses comparisons, from the five individuals.

6 individual 1.2 mm in diameter DBS disks can be obtained from a 15 µl Whatman DMPK-C DBS. Highly reproducible quantitative analysis of 92 different proteins from one such disk

can be obtained with Proseek® Multiplex^{96x96}. This results in 552 possible quantitative protein analyses from one single 15 µl DBS obtained from for example a heel stick from a premature infant. This could possibly enable ethical studies of premature infants, due to extensive data generation from minimal sampling, possibly leading to increased survival rates in the future.

7 Acknowledgement

8 References

1. Guthrie R, Susi A. 1963. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics, 32, 338–343.
2. Parker S P, Cubbit W D. 1999. The use of dried blod spot in epidemiological studies. Clinical pathology, 52, 633-639.
3. PKU laboratory, Karolinska university hospital, Stockholm, Sweden. http://www.karolinska.se/PKU (2013-12-19).
4. McDade T W, et al. 2007. What a drop can do: dried blood spots as a minimally invasive method for integrating biomarkers into population-based research. Demography, 44, 899-925.
5. Fiscus S A, et al. 1998. Quantitation of human immunodeficiency virus type 1 RNA in plasma by using blood dried on filter paper. Clinical microbiology, 36, 258-260.
6. Lin Y-Q, et al. 2011. Combination of ELISA and dried blood spot technique for the quantification of large molecules using exanatide as a model. Pharmacological and toxicological methods, 64, 124-128.
7. Barfield M, et al. 2008. Application of dried blood spots combined with HPLC-MS/MS for the quantification of acetaminophen in toxicokinetic studies. Chromatography B, 870, 32-27.
8. Spooner N, et al. 2009. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Analytical Chemistry, 81, 1557-1563.
9. ACTG/IMPAACT Lab Tech Committee. 2012. Processing of Dried Blood Spots Standard Operating Procedure. LTC-SOP-55 v2.0.
10. Lai Y, et al. 2005. Enzyme-linked immunosorbent assays (ELISAs). Critical Care Medicine, 33, 343-434.
11. Pla-Roca M, et al. 2012. Antibody colocalization microarray: a scalable technology for multiplex protein analysis in complex samples. Molecular & Cellular Proteomics, 11, M111 011460.
12. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, et al. (2002) Protein detection using proximity-dependent DNA ligation assays. Nature biotechnology 20: 473-477.
13. Gullberg M, Gustafsdottir SM, Schallmeiner E, Jarvius J, Bjarnegard M, et al. (2004) Cytokine detection by antibody-based proximity ligation. Proceedings of the National Academy of Sciences of the United States of America 101: 8420-8424.
14. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S (2011) Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic acids research Submitted.
15. Findlay J W A, et al. 2000. Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. Pharmaceutical and Biomedical analysis, 21, 1249-1273.
16. DeSilva B, et al. 2003. Recommendations for the bioanalytical method validation of ligand-bindning assays to support pharmacokinetic assessment of macromolecules. Pharmaceutical Research, 20, No. 11.

17. Food and Drug Administration, U.S. Department of Health and Human Services, Center for Drug Evaluation and Research, Center for Veterinary Medicine, 2001. Guidance for industry bioanalytical method validation.
http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf (2013-09-18)

18. Spooner N, et al. 2009. Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method. Analytical Chemistry, 81, 1557-1563.

19. Eurogentec. qPCR guide. http://www.eurogentec.com/uploads/qPCR-guide.pdf (2013-12-19).

20. Life technologies, Applied Biosystems. Real time PCR: Understanding C_t.
http://www.lifetechnologies.com/content/dam/LifeTech/migration/en/filelibrary/nucleic-acid-amplification-expression-profiling/pdfs.par.70657.file.dat/understanding%20ct%20application%20note.pdf (2013-12-19). Application note.

21. Blessborn D, et al. 2013. Heat stabilization of blood spot samples for determination of metabolically unstable drug compounds. Bioanalysis, 5, 31-39.

22. Hedges JC, et al. 2000. Mitogen-activated protein kinases resulate cytokine gene expression in human airway myocytes. Cell Molecular Biology, 23, 86-94.

23. Arora R, et al. 2013. Hematocrit and its impact on quantitative bioanalysis using dried blood spot technology. Application note by Agilent Technologies Inc., 2013 Printed in the US March 1, 2013, 5991-0099EN.

24. Skogstrand K, et al. 2008. Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples. Immunological Methods, 336, 78-84.

25. Göhring K, et al. 2010. Influence of different extraction methods and PCR techniques on the sensitivity of HCMV-DNA detection in dried blood spot (DBS) filter cards. Clinical Virology, 48, 278-281.

26. Skogstrand K, et al. 2005. Simultaneous measurement of 25 inflammatory markers and neutrophins in neonatal dried blood spots by immunoassay with xMAP technology. Clinical chemistry, 51:10, 1854-1866.

27. Ramakrishnan L, 2008. Analysis of the use of dried blood spot measurements in disease screening. Diabetes science and technology, 2, issue 2.

28. E-mail correspondence (2013-08-27) with Thorkild Tylleskär, Professor at the Department of Global Public Health and Primary Care, University of Bergen.

9 Supplementary material

9.1 Formulas

Replicate number	j
Number of run	i
Number of replicates in the i:th run	n_i
Number of cycles in qPCR	n_c
Measured result (Cycle threshold value)	C_t
Linearized measured result (amplicon)	$z = 2^{(n_c - C_t)}$
Target concentration	μ_T
Average value for the i:th run	$\bar{z}_{i.} = \frac{1}{n_i}\sum_{j=1}^{n} z_{ij}$
Standard deviation for the i:th run	$s_i = \sqrt{\frac{1}{(n_i-1)}\sum_{j=1}^{n}(z_{ij} - \bar{z}_{i.})^2}$
Precision (percentage coefficient of variance, %CV)	$\%CV = 100(s_i/\bar{z}_{i.})$
Accuracy (percentage relative error, %RE)	$\%RE = 100(\bar{z}_{i.} - \mu_T)/\mu_T$
Total error	$\%TE = \%CV + |\%RE|$

9.2 Elution buffer compatibility with Proseek® analysis

9.2.1 IL-8 spike distinguishing properties
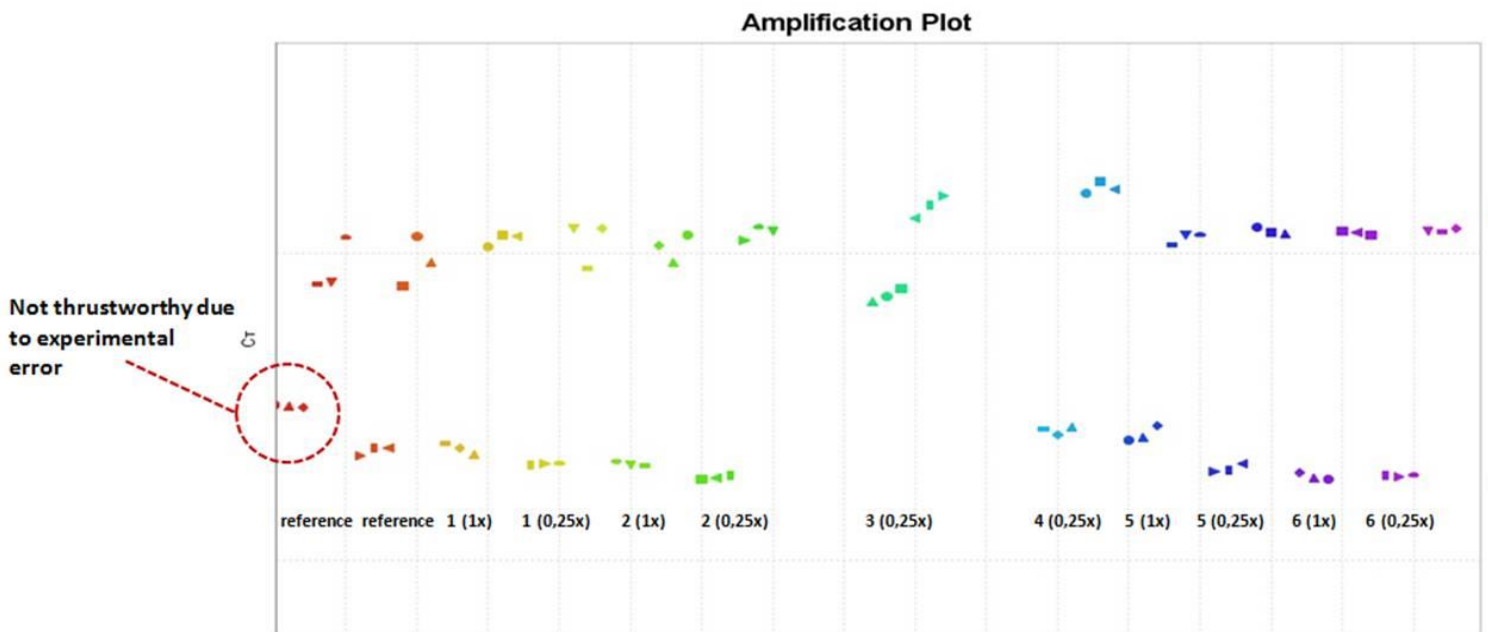
Figure 21. Ct values from triplicate Proseek® analysis with the different conceivable elution buffers. The first three data points for each colour (the lower ones) are IL-8 buffer backgrounds. The following three data points of each colour (the higher ones) are 100 pM spiked IL-8 samples in the corresponding buffer. "Reference" is Proseek® calibrator diluent. 1x is concentrated buffer and 0.25x is buffer titrated to 25 % in Proseek® assay diluent. The numbers 1 – 6 corresponds to elution Buffer 1 – 6.

9.2.2 IL-8 standard curves

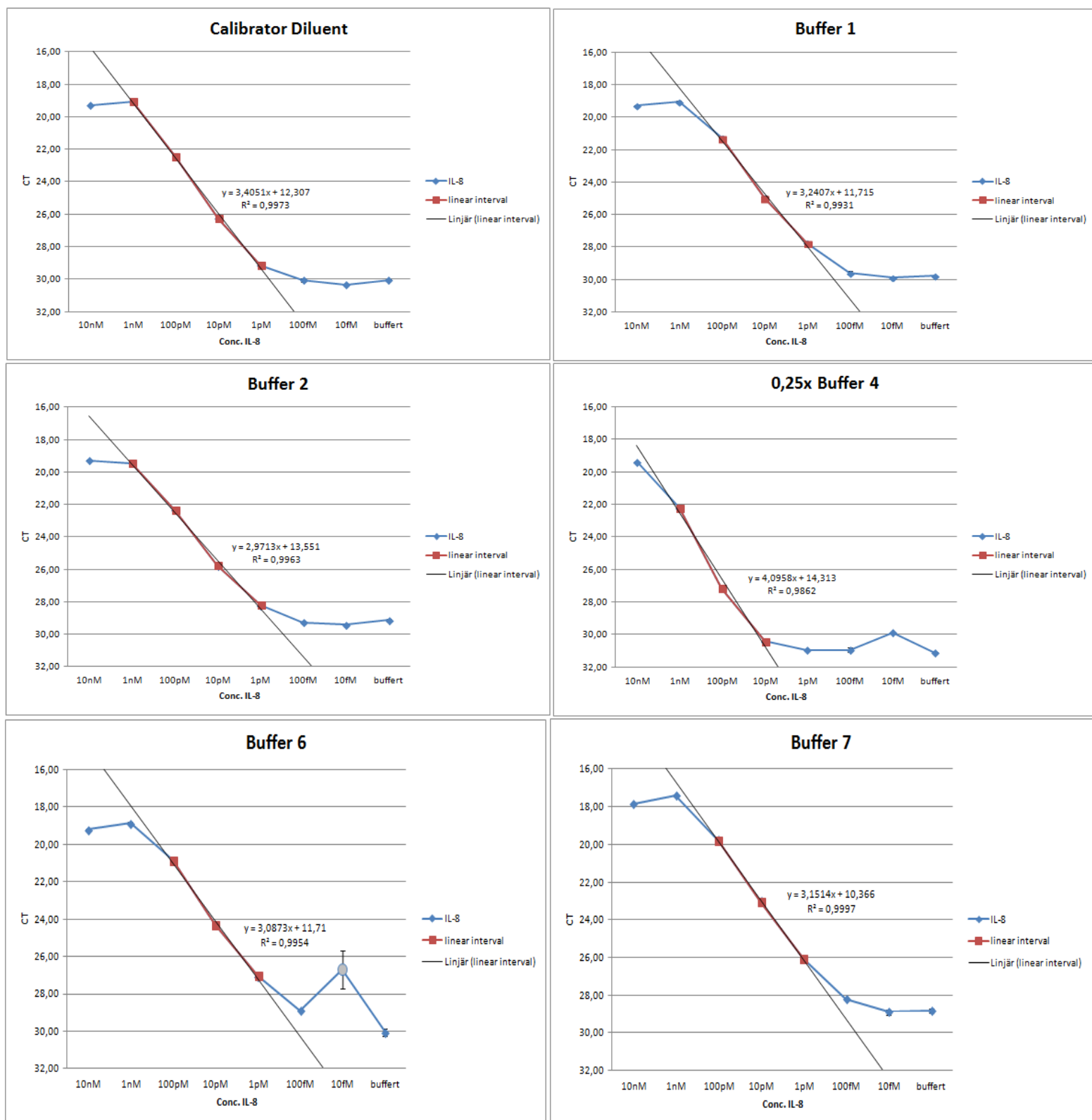**Figure 22.** IL-8 standard curves (10 nM – 100 fM plus reference) were prepared in the different conceivable elution buffers and analyzed with Proseek®. The red marked part of the plots is interpreted as the linear range and these data is used for the trend line calculations.

9.3 Inter-spot elution accuracy

Table 17. Mean Ct (triplicates) from Proseek® analysis of endogenous IL-8 levels from three different DBS elutions (A, B and C) with Calibrator diluent or Buffer 2 from PE Grade 226 and Agilent Bond Elut DMS.

Elution	PE Grade 226	Agilent Bond Elut DMS
Calibrator diluent elution A	28.31	26.73
Calibrator diluent elution B	28.52	25.75
Calibrator diluent elution C	28.59	25.77
Buffer 2 elution A	23.76	25.20
Buffer 2 elution B	23.85	24.81
Buffer 2 elution C	23.80	25.50

9.4 Combined elution and probe incubation

Table 18. The table presents amplicon values (linearized Ct values calculated as $2 \wedge (40 - Ct)$), for 8 and 40 µl Buffer 1 standard elution procedures, from the Proseek® analysis of 100 pM IL-8 DBS on PE Grade 226 and Agilent Bond Elut DMS. The calculated ratios at the bottom of the table are 8 µl amplicon values divided by 40 µl amplicon values. The theoretical ratio, supposing equal efficiency regardless elution volume, is 5 (40 / 8).

Elution	PE Grade 226	Agilent Bond Elut DMS
8 µl Buffer 1 elution	48942	115783
40 µl Buffer 1 elution	15715	29635
Ratio	**3.1**	**3.9**

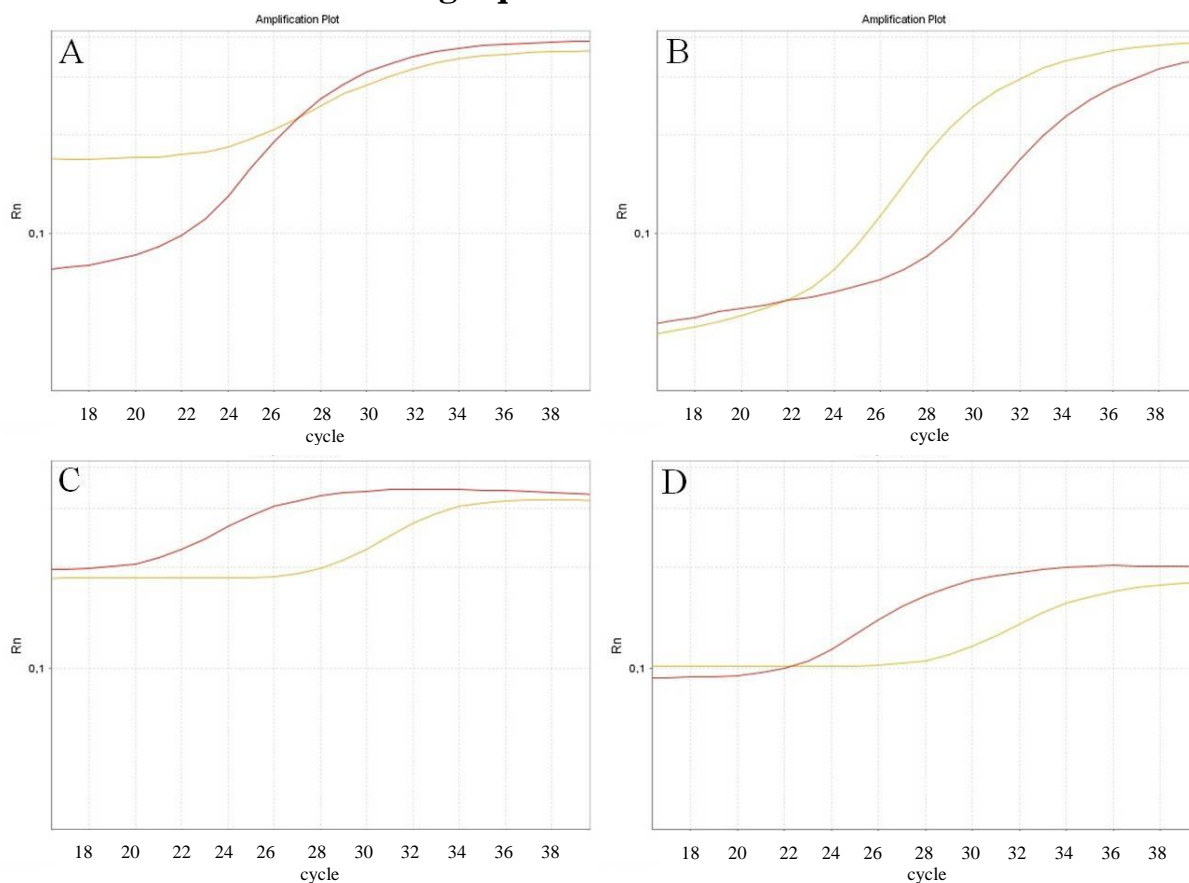## 9.5 DBS disks in wells through qPCR detection

Figure 23. Raw qPCR amplification plots for the IL-8 Proseek® analysis of 3 mm in diameter DBS disks (**A** and **B**) and 3 mm in diameter blank filter disks (**C** and **D**). Red curves are 100 pM IL-8 spiked DBS and 100 pM spiked Buffer 1 respectively. Yellow curves are endogenous DBS levels of IL-8 and non-spiked Buffer 1 respectively. **A** and **C** are analyzed with PE Grade 226. **B** and **D** are analyzed with Agilent Bond Elut DMS. 6 µl probe mix and 2 µl Buffer 1 were used for the combined elution and incubation. The DBS´s and blank filter disks were left in the wells through the complete analysis including the final qPCR detection.

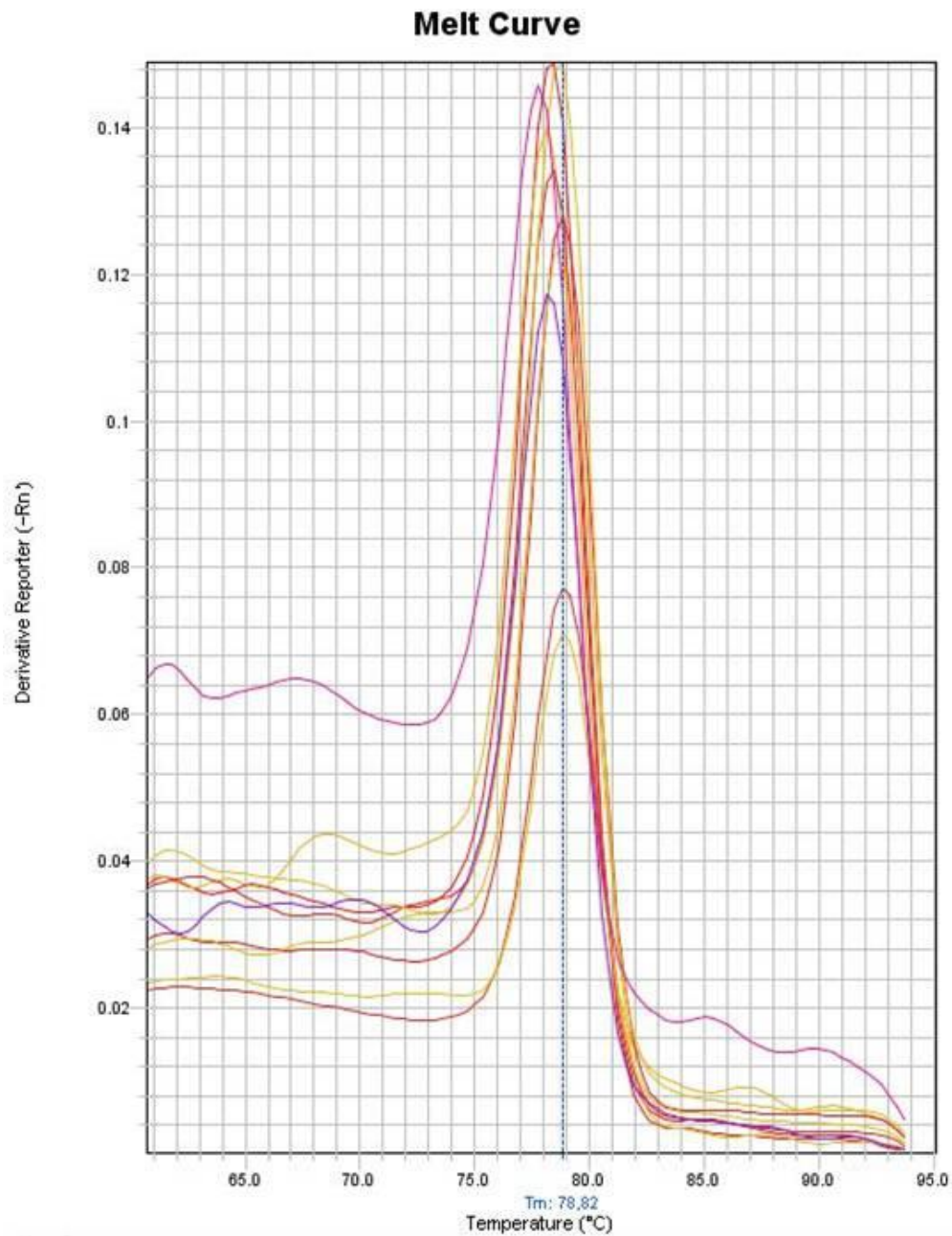**Figure 24.** Combined melt curve corresponding to all the Proseek® analysis presented in Figure 22 (3 mm in diameter DBS disks and blank filter disks left in the wells through the entire Proseek® analysis). The melt curve indicates the amount of amplified DNA with different lengths in terms of decreasing fluorescence with temperature (DNA amplicons with different lengths melts at different temperatures).

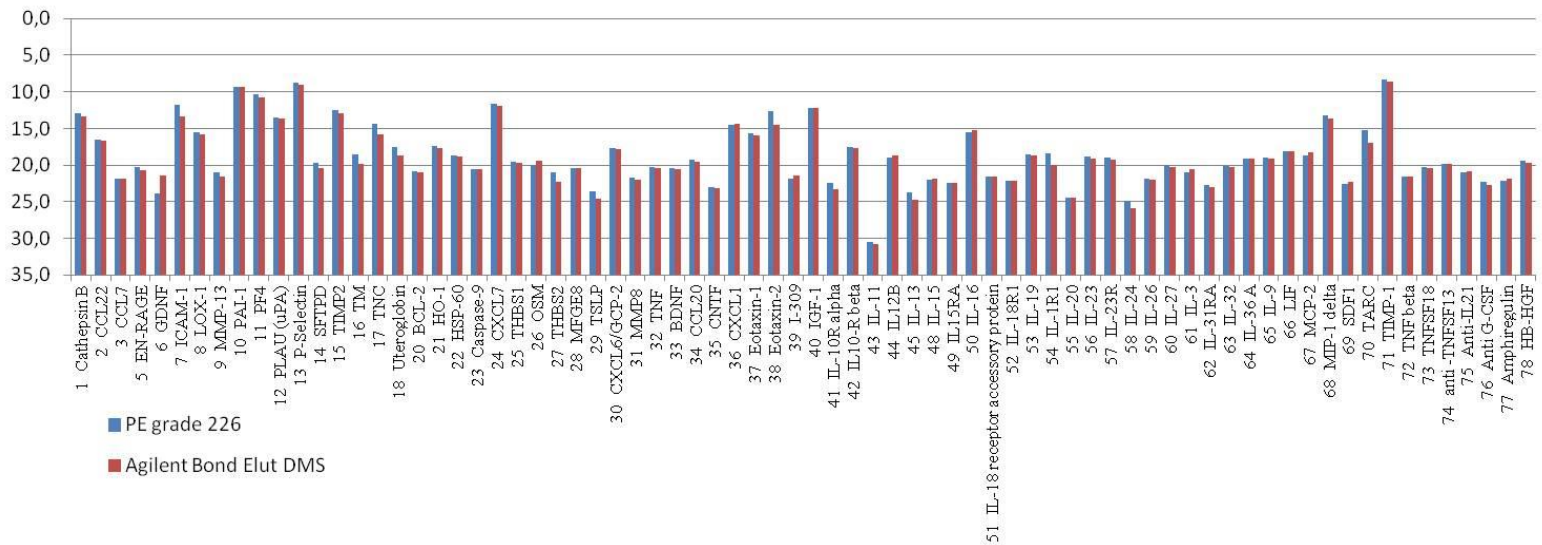## 9.6 DBS and Proseek® Multiplex$^{96 \times 96}$ inflammation panel

Figure 25. Mean Ct values (duplicates) for the Proseek® Multiplex$^{96 \times 96}$ exploratory inflammation analysis of endogenous EDTA DBS from PE Grade 226 and Agilent Bond Elut DMS. 1.2 mm in diameter DBS were analyzed for the Buffer 1 standard elution procedure for PE Grade 226 and Agilent Bond Elut DMS respectively.

9.7 DBS and Proseek® Multiplex[96x96] Oncology I evaluation

9.7.1 Experiment setups

Run 1 - stdandard elution

		Whatman DMPK-C										Agilent Bond Elut DMS	
		1:1		1:10		1:100	1:1		1:10		1:100		
		1	2	3	4	5	6	7	8	9	10	11	12
DBS venous	A	Individual 1					Individual 2					Capillary 1:1	
DBS capillary	B	Individual 1					Individual 2					EDTA 1:1	
DBS EDTA	C	Individual 1					Individual 2					Capillary	ICP
DBS Citrate	D	Individual 1					Individual 2					1:10	
DBS Heparin	E	Individual 1					Individual 2					1:100	
Plasma EDTA	F	Individual 1					Individual 2					EDTA	
Plasma Citrate	G	Individual 1					Individual 2					1:10	Neg. Ctrl.
Plasma Heparin	H	Individual 1					Individual 2					1:100	

Run 2 - stdandard elution

		Whatman DMPK-C										Agilent Bond Elut DMS	
		1:1		1:10		1:100	1:1		1:10		1:100		
		1	2	3	4	5	6	7	8	9	10	11	12
DBS venous	A	Individual 3					Individual 4					Kapillärt 1:1	
DBS capillary	B	Individual 3					Individual 4					EDTA 1:1	
DBS EDTA	C	Individual 3					Individual 4					Kapillärt	ICP
DBS Citrate	D	Individual 3					Individual 4					1:10	
DBS Heparin	E	Individual 3					Individual 4					1:100	
Plasma EDTA	F	Individual 3					Individual 4					EDTA	
Plasma Citrate	G	Individual 3					Individual 4					1:10	Neg. Ctrl.
Plasma Heparin	H	Individual 3					Individual 4					1:100	

Run 3 - filter in well through pre- amp.

			Whatman DMPK-C										Agilent Bond Elut DMS	
			1	2	3	4	5	6	7	8	9	10	11	12
1,2 mm disk	DBS venous	A	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5		blank	
	DBS capillary	B	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5		blank	
	DBS EDTA	C	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			ICP
	Agilent capillary	D	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			
1/2 1,2 mm disk	DBS venous	E	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			
	DBS capillary	F	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			Neg. Ctrl.
	DBS EDTA	G	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			
	Agilent capillary	H	Individual 1		Individual 2		Individual 3		Individual 4		Individual 5			

Figure 26. The analysis of the 8 different sample types from the 5 individuals were divided into 3 Proseek® Multiplex[96x96] Oncology I runs. The plate setups for the three runs were those presented in the figure. ICP were, for all runs, positioned at the same place in the well to achieve as accurate between run normalization as possible.

9.7.2 Non-hooked assays with distinct endogenous level

The following 20 assays didn´t hook but showed distinct endogenous levels for all different sample types (for example capillary DBS and heparin plasma) when analyzed with Proseek® Multiplex[96x96] Oncology I:

- IL-8
- VEGF
- CD40L
- EGF
- Osteoprotegerin
- MCP-1
- Galectin-3
- TNF RI
- PDGF-BB
- IL-2 R alpha
- Fas
- CCL19
- EMMPRIN
- EpCAM
- CCL24
- uPAR
- Flt-3 Ligand
- CCL21
- Caspase-3
- CD69